



Signaling Flow Cytometry

Find what you didn't know was missing

What's Really Going on in Your Cells?

Flow cytometry uniquely enables rapid analysis of multiple endpoints, including protein expression and intracellular signaling in heterogeneous populations of cells. As a result, you can use this technique to gain deep mechanistic insights into biological processes in normal and pathologic states.

Already widely recognized as a premier provider of antibodies for cell signaling analysis, Cell Signaling Technology (CST) offers a diverse selection of antibodies and reagents for flow cytometry, supporting both quantitative analysis of intracellular signaling pathways and phenotyping.

Our expertise in antibody design, conjugation, and flow cytometry enables us to develop and validate antibodies that you can use in your flow cytometry experiments with confidence. And the same scientists who develop and validate our flow cytometry products also provide technical support to ensure your experiments are successful.

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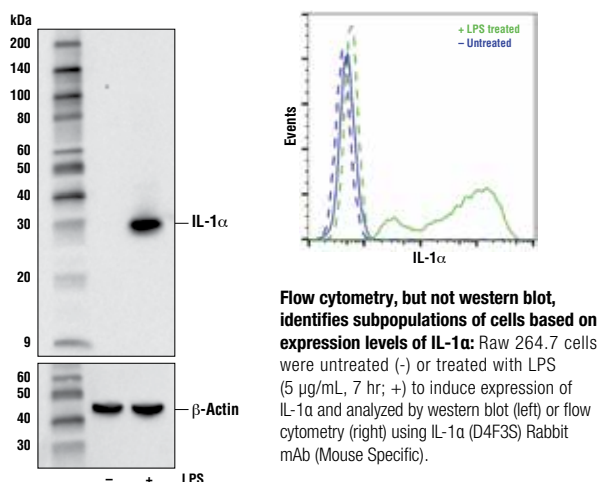
To learn more about the benefits of signaling flow cytometry, watch our short video at [cst-science.com/flow](https://www.cst-science.com/flow).

Cell-by-cell Signaling Analysis Powered by CST Antibodies

Why should you consider flow cytometry for signaling?

Flow cytometry enables quantitative analysis of protein expression, signaling states, and physical characteristics (cell size/granularity) at the single-cell level. Modern flow cytometers are capable of collecting data on multiple proteins from thousands of cells per second in a heterogeneous mixture. While flow cytometry is commonly employed to identify cell types using phenotypic markers expressed on the cell surface, it can also be used to measure intracellular signaling events.

Researchers pursuing hypotheses about signaling events traditionally start with western blot. In this technique, lysates are pooled and analyzed one protein at a time, and infrequent signaling events may be missed due to insufficient detection sensitivity. Western blotting has limited capability to perform multiparameter quantitative analysis. Another drawback is that workflows can take a day or more to complete.



Flow cytometry is an ideal solution to avoid these shortcomings, enabling multiplexed and quantitative analysis of signaling events with greater sensitivity and precision than western blot, in just a few hours. Importantly, by collecting discrete data points from individual cells, flow cytometry enables researchers to identify and quantify subpopulations of cells that exhibit specific signaling responses, to explore the range of responses in those cells, and to correlate multiple quantitative readouts. In short, signaling flow cytometry enables you to ask new research questions and characterize important signaling events from the plasma membrane to the nucleus.

Advantages of Flow Cytometry Over Western Blot for Analysis of Signaling Events

	Flow Cytometry	Western Blot
Study single cell analysis	yes	no
Ability to detect infrequent or rare signaling events	high	low
Identification of subpopulations in which signaling changes occur	yes, simultaneous	only following cell population separation/enrichment steps
Multiparameter analysis	yes	no
Throughput	high	low
Assay duration (sample prep through analysis)	2-4 hours	1-2 days depending on length of primary antibody incubation
Signaling quantification	yes, simultaneous	varies by set-up: traditional film method, no; digital imager, yes

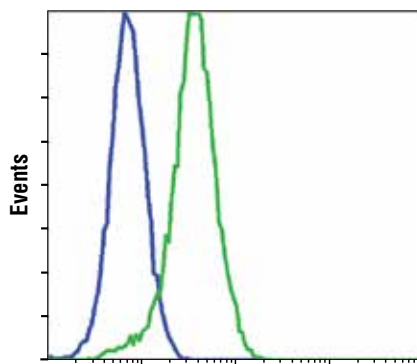
New to flow cytometry? Scientists from our team of flow cytometry experts are available to provide consultation and technical support, from experimental design to analysis. Visit cst-science.com/support.

A Powerful Technique

Intracellular analysis of signal transduction using activation state-specific antibodies

Antibodies that specifically recognize proteins with post-translational modifications (PTMs) – including phosphorylation, methylation, acetylation, and cleavage – can enable direct readout of protein activation states. The use of these PTM-specific antibodies has proven to be a powerful approach for characterizing cellular activities and understanding signal transduction in mechanistic studies of normal and disease states. Many researchers are familiar with PTM-specific antibodies used in western blot experiments; PTM readout in flow cytometry uses the same principles. However, flow cytometry, but not western blot, enables the user to assess differential responses to treatment, or endogenous variation in signaling, in heterogeneous populations at the cell level.

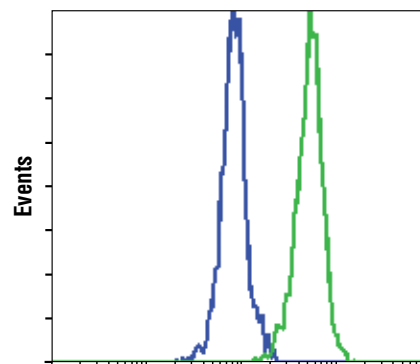
Phosphorylation



**Phospho-Stat1 (Tyr701) (58D6)
(Alexa Fluor® 647 Conjugate)**

Analysis of Jurkat cells, untreated (blue) or treated with hIFN- α 1 (green), using Phospho-Stat1 (Tyr701) (58D6) Rabbit mAb (Alexa Fluor® 647 Conjugate).

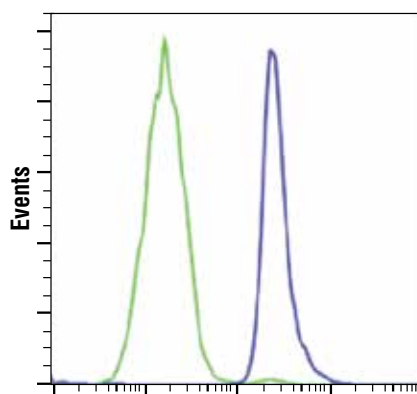
Acetylation



**Acetyl-Histone H3 (Lys9) (C5B11)
(Alexa Fluor® 488 Conjugate)**

Analysis of HeLa cells, untreated (blue) or SAHA-treated (green), using Acetyl-Histone H3 (Lys9) (C5B11) Rabbit mAb (Alexa Fluor® 488 Conjugate).

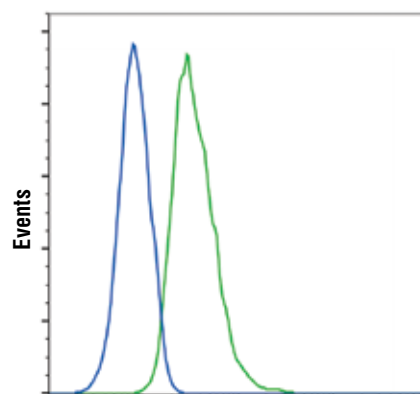
Methylation



**Di-Methyl-Histone H3 (Lys27) (D18C8)
(Alexa Fluor® 647 Conjugate)**

Analysis of Jurkat cells, untreated (blue) or treated with GSK343 (5 μ M, 4d; green), using Di-Methyl-Histone H3 (Lys27) (D18C8) XP® Rabbit mAb (Alexa Fluor® 647 Conjugate).

Cleavage



**Cleaved Caspase-9
(Asp315) (D8I9E) (PE Conjugate)**

Analysis of Jurkat cells, untreated (blue) or treated with Etoposide (25 mM, overnight; green), using Cleaved Caspase-9 (Asp315) (D8I9E) Rabbit mAb (PE Conjugate).

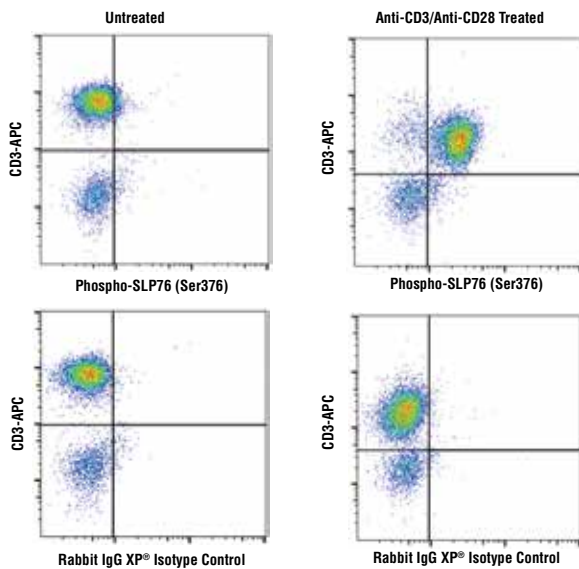
CST provides a comprehensive catalog of flow-validated, PTM-specific antibodies to important targets to accelerate your research progress.

A More Complete Cellular Profile, Inside and Out

Immunophenotyping, or the use of antibodies to identify immune cells (and other cell types) by surface labeling, is one of the more frequent uses of flow cytometry. However, the majority of critical biological processes happen inside the cell. By limiting experiments to the cell surface, you may be missing out on important mechanistic insights into immune cell modulation, epigenetic landscape changes, or aberrant signaling activities in disease models, to provide just a few examples. Expanding your flow cytometry analysis to include intracellular signaling enables you to pursue new research aims and pinpoint cellular drivers of disease.

Multiplex surface markers and intracellular signals to gain unique insights

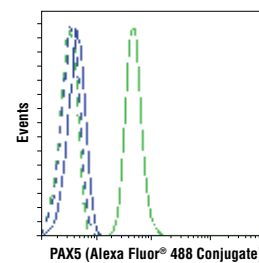
Combining immunophenotypic analysis with signaling readouts enables analysis of biological responses across various cell populations to gain insight into critical cell types. For example, immune system response to experimental treatments or disease states can be profiled and correlated with expression of receptors or other markers. Multiplexing using flow cytometry can also speed research findings.



Analysis of human PBMCs, treated and stained as shown, using Phospho-SLP-76 (Ser376) (D7S1K) XP® Rabbit mAb (top row) or concentration-matched Rabbit (DA1E) mAb IgG XP® Isotype Control (bottom row) with an Alexa® 488-conjugated secondary antibody. Samples were co-stained with a CD3 antibody.

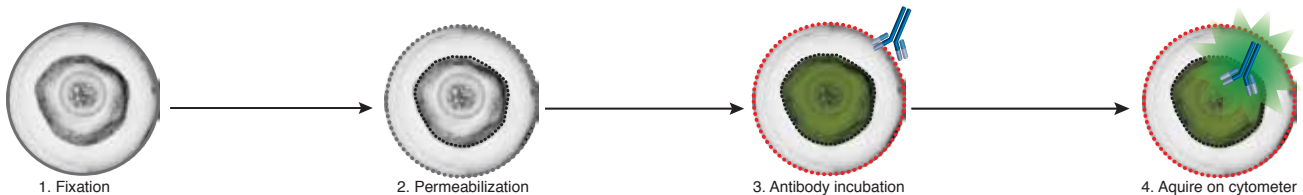
Make your move to intracellular flow cytometry

Transitioning to intracellular protein detection can feel intimidating, whether you are new to flow cytometry or are experienced at running live-cell assays. Multiplexing with antibodies that recognize intracellular and extracellular targets can be readily done, but it is important to consider how working conditions (ie, buffers, blocking steps, permeabilizing agents) may affect antibody binding and specificity when designing experiments and choosing a protocol. For example, CD19 is commonly used to identify B cells, but it is sensitive to fixation. For analysis of fixed samples where CD19 staining is not an option, PAX5 may be used to identify B cells.



Analysis of Pax5-negative (Jurkat, blue) and Pax5-positive (Ramos, green) cells using PAX5 (D19F8) XP® Rabbit mAb (Alexa Fluor® 488 Conjugate).

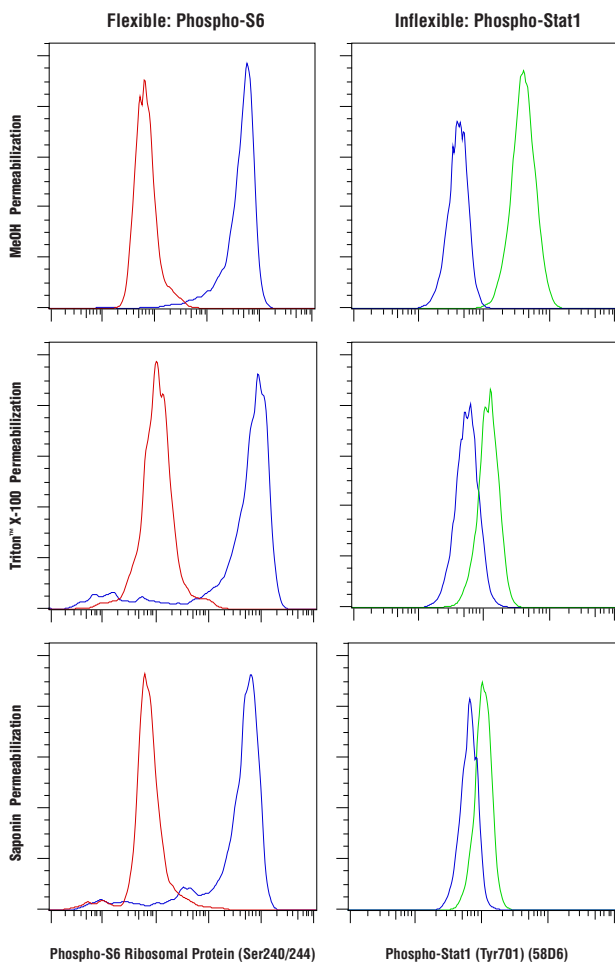
General workflow for detection of intracellular targets



As you navigate setting up intracellular assays, take advantage of our experience in flow cytometry protocol design and antibody validation. If you have questions or experience challenges at any point, support from the Flow Cytometry Group is available at cst-science.com/support.

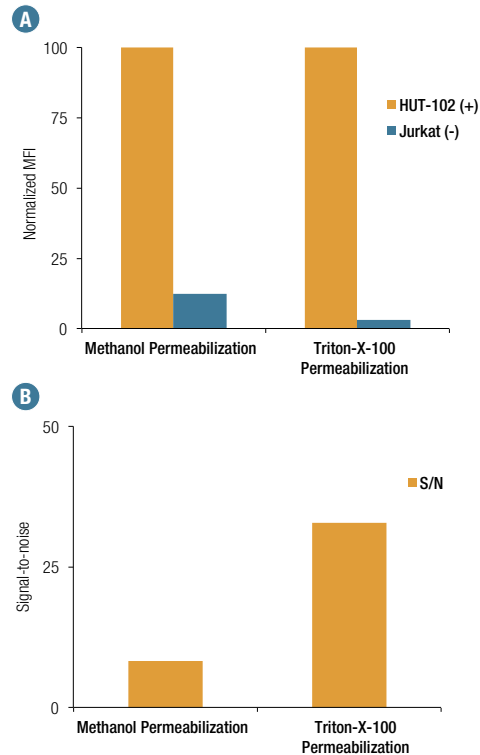
Protocol Optimization for Antibody Performance

Antibody performance in flow cytometry may be affected by fixation and permeabilization reagents, antibody dilution, and buffers used for antibody incubation and wash steps. While CST provides a recommended protocol for every antibody, when performing multiplexed extra- and intracellular staining for flow cytometry, alternative protocols may need to be used to accommodate all antibodies in the panel. Our experts in flow cytometry are available to assist with your experimental design. As illustrated below, phosphorylated S6 ribosomal protein may be detected using a variety of permeabilization protocols, while detection of phosphorylated STAT1 requires methanol to enable antibody binding.



Analysis of Jurkat cells, untreated (blue), inhibited with LY294002 (red), or stimulated with Interferon- α (green), fixed with 4% formaldehyde and permeabilized as indicated, using Phospho-S6 Ribosomal Protein (Ser240/244) (D68F8) XP® Rabbit mAb (Alexa Fluor® 647 Conjugate) (left) or Phospho-Stat1 (Tyr701) (58D6) Rabbit mAb (Alexa Fluor® 488 Conjugate) (right).

In any antibody-based application, the specific binding of the antibody to its target epitope, and not to off-target sites, is crucial to obtaining biologically accurate data. The optimal staining protocol for each flow-validated antibody is determined by CST scientists and provided to you on the product page on cellsignal.com.



Multiple protocol variables tested to maximize S/N. When using methanol permeabilization, OX40 (D1S6L) Rabbit mAb exhibits significant nonspecific binding in Jurkat cells (negative control) (A). However, nonspecific binding is significantly reduced when Triton™ X-100 is used for permeabilization, generating a much higher S/N ratio for the same concentration of the antibody (B).

To find the optimized protocol for a particular antibody, consult the appropriate product page at cellsignal.com. Many CST antibodies will work with other protocol variations as well; contact Technical Support for help with troubleshooting, experimental set-up, or protocol questions. Visit cst-science.com/support.

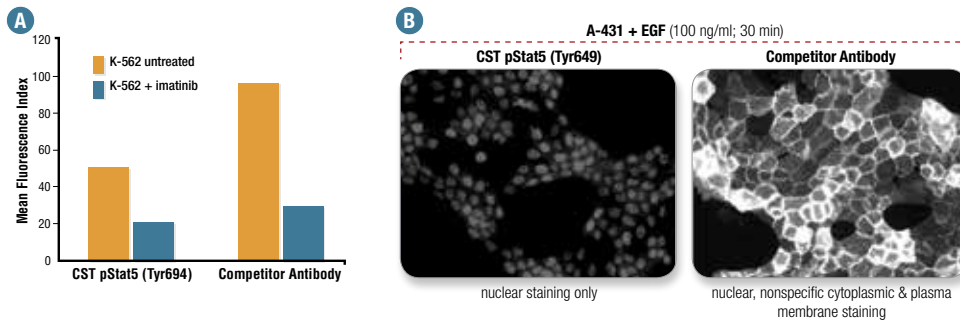
CST Antibodies for Flow Cytometry

Be confident in your results

At CST, flow-validated products undergo rigorous testing in biologically relevant models, ensuring specificity and optimal signal-to-noise (S/N) for both unconjugated and conjugated antibodies. Cross-platform validation further confirms antibody specificity. In addition, all antibodies have been tested for stability and lot-to-lot reproducibility to ensure reliable performance over the course of your experiments. Our industry-leading validation of antibodies for flow cytometry make it possible to examine complex intracellular signaling cascades in cell lines, dissociated tissues, aspirates, or hematology specimens, and trust the accuracy of your results. For more on how CST validates antibodies for flow cytometry, visit cst-science.com/flow-validation.

Validation across multiple applications

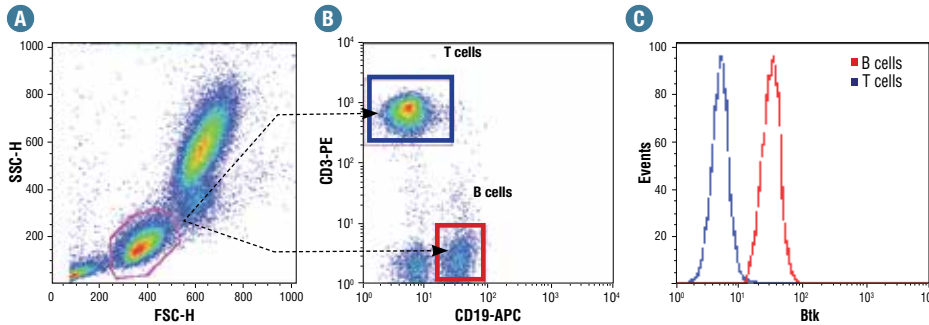
Comparative analysis in flow cytometry and immunofluorescence confirms that the Phospho-Stat5 (Tyr694) antibody from CST detects the target specifically, correctly localizing to the nuclear compartment of the cell.



Flow cytometric analysis suggests a brighter signal from a competitor's Phospho-Stat5 (Tyr694) antibody compared to CST Phospho-Stat5 (Tyr694) (D47E7) XP[®] Rabbit mAb (**A**). However, immunofluorescent analysis reveals that the competitor antibody inappropriately stains the cytoplasm and plasma membrane, while with Phospho-Stat5 (Tyr694) (D47E7) XP[®] Rabbit mAb demonstrates only the appropriate nuclear staining (**B**).

Specificity in different cell populations

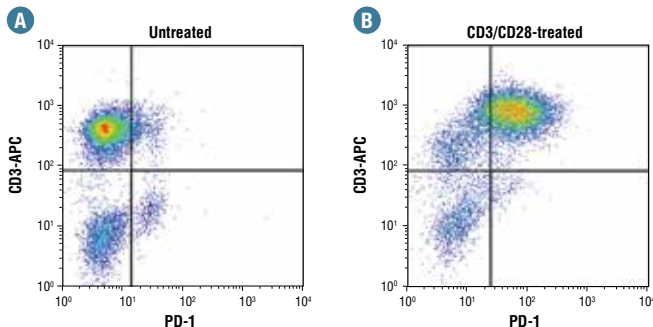
Btk (D3H5) Rabbit mAb selectively isolates B cells from human whole blood.



Human whole blood was fixed, lysed, and permeabilized as per the CST Flow Alternate Protocol and stained using Btk (D3H5) Rabbit mAb. Cells were gated as shown in (**A**). Samples were co-stained using CD3-PE and CD19-APC to distinguish T and B cell subpopulations, respectively (**B**). B (red) and T (blue) cell population gates were applied to a histogram depicting the mean fluorescence intensity of Btk (**C**). Anti-rabbit IgG (H+L), F(ab')₂ Fragment (Alexa Fluor[®] 488 Conjugate) was used as a secondary antibody.

Activator/Inhibitor treatment of cell populations

PD-1 (D4W2J) XP[®] Rabbit mAb selectively detects increased PD-1 expression in CD3-positive PBMCs treated with anti-CD3/CD28.



Flow cytometric analysis of human PBMCs which were untreated (**A**) or treated with anti-CD3/CD28 (72 hr) (**B**), using PD-1 (D4W2J) XP[®] Rabbit mAb and co-stained with a CD3 antibody.

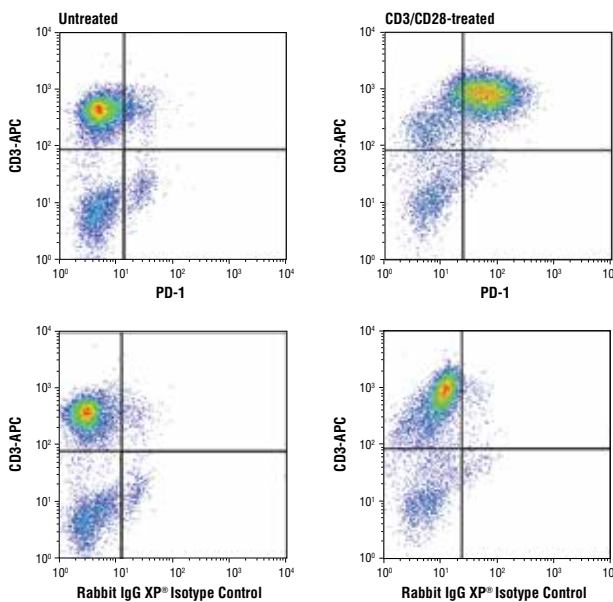
Tools to Accelerate Your Flow Cytometry Success

Can antibodies from CST support your research?

CST has an extensive catalog of antibodies to support flow cytometry experiments in a wide variety of research fields, such as immunology, oncology, epigenetics, cell biology, metabolism, and developmental biology. Antibodies validated for flow cytometry can be used to monitor cellular functions, such as DNA damage, apoptosis, cell cycle and proliferation, and more. Examples relevant to several of these topics are included throughout this brochure; additional data are shown below. For a full list of flow validated antibodies from CST, visit cst-science.com/flow-products.

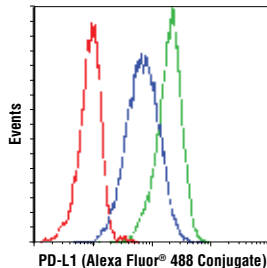
Immuno-Oncology

PD-1 expression can be used to identify T cells that have been activated through engagement with an antigen presenting cell. PD-1 is upregulated following T cell activation, as illustrated with human PBMCs stimulated with anti-CD3/anti-CD28 for 3 days. T cells (CD3⁺) show increased PD-1 expression in the treated condition, with little/no PD-1 expression in the untreated condition.



PD-1: Analysis of human PBMCs, treated and stained as shown, using PD-1 (D4W2J) XP[®] Rabbit mAb (top row) or concentration-matched Rabbit (DA1E) mAb IgG XP[®] Isotype Control (bottom row) with an Alexa Fluor[®] 488 Conjugate secondary antibody. Samples were co-stained with CD3 (UCHT1) Mouse mAb (APC Conjugate)

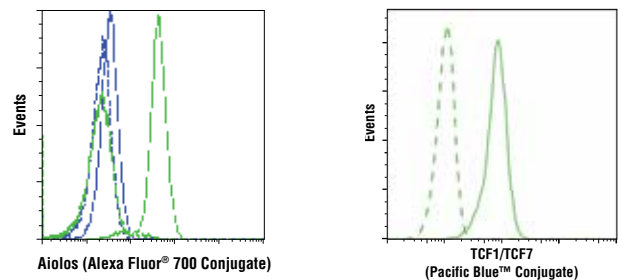
Some tumors express PD-L1 to downregulate the immune system response. The PD-L1 (D8T4X) antibody from CST enables you to determine whether PD-L1 is expressed in your system of interest, and is equally functional on live, fixed, and fixed/permeabilized cells.



PD-L1: Analysis of SUP-M2 cells (green), MDA-MB-231 cells (blue), and Ramos cells (red) using PD-L1 (Extracellular Domain Specific) (D8T4X) Rabbit mAb (Alexa Fluor[®] 488 Conjugate).

Transcription Factors

Expression levels of transcription factors play key roles in many biological functions. Levels of TCF1/TCF7 vary throughout T cell development, and in response to infection. TCF1/TCF7 is abundant in naive CD8⁺ T cells, but is downregulated upon differentiation into effector T cells. The transcription factor Aiolos promotes B cell and T cell differentiation, with higher expression in mature lymphocytes.

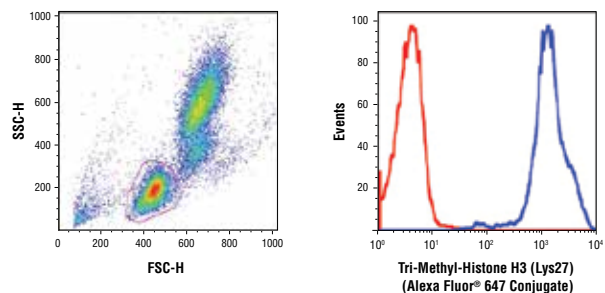


Aiolos: Analysis of U-937 cells (blue) and RPMI 8226 cells (green) using Aiolos (D1C1E) Rabbit mAb (Alexa Fluor[®] 700 Conjugate) (solid lines) or a concentration-matched Rabbit (DA1E) mAb IgG XP[®] Isotype Control (Alexa Fluor[®] 700 Conjugate) (dashed lines).

TCF1/TCF7: Analysis of Jurkat cells using TCF1/TCF7 (C63D9) Rabbit mAb (Pacific Blue™ Conjugate) (solid line) compared to concentration-matched Rabbit (DA1E) mAb IgG XP[®] Isotype Control (Pacific Blue™ Conjugate) (dashed line).

Chromatin Modulation

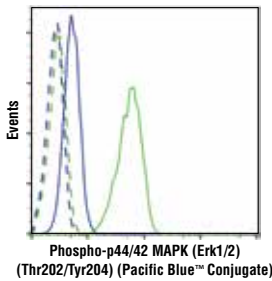
Flow cytometry is ideally suited to quantify changes to epigenetic signatures in the immune system or dissociated tissue. Broad shifts in the methylation or acetylation of key amino acid residues on histone proteins indicate underlying changes in the biological activity of the cell.



Tri-Methyl-Histone H3 (Lys27): Analysis of human whole blood using Tri-Methyl-Histone H3 (Lys27) (C36B11) Rabbit mAb (Alexa Fluor[®] 647 Conjugate). The forward/side-scatter lymphocyte gate was applied to generate a histogram of Tri-Methyl-Histone H3 (Lys27) (blue) and concentration-matched Rabbit (DA1E) mAb IgG XP[®] Isotype Control (Alexa Fluor[®] 647 Conjugate) (red).

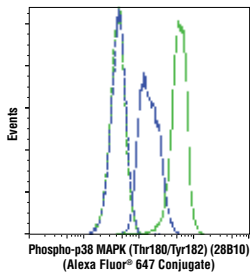
Growth and Stress

The p44/42 MAPK (Erk1/2) signaling pathway can be activated in response to a diverse range of extracellular stimuli including mitogens, growth factors, and cytokines. Utilize positive and negative control treatments such as TPA and U0126 to establish the range of Erk1/2 phosphorylation in your system, and quantify it accurately using phospho-Erk1/2 antibodies and conjugates.



Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204): Analysis of Jurkat cells, treated with U0126 (10 μ M, 2 hr; blue) or with TPA (12-O-Tetradecanoylphorbol-13-Acetate) (200 μ M, 30 min; green), using Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (197G2) Rabbit mAb (Pacific Blue™ Conjugate) (solid line) or concentration-matched Rabbit (DA1E) mAb IgG XP® Isotype Control (Pacific Blue™ Conjugate) (dashed line).

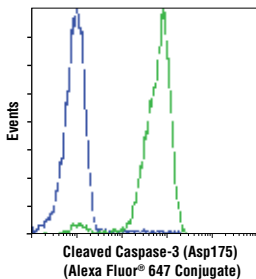
Phosphorylation of p38 is a key indicator of cellular stress. CST's antibodies against phospho-p38 deliver a sensitive, specific readout to assess the impact of your experimental treatments on the stress response, and can be used to monitor unintended cellular stress.



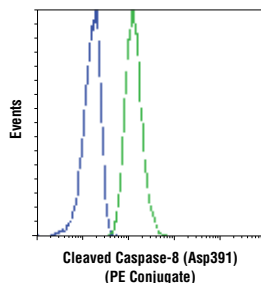
Phospho-p38 MAPK (Thr180/Tyr182): Analysis of THP-1 cells, untreated (blue) or treated with Anisomycin (25 μ g/ml, 30 min; green) using Phospho-p38 MAPK (Thr180/Tyr182) (28B10) Mouse mAb (Alexa Fluor® 647 Conjugate) (solid lines) or concentration-matched Mouse (MOPC-21) mAb IgG1 Isotype Control (Alexa Fluor® 647 Conjugate) (dashed lines).

Apoptosis

A critical step in apoptosis, or programmed cell death, is the cleavage and subsequent activation of caspase proteins. This can easily be quantified in your fixed samples with our cleavage-specific antibodies.



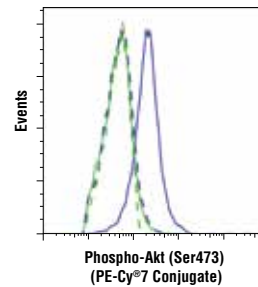
Cleaved Caspase-3 (Asp175): Analysis of Jurkat cells, untreated (blue) or treated with Etoposide (green), using Cleaved Caspase-3 (Asp175) (D3E9) Rabbit mAb (Alexa Fluor® 647 Conjugate).



Cleaved Caspase-8: Analysis of Jurkat cells, untreated (blue) or treated with Etoposide (green), using Cleaved Caspase-8 (Asp391) (18C8) Rabbit mAb (PE Conjugate).

Signaling

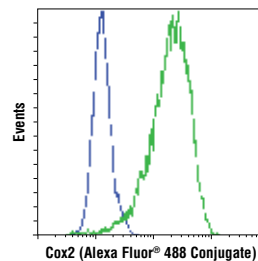
Akt is a crucial signaling node and protein kinase that actively controls survival and apoptosis. Many cellular functions, from T cell activation to nutrient level sensing, involve the phosphorylation or dephosphorylation of Akt. When utilized as a readout in heterogeneous populations analyzed by flow cytometry, phospho-Akt antibodies from CST empower researchers to rapidly discover subpopulations that exhibit varying cellular response to experimental stimuli.



Phospho-Akt (Ser473): Analysis of Jurkat cells, untreated (green) or treated with LY294002, Wortmannin, and U0126 (blue), using Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb (PE-Cy®7 Conjugate) (solid lines) or concentration-matched Rabbit (DA1E) mAb IgG XP® Isotype Control (PE-Cy®7 Conjugate) (dashed lines).

Cell Metabolism

Changes in expression of metabolic proteins may occur as the cell responds to specific stimuli, and this change can be easily quantified by flow cytometry. Cox2 expression is induced when cells encounter lipopolysaccharides from bacterial invaders.



Cox2: Analysis of Raw 264.7 cells, untreated (blue) or LPS-treated (1 μ g/ml, 24 hr; green), using Cox2 (D5H5) XP® Rabbit mAb (Alexa Fluor® 488 Conjugate).

For a full list of flow validated antibodies from CST visit cst-science.com/flow-products.

Antibodies Customized to Meet Your Needs

Conjugated antibodies

CST offers a range of primary antibodies directly conjugated with Alexa Fluor®, PE, APC, CyDye™, tandems, and other fluorophores, all optimized for flow cytometry assays. Conjugated antibodies are tested and validated by our in-house team of scientists for specificity, sensitivity, and reproducibility.

In addition, CST offers a range of fluorophore-conjugated secondary antibodies compatible with our primary antibodies. While direct staining with primary antibody conjugates is most commonly used for flow cytometry, indirect staining may also be used. Indirect staining requires two incubation steps, first with unlabeled primary antibody and second with a compatible fluorophore-conjugated secondary antibody.

Direct Versus Indirect Staining for Flow Cytometry

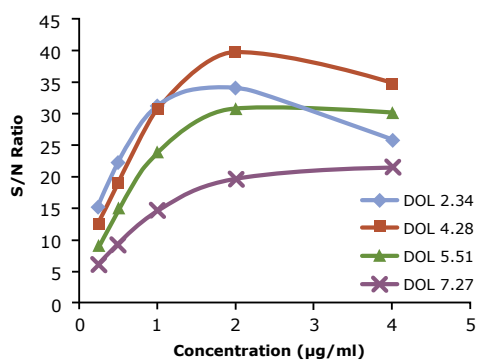
Method	Advantages
Direct: Directly conjugated antibodies cst-science.com/flow-conjugates	<ul style="list-style-type: none">• Direct multiplexing of antibodies from same host is possible• Faster protocol due to fewer incubation and wash steps
Indirect: Conjugated secondaries + unconjugated primary antibodies cst-science.com/flow-secondaries cst-science.com/flow-primaries	<ul style="list-style-type: none">• May improve detection of targets present in low abundance, as provides higher sensitivity due to secondary amplification (multiple secondary antibodies binding each primary antibody)• Useful for initial validation of a primary antibody when a direct conjugate is not yet available

Custom antibody conjugation services

Are you looking for a conjugated antibody that isn't in our catalog? CST makes custom antibody conjugates to meet your research needs.

- ▶ **Flexible Service Offerings:** Tiered options range from basic antibody conjugation to full validation and stability testing of conjugates
- ▶ **Validation:** Conjugates are tested in key applications using biologically relevant cell systems and controls
- ▶ **Optimization:** Flexible conjugation chemistry, removal of free dye and/or antibody purification, and identification of optimal degree of labeling (DOL) to ensure the best signal-to-noise ratio
- ▶ **Support:** Consultation and technical support from the same scientists who produce our commercially available conjugates

Degree of labeling to ensure specificity and maximal signal



Conjugation to certain fluorescent dyes (eg, Alexa Fluor® and Pacific Blue™) requires optimization by degree of labeling (DOL) testing to identify the optimal antibody:dye molecular ratio, resulting in conjugates with maximum specific signal intensity.

For more information on CST custom conjugation offerings, visit cst-science.com/flow-customs.

Companion Reagents for Flow Cytometry

CST provides a wide selection of products to support your flow cytometry protocols. In addition to antibodies directed at intracellular and signaling targets, this includes antibodies directed at extracellular markers, isotype control and secondary antibodies, cellular dyes, buffer reagents, and chemical activators and inhibitors. These reagents are also used in-house for antibody validation in flow cytometry analysis.

Immune cell phenotyping markers

Conjugated antibodies toward surface markers, such as CD markers, can be used independently or multiplexed with other antibodies to phenotype cells within heterogenous populations. For a full product listing, visit cst-science.com/flow-markers.

Isotype controls

Isotype controls are used to estimate nonspecific binding of primary antibodies due to Fc receptor binding or other protein-protein interactions. In order to provide meaningful comparison data, choose an isotype control antibody that has the same immunoglobulin type and fluorescent dye (if applicable) as the test antibody. To view a full product listing, visit cst-science.com/flow-isotypes.

Secondary antibodies

Fluorophore-conjugated secondary antibodies are available to support your flow cytometry assays. While direct conjugates are usually preferred, secondary antibodies may be used for indirect detection. Choose a secondary antibody that is directed against the host species of the primary antibody. To browse secondary antibodies, visit cst-science.com/flow-secondaries.

Activators and inhibitors

For signaling readouts, having positive and negative controls is essential to understand the range of biological response to stimuli. CST provides chemical modulators to enable you to assess the dynamic range of your system, which is useful for measuring changes in activation states and total protein expression. For more information, visit cst-science.com/activators-inhibitors.

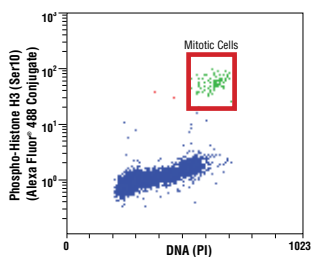
Buffers and reagents

Minimize experimental variables by choosing the same buffers and reagents used by our scientists during antibody validation and protocol optimization. For companion reagent product listings, visit cst-science.com/flow-buffers.

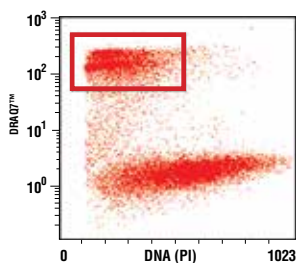
Cellular Dyes

Discrimination between live and dead cells is a critical step to exclude false positives in your cell populations when analyzing flow cytometry results. CST offers reagents to allow you to perform this discrimination in both live cell and fixed cell assays along with other cellular dyes.

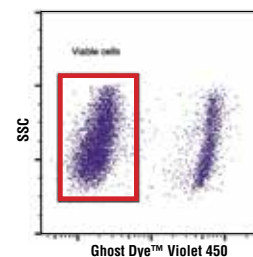
Visit cst-science.com/flow-dyes for more information.



Dead-Cell Staining: Analysis of Jurkat cells using Phospho-Histone H3 (Ser10) (D2C8) XP[®] Rabbit mAb (Alexa Fluor[®] 488 Conjugate) compared to propidium iodide (DNA content). The box indicates phospho-histone H3 positive cells.



Live-Cell Viability Staining: Analysis of live Jurkat cells treated with Staurosporine using DRAQ7™. Gated population represents DRAQ7™-positive apoptotic cells.



Fixed-Cell Viability Staining: Analysis of live and fixed/permeabilized human peripheral blood mononuclear cells, combined and stained with Ghost Dye™ Violet 450 Viability Dye. Viable gate is indicated.



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