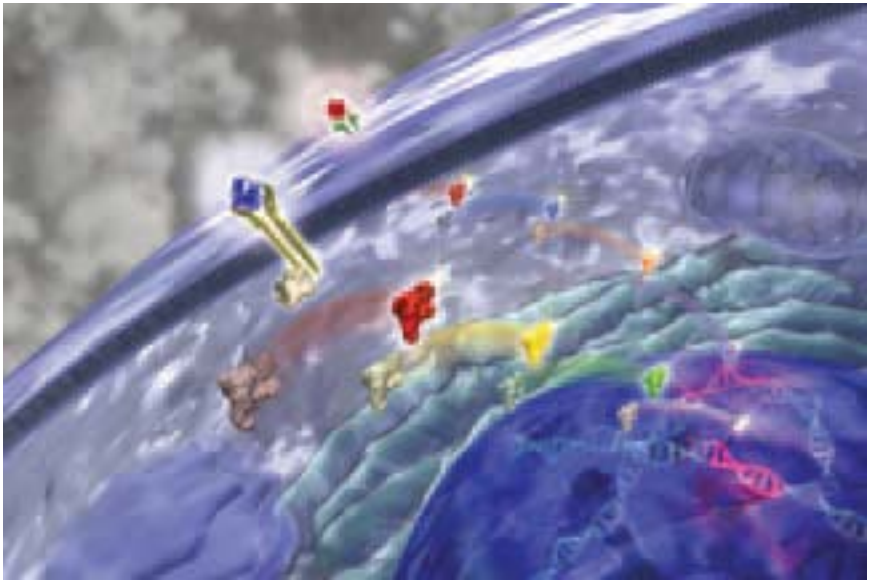
Techniques for Phospho Protein Analysis

Application Handbook 1st Edition



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Introduction to Phospho Protein Analysis

Phosphorylation of tyrosine, serine, and threonine residues is critical for the control of protein activity involved in various cellular events. An assortment of kinases and phosphatases regulate intracellular protein phosphorylation in many different cell signaling pathways, such as T and B cell signaling, those regulating apoptosis, growth and cell cycle control, plus those involved with cytokine, chemokine, and stress responses. Historically, phospho protein detection has been performed using techniques such as radiometric kinase assays and phosphoamino acid labeling. However, the advent of phospho specific antibodies has facilitated the use of more straightforward techniques such as Western blotting, immunoprecipitation, and immunofluorescence microscopy. These techniques, however, have several shortcomings in that they require a relatively large amount of sample, are time consuming, do not produce truly quantitative results, and are not conducive to multiparameter analysis.

Emerging technology from Garry Nolan, PhD at Stanford University has revolutionized historical approaches to phosphoprotein analysis. This technology combines phospho specific antibodies with the power of flow cytometry to enhance phospho protein study in ways not before possible. Flow cytometry requires only a small sample size and is ideal for performing rapid, quantitative, multiparameter analyses of single cells and distinct cell subpopulations. As a worldwide leader in flow cytometry reagents and analysis, BD Biosciences – Pharmingen has joined Dr. Nolan in a collaborative effort to make the technology and reagents available to the research community. Thus, we have launched BD™ Phosflow reagents to enable intracellular phosphoprotein analysis of single cells or subpopulations using flow cytometry.

Furthermore, we have extended this approach by employing flow cytometry using the BD™ Cytometric Bead Array (CBA) to analyze protein phosphorylation in cell and tissue lysates. The new BD™ CBA Flex Set system is an open and configurable system designed to be the easiest method of creating multiplex assays. With our proprietary conjugation chemistry, pair optimization strategies, and direct PE detection reagents, you can be assured consistent and superior assay performance in complex biological samples. Each antibody pair we develop is evaluated for dynamic range, sensitivity, and parallel titration to native biological samples. By avoiding a streptavidin-biotin-PE detection method employed by other assays, our direct PE detection reagents minimize the risk of increased background often caused by endogenous biotin in tissue- or cell lysate samples. Thus, BD CBA Flex Sets provide a reliable and flexible method for quantitative detection of multiple phospho proteins in a single lysate sample. BD CBA Flex Set beads can be used on any dual laser flow cytometer (BD FACSCalibur™, BD FACSCanto™, BD FACSAria™, BD™ LSR II, etc). However, the BD FACSArray™ bioanalyzer, which is a plate based, low cost, dual laser system, is paticularly well suited to this task.

With the addition of the instrument platforms BD™ Pathway and BD™ CARV II for automated life cell confocal imaging, BD Biosciences now also offers complete solutions for high content analysis of protein phosphorylation. High content cell imaging enables researchers to visualize protein phosphorylation in its microenvironment and to study protein protein interaction and translocation events simultaneously.

A large selection of BD Transduction Laboratories™ and BD Pharmingen™ high quality monoclonal antibodies has been evaluated for immunofluorescence microscopy and is the basis for new applications for this energizing and exiting platform technology.

BD Biosciences offers a variety of antibodies for the detection of phosphorylation motifs and produces quality antibodies that will meet and exceed your research needs, whether your experiment calls for Western blotting, immunoprecipitation, immunofluorescence microscopy, or flow cytometry. On the pages that follow, you'll find selected protocols and sample data that will help you to establish these exiting new technologies in your own laboratory.

Table 1. BD Biosciences Phospho Protein Profiling Technologies

	Intracellular Flow Cytometry	High Content Cell Imaging	Cytometric Bead Array	Western Blot & Immunoprecipitation
Sample Type	• Suspension and adherent cells	• Tissue, adherent cells, immobilized suspension cells	• Lysates from cells or tissues	• Lysates from cells or tissues
BD Biosciences Reagents	• BD Phosflow directly conjugated phospho antibodies	• BD Transduction Laboratories and BD Pharmingen phospho antibodies	• BD CBA Flex Sets	• BD Transduction Laboratories and BD Pharmingen phospho antibodies
BD Biosciences Instrumentation	• BD FACSArray™ Bioanalyzer • BD FACSCalibur™ • BD™ LSR II • BD FACSCanto™ • BD FACSAria™	• BD Pathway™ • BD™ CARV II	• BD FACSArray Bioanalyzer • BD FACSCalibur™ • BD™ LSR II • BD FACSCanto™ • BD FACSAria™	• NA
Key advantages	• Multiplexed activation state analysis on single cell level • Enables phenotyping of small subpopulations using validated cell surface markers • Small sample volume • 96 well plate compatible • 3 hours to results	• Multiplexed activation state analysis on single cell level • Enables (co-) localization of phosphorylation events within its subcellular context • Small sample volume • 96 well plate compatible • 3 hours to results	• Highly multiplexed • Quantification of phophorylation events • Small sample volume • 96 well plate compatible • 4 hours to results	• Provides molecular weight information which can compensate for less specific antibodies • Highly Multiplexed capabilities with BD Powerblot (>1000 proteins analyzed simultaneously)
Limitations	• Less applicable for tissues	• Sophisticated data acquisition and analysis required	• Subpopulation analysis requires cell sorting/purification	• Time consuming • Doesn't support higher throughput • Higher sample consumption • Semi-quantitative • Subpopulation analysis requires cell sorting/purification

Chapter 1

BD™ Phosflow Phosphorylation State Analysis in Single Cells

Introduction

Until recently, the study of intracellular signaling pathways in small cell populations was limited by the inflexibility of available techniques. Traditional methods such as Western blot, immunoprecipitation, and immunofluorescence microscopy did not allow researchers to correlate intracellular signaling events with discrete subpopulations of cells. Multi-parameter flow cytometry provides a flexible and powerful addition to these techniques. Flow cytometry is not only rapid, sensitive, and quantitative, but it also enables the differential evaluation of intracellular signaling events directly in complex primary samples such as whole blood or peripheral blood mononuclear cells (PBMCs). This is particularly useful for immune cell function analysis, where it is necessary to study intracellular signaling pathways in their most native context.

BD™ Phosflow reagents have been developed in collaboration with the laboratory of Garry Nolan at Stanford University. This cutting edge technology enables measurement of activation states of multiple proteins on a single cell level. By combining surface marker immunophenotyping and intracellular signal assessment, it is possible to obtain mechanistic and kinetic information of subset-specific signaling. By choosing BD Phosflow reagents you will obtain high quality antibodies conjugated to a variety of fluorophores facilitating three-, four-, five-, and six-color experiments. Understanding the kinetics of cellular responses based on kinase activity would be beneficial to many fields of immunology since cellular subset analysis is not possible by conventional techniques.

Furthermore, the complexity of cellular subsets within the largely attributed groups of T cells, B cells, NK cells, and monocytes illustrates that subsets can differentially function in immune responses as is the case with naïve and memory T cells. Thus, it is important to study intracellular signaling mechanisms both in isolation and within the context of other contacting cells.

For your convenience, BD Biosciences has validated directly labeled antibodies for multi-parameter analysis of phosphorylation events in T and B cells in human whole blood and PBMCs and mouse lymphoid organs.

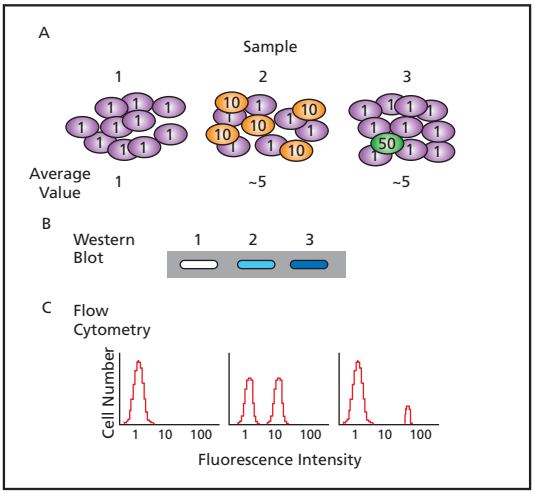

Figure 1. A theoretical experiment comparing Western blot and flow cytometry with three samples and a protein of interest at 1, 10, or 50 copies per cell. Sample 2 and 3 look the same via Western blot, but when stained with fluorescently labeled antibodies, the differences between the samples become more relevant. (Source: P.O. Krutzik *et al*. Clinical Immunology 110 (2004) 206–221)

WESTERN BLOT	FLOW CYTOMETRY
Population analysis Obtain average value of multiple cells	**Single cell analysis** Collect data for each individual cell
Homogeneous sample Limited to cultured or purified cells	**Heterogeneous cell types** Complex primary samples, such as immune cells
One parameter Obtain data sets individually	**Multi-parameter** Correlate multiple markers simultaneously
Large number of cells Requires *in vitro* derived cultures	**Small number rare cell subsets** Direct analysis of rare cell types
Time consuming for large sample sets Not amenable to large screening efforts	**Rapid and scalable** Performed in 96-well plates and in parallel
Protein size and antibody specificity Antibody selectivity for target is clearly visible	**Antibody must be validated** Antibody must have high affinity and selectivity

Table 1. Comparison of phospho specific flow cytometry and traditional Western blotting (Source: P.O. Krutzik *et al*. Clinical Immunology 110 (2004) 206–221)

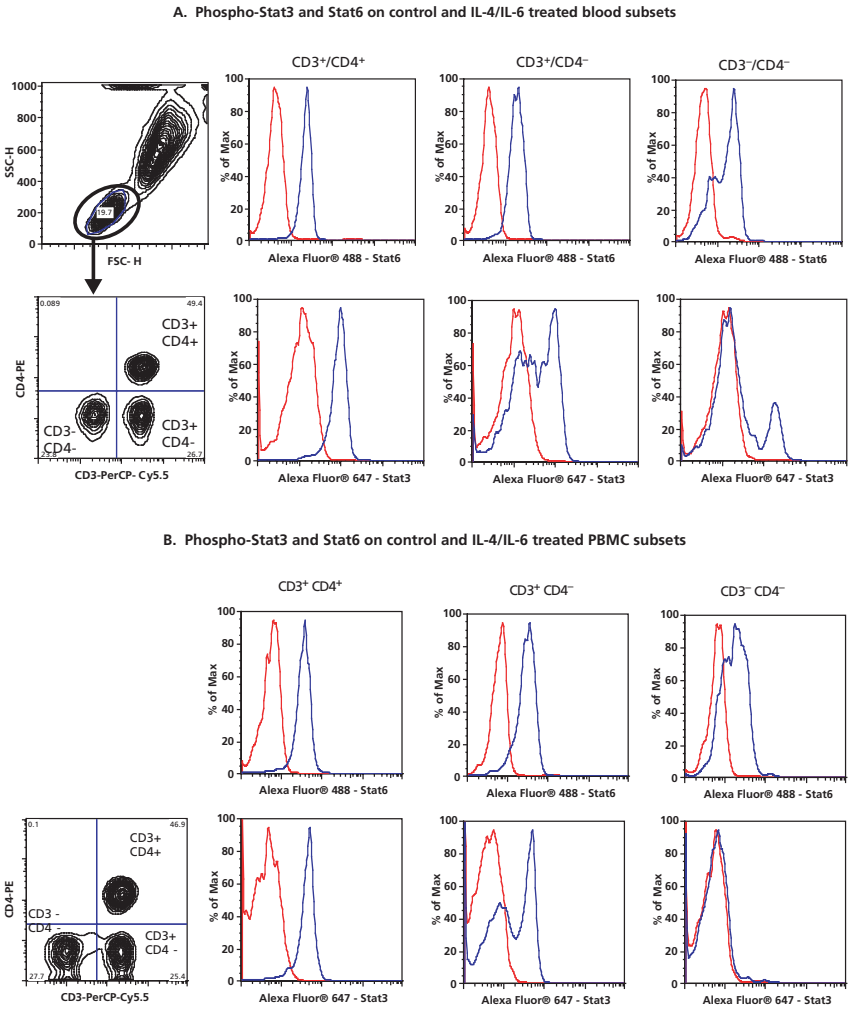

Figure 2. Multicolor analysis of human whole blood (A), or human PBMCs (B), stained with PerCP-Cy5.5 anti-human CD3, PE anti-human CD4, Alexa Fluor® 488 anti-Stat 6 (pY641), and Alexa Fluor® 647 anti-Stat 3 (pY705).

(A) Human whole blood was either left untreated (red histograms) or treated with IL-6 + IL-4 (blue histograms and CD3, CD4 contour plot), 100 ng/ml each for 15 minutes at 37°C. The cells were then fixed using BD™ Phosflow Lyse/Fix Buffer for 10 minutes at 37°C, and followed by Perm Buffer III for 30 minutes on ice. Lymphocytes were gated as shown in the scatter profile.

(B) Human PBMCs were either left untreated (red histograms) or treated with IL-6 + IL-4 (blue histograms and CD3, CD4 contour plot), 100 ng/ml each for 15 minutes at 37°C. The cells were then fixed using BD Cytofix™ Buffer for 10 minutes at 37°C, and followed by BD™ Phosflow Perm Buffer III for 30 minutes on ice.

In ***Figure 2*** we are comparing flow cytometry data obtained from PBMCs with results from whole blood samples. Human whole blood or PBMCs were stimulated with IL-4 and IL-6 for 15 min at 37°C, fixed and permeabilized, incubated with directly conjugated monoclonal antibodies against CD3, CD4, phosphorylated Stat6 and Stat3, and subsequently analyzed using a BD FACSCalibur™ flow cytometer (for detailed protocols please see pages 15–25).

As shown in ***Figure 2,*** Stat6 (pY641) phosphorylation is increased in all stimulated $CD3^+CD4^+$ cells (containing helper T cells) and $CD3^+CD4^-$ cells (containing cytotoxic T cells). In $CD3^-CD4^-$ cells (containing B cells, NK cells) we observed sub-populations with different levels of Stat6 (pY641) phosphorylation upon activation with IL-4. The results obtained are consistent in PBMCs and whole blood samples.

Stat3 (pY705) phosphorylation is increased consistently in all $CD3^+CD4^+$ cells. In $CD3^+CD4^-$ cells we detected different levels of Stat3 phosphorylation after activation with IL-6. In stimulated PBMCs, we did not see an increased Stat3 phosphorylation for $CD3^-CD4^-$ cells. However, when analyzing samples from whole blood, we identified a small population of $CD3^-CD4^-$ cells responding with an increased Stat3 (pY705) phosphorylation.

Using the BD Phosflow whole blood protocol enables phosphorylation-state analysis in single cells under the most native conditions possible *in vitro*, without involving time consuming and disturbing cell purification procedures.

In ***Figure 2*** we have shown how BD Phosflow technology can be used as a powerful tool to study phosphorylation events in complex primary samples like human whole blood or PBMCs.

Likewise, BD Phosflow mouse technology can be performed on target cells by using a combination of antibodies to phospho proteins and a wide variety of antibodies against well-defined mouse cell surface markers.

For multicolor staining of mouse target cells, we prefer to use a one-step procedure to process the cells, ie., to stimulate the whole mouse spleen cells prior to lysis of red cells. We found no significant difference in terms of surface and phospho staining intensity when comparing cells stimulated before or after red-cell lysis. However, the one-step procedure saves time and increases cell yield. It is virtually identical to the protocol described for human whole blood with only minor modifications. (see ***BD™ Phosflow Protocol for Mouse Cells*** pages 31–32)

Many cell surface markers are sensitive to fixation/permeabilization procedures and lose recognition by relevant antibodies afterwards. For your convenience, many antibodies conjugated to various fluorochromes have been tested under several conditions. The results are summarized on pages 26–30 for human and 33–35 for mouse.

An example of multicolor flow cytometric analysis of phosphorylation events in mouse leukocytes is shown in ***Figure 3.*** Spleen cells were stimulated for 15 minutes with mouse GM-CSF, IFN-γ, and IL-6 prior to lysis of red blood cells and processed according to the BD Phosflow Protocol III for Mouse Cells. They were blocked with BD FcBlock™ antibody (Cat. No. 553141 or 553142) for

15 minutes, and were then labeled with directly conjugated monoclonal antibodies against mouse CD45R/B220 (Cat. No. 557683), mouse CD11b (Cat. No. 550993), Stat3 (pY705) (Cat. No. 557814), and Stat5 (pY694) (Cat. No. 612567). As shown, Stat5 phosphorylation is mainly localized in the $CD11b^+$ $B220^-$ cell population. However, the increased phosphorylation of Stat3 is demonstrated in both $B220^+$ and $B220^-$ spleen cells.

In another example, mouse spleen cells were stimulated with LPS and PMA and were subsequently labeled with PerCP-Cy5.5 anti-mouse CD11b (Cat. No. 550993), Alexa Fluor® 488 anti-mouse CD45R/B220 (Cat. No. 557669), PE anti-p38 MAPK (pT180/pY182) (Cat. No. 612565), and Alexa Fluor 647® anti-ERK1/2 (p44) (pT202/pY204) (Cat. No. 612593). **Figure 4** demonstrates that phosphorylation of p38 MAPK occurs mainly in the $CD11b^+$ $B220^-$ cell population, while ERK1/2 is phosphorylated in $B220^+$ and $B220^-$ spleen cells.

Our third example shows the phosphorylation of ZAP70 and c-Cbl in thymocytes that were stimulated via the T cell receptor (TCR). Antibodies to mouse CD3ε (clone 145-2C11) (Cat. No. 553057) and CD4 (clone GK1.5) (Cat. No. 553726) were first allowed to bind to the cells for 30 minutes at 4°C. (Cross-linking of CD4 with the GK1.5 antibody activates the Src family kinase Lck that phosphorylates ZAP70.[12]) Then the 145-2C11 antibody bound to the TCR complex was cross-linked by the addition of purified anti-Hamster IgG Cocktail (Cat. No. 554009) for 15 minutes at 4°C. The antibody-coated cells were subsequently activated by incubation for 3-5 minutes at 37°C. Afterwards the cells were labeled with directly conjugated antibodies to CD4 (clone RM4-5, Cat. No. 557667), CD8a (Cat. No. 551162), ZAP70 (pY319) (Cat. No. 557817), and c-Cbl (pY700) (Cat. No. 558087). ***Figure 5*** demonstrates that ZAP70 and c-Cbl were phosphorylated in the $CD4^+$ $CD8a^+$ thymocytes that were stimulated by anti-CD3ε and anti-CD4 antibodies.

Overall, these examples demonstrate that the phosphorylation states of mouse leukocytes can be evaluated quickly and precisely by multicolor flow cytometry. This powerful technology will greatly facilitate our understanding of signaling mechanisms in leukocyte subpopulations.

BD Phosflow activation state analysis is not limited to leucocytes and other suspension cells. BD Biosciences has developed a protocol that enables the detection of phosphorylation events in adherent cells using intracellular flow cytometry. In the experiment shown in ***Figure 6,*** NIH3T3 cells were analyzed for Platelet Derived Growth Factor Receptor (PDGFR) phosphorylation. To our experience the trypsinization step used in this procedure to bring cells into suspension right after the appropriate stimulation does not effect the phosphorylation pattern in the cells tested.

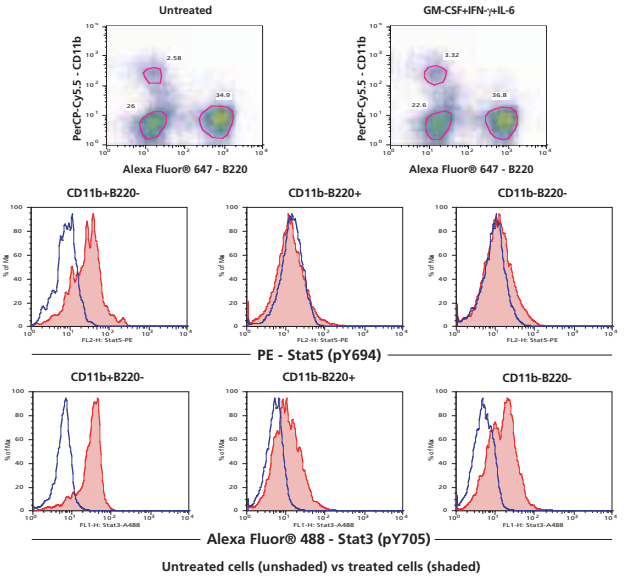

Figure 3. Multicolor Analysis of C57 BL/6 Spleen Cells Stained with PerCP-Cy5.5 anti-CD11b, PE anti-Stat5, Alexa Fluor® 488 anti-Stat3, and Alexa Fluor® 647 anti-CD45R/B220.

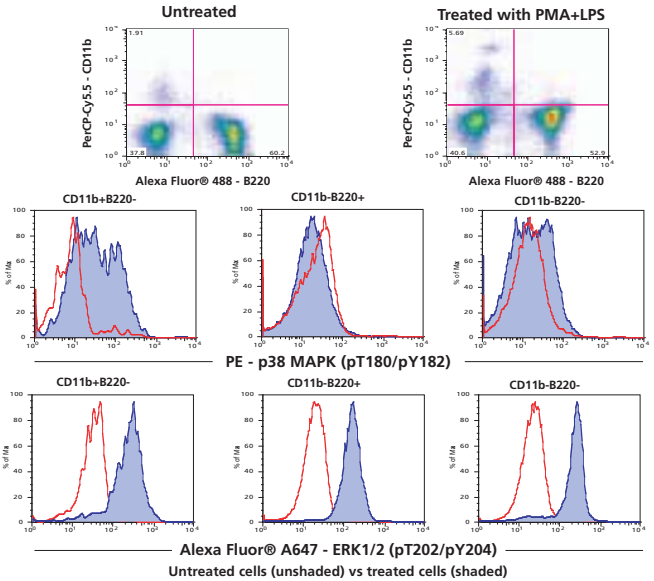

Figure 4. Multicolor Analysis of C57 BL/6 Spleen Cells Stained with PerCP-Cy5.5 anti-CD11b, PE anti-p38, Alexa Fluor® 488 anti-CD45R/B220, and Alexa Fluor® 647 anti-ERK1/2.

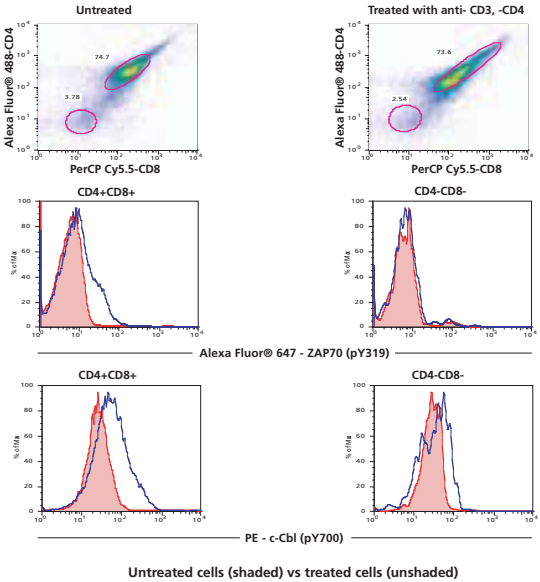

Figure 5. Multicolor analysis of C57 BL/6 thymocytes stimulated with anti-CD3 and anti-CD4.

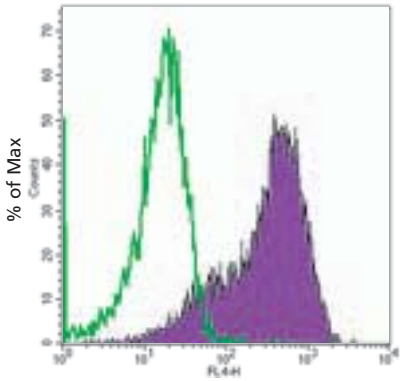

Figure 6. Flow Cytometric Analysis of Phospho-PDGF Receptor (Y857)-Alexa647: NIH3T3 cells (mouse embryonic fibroblast cells ATCC CRL-1658) were serum starved overnight, they were then either left unstimulated or stimulated with 200ng/ml of Platelet Derived Growth Factor (PDGF, Cat. No. 354051) at 37˚C for 2 min. Cells were fixed with BD Phosflow Fix Buffer I (Cat. No. 557870) at 37˚C for 10 min and permeabilized in BD Phosflow Perm Buffer III (Cat. No. 558050) on ice for at least 30 min and stained with 0.032 ug/test of Alexa Fluor®-Phospho-PDGFR(pY857) (clone J24-618, Cat. No. inquire). Cells were then analyzed by flow cytometry.

References

BD™ Phosflow technology and reagents have been developed in collaboration with the laboratory of Garry Nolan at Stanford University.

To learn more about how to apply intracellular flow cytometry for phospho protein analysis in single cells, please refer to the following selection of recent publications:

1. Sachs, K., Perez, O., Pe'er, D., Lauffenburger, D.A., Nolan, G.P. Causal Protein-Signaling Networks Derived from Multiparameter Single-Cell Data. *Science* 308:523-529 (2005).
2. Irish JM, Hovland R, Krutzik PO, Perez OD, Bruserud O, Gjertsen BT, Nolan GP. Single cell profiling of potentiated phospho protein networks in cancer cells. *Cell*. 2004 Jul 23; 118(2):217-28.
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13. Thien CB, Scaife RM, Papadimitriou JM, Murphy MA, Bowtell DD, Langdon WY. A mouse with a loss-of-function mutation in the c-Cbl TKB domain shows perturbed thymocyte signaling without enhancing the activity of the ZAP-70 tyrosine kinase. *J. Exp. Med.* 2003 Feb 17; 197(4):503-13.

BD™ Phosflow Protocols for human PBMCs

Protocol I (Detergent Method)

Fix Buffer used:

BD™ Phosflow Fix Buffer I (Cat. No. 557870)

Perm Buffer used:

BD™ Phosflow Perm/Wash Buffer I (Cat. No. 557885)

1. Prepare PBMCs from human blood.
2. Pre-warm the BD™ Phosflow Fix Buffer I in a 37°C water bath for 5–10 minutes before use.
3. (Optional) Culture PBMCs in RPMI with 5% human serum at 37°C in a CO_2 incubator for 2 hrs. (This step may help to improve the signal for some phospho proteins).
4. Treat cells with appropriate stimulators.

   Note: Methods of activation vary and should be determined by the researcher. Some examples tested in house are given on page 30. Visit **www.bdphosflow.com** for updates.)
5. Fix cells immediately in order to maintain phosphorylation state. Rather than spinning down the cells, we recommend fixing the cells by adding an equal volume of pre-warmed BD Phosflow Fix Buffer I to the cell suspension.
6. Incubate cells at 37°C for 10 minutes.
7. (The fixed cells can be frozen in –80°C directly at this step for later usage. The cells should be thawed at 37°C and washed immediately, to continue with the following procedure).
8. Pellet by centrifugation (300 × g) for 5–10 minutes and remove supernatant.
9. Wash the cells twice with BD Phosflow Perm/Wash Buffer I. Centrifuge at 300 × g for 5-10 minutes and remove supernatant.
10. Resuspend cells in BD Phosflow Perm/Wash Buffer I at 1×10^7 cells/ml.
11. Aliquot optimal concentrations of fluorochrome-conjugated antibodies to each tube, and add 100µl (1×10^6) of fixed and permeabilized cells.
12. Incubate at room temperature for 30 minutes in the dark.
13. Wash once with 2ml of BD Phosflow Perm/Wash Buffer I. Centrifuge at 300 × g for 5-10 minutes and remove supernatant. Resuspend the cells in 500µl of BD Pharmingen™ Stain Buffer (Cat. No. 554656) prior to flow cytometric analysis.

BD™ Phosflow Protocols for Human PBMCs

Protocol II and III (Mild or Harsh Alcohol Method)

Fix Buffer used:

BD Cytofix™ Buffer (Cat. No. 554655)

Perm Buffer used:

Depending on the surface markers and phospho antibody conjugates you are using, either BD™ Phosflow Perm Buffer II (Cat. No. 558052) or III (Cat. No. 558050) is recommended. Please refer to our phospho antibody and CD marker reference charts (pages 26–30) for details.

1. Prepare PBMCs from human blood.
2. Pre-warm the BD Cytofix™ Buffer in a 37°C water bath for 5-10 minutes before use.
3. (Optional) Culture PBMCs in RPMI with 5% human serum at 37°C in a CO_2 incubator for 2 hrs. (This step may help to improve the signal for some phospho proteins).
4. Treat cells with appropriate stimulators.

   Note: Methods of activation vary and should be determined by the researcher. Some examples tested in house are given on page 30. Visit **www.bdphosflow.com** for updates.
5. Fix cells immediately in order to maintain phosphorylation state. Rather than spinning down the cells, we recommend fixing the cells by adding an equal volume of pre-warmed BD Cytofix Buffer to the cell suspension.
6. Incubate cells at 37°C for 10 minutes.
7. (The fixed cells can be frozen in –80°C directly at this step for later usage. The cells should be thawed at 37°C, and washed immediately, to continue with the following procedure).
8. Pellet by centrifugation (300 × g) for 5–10 minutes and remove supernatant.
9. Vortex or mix to disrupt the pellet. Permeabilize the cells by adding 1ml of BD™ Phosflow Perm Buffer (for 1-10×10^6 cells) and incubating for 30 minutes on ice. Depending on the surface markers and phospho specific antibody conjugates you are using, either BD Phosflow Perm Buffer II (Cat. No. 558052) or III (Cat. No. 558050) is recommended. Please refer to our phospho antibody and CD marker reference charts (pages 26–30) for details.

   Note: Longer incubation times in this permeabilization buffer may decrease the signal intensity of surface marker staining.
10. Wash the cells twice with BD Pharmingen™ Stain Buffer (Cat. No. 554656). Centrifuge at 300 × g for 5-10 minutes and remove supernatant.

11. Resuspend the cells in BD Pharmingen™ Stain Buffer at 1×10^7 cells/ml
12. Aliquot optimal concentration of fluorochrome-conjugated antibodies to each tube, and add 100µl (1×10^6) of fixed and permeabilized cells.
13. Incubate at room temperature for 30 minutes in the dark.
14. Wash once with 2ml of BD Pharmingen™ Stain Buffer. Centrifuge at 300 × g for 5-10 minutes and remove supernatant. Resuspend the cells in 500µl of the same buffer prior to flow cytometric analysis.

BD™ Phosflow Protocols for CD3/CD28 Activation of Human PBMCs

Protocol I (Detergent Method)

Fix Buffer used:

BD™ Phosflow Fix Buffer I (Cat. No. 557870)

Perm Buffer used:

BD™ Phosflow Perm/Wash Buffer I (Cat. No. 557885)

1. Prepare PBMCs from human blood.
2. Pre-warm the BD™ Phosflow Fix Buffer I in a 37°C water bath for 5-10 minutes before use.
3. (Optional) Culture the PBMCs in RPMI with 5% human serum at 37°C in a CO_2 incubator for 2 hrs. (This step may help to improve the signal for some phospho proteins).
4. Resuspend the PBMCs in cold PBS with 1%FCS (1×10^6cells/50µl), add anti-CD3 (Cat. No. 555329) and anti-CD28 (Cat. No. 555725, 1µg each for 1×10^6 cells), and incubate on ice for 20 minutes.
5. Wash the cells with cold PBS with 1%FCS, and spin at 4°C (250 × g) for 5 minutes.
6. Resuspend the PBMCs in cold PBS with 1%FCS (1×10^6 cells/50µl), add goat anti-mouse Ig (2ug of Cat. No 553998 for 1×10^6 cells), and incubate on ice for 20 minutes.
7. Wash the cells with cold PBS with 1%FCS, spin at 4°C (250 × g) for 5 minutes.
8. Resuspend the cells in pre-warmed (37°C) PBS with 1%FCS (1×10^6 cells/50µl), incubate at 37°C for 2-5 minutes (The incubation time depends on the phospho protein tested and should be determined by the researcher).

9. Fix cells immediately in order to maintain phosphorylation state. Rather than spinning down the cells, we recommend fixing the cells by adding an equal volume of pre-warmed (37°C) BD Phosflow Fix Buffer I (Cat. No. 557870) to the cell suspension.
10. Incubate cells at 37°C for 10 minutes.
11. (The fixed cells can be frozen in –80°C directly at this step for later usage. The cells should be thawed at 37°C, and washed immediately, to continue with the following procedure).
12. Pellet the cells by centrifugation (300 × g) for 5–10 minutes and remove supernatant.
13. Wash the cells twice with BD™ Phosflow Perm/Wash Buffer I. Centrifuge at 300 × g for 5-10 minutes and remove supernatant.
14. Resuspend the cells in BD Phosflow Perm/Wash Buffer I at 1×10^7 cells/ml.
15. Add 10µg of normal mouse Ig for each 100µl of cells. Incubate at room temperature for 10 minutes.
16. Aliquot optimal concentrations of fluorochrome-conjugated antibodies to each tube and add 100µl of the fixed and permeabilized cells.
17. Incubate at room temperature for 30 minutes in the dark.
18. Wash once with 2ml of BD Phosflow Perm/Wash Buffer I. Centrifuge at 300 × g for 5-10 minutes and remove supernatant. Resuspend the cells in 500µl of BD Pharmingen™ Stain Buffer (Cat. No. 554656) prior to flow cytometric analysis.

BD™ Phosflow Protocols for CD3/CD28 Activation of Human PBMCs

Protocol II and III (Mild or Harsh Alcohol Method)

Fix Buffer used:

BD Cytofix™ Buffer (Cat. No. 554655)

Perm Buffer used:

Depending on the surface markers and phospho antibody conjugates you are using, either BD™ Phosflow Perm Buffer II (Cat. No. 558052) or III (Cat. No. 558050) is recommended. Please refer to our phospho antibody and CD marker reference charts (page 26–30) for details.

1. Prepare PBMCs from human blood.
2. Pre-warm the BD Cytofix™ Buffer in a 37°C water bath for 5-10 minutes before use.
3. (Optional) Culture the PBMCs in RPMI with 5% human serum at 37°C in a CO_2 incubator for 2 hrs. (This step may help to improve the signal for some phospho proteins).
4. Resuspend the PBMCs in cold PBS with 1%FCS (1×10^6 cells/50µl), add anti-CD3 (Cat. No. 555329) and anti-CD28 (Cat. 555725, 1µg each for 1×10^6 cells), and incubate on ice for 20 minutes.
5. Wash the cells with cold PBS, and spin at 4°C (250 × g) for 5 minutes.
6. Resuspend the PBMCs in cold PBS with 1%FCS (1×10^6 cells/50µl), add goat anti mouse Ig (2ug of Cat. No 553998 for 1×10^6 cells), and incubate on ice for 20 minutes.
7. Wash the cells with cold PBS with 1%FCS, and spin at 4°C (250 × g) for 5 minutes.
8. Resuspend the cells in pre-warmed (37°C) PBS with 1%FCS (1×10^6 cells/50µl), incubate at 37°C for 2-5 minutes (The incubation time depends on the phospho protein tested and should be determined by the researcher).
9. Fix the cells immediately in order to maintain phosphorylation state. Rather than spinning down the cells, we recommend fixing the cells by adding an equal volume of pre-warmed (37°C) BD Cytofix Buffer to the cell suspension.
10. Incubate the cells at 37°C for 10 minutes.
11. (The fixed cells can be frozen in –80°C directly at this step for later usage. The cells should be thawed at 37°C, and washed immediately, to continue with the following procedure).
12. Pellet by centrifugation (300 × g) for 5–10 minutes and remove supernatant.

9. Vortex or mix to disrupt the pellet. Permeabilize the cells by adding 1ml of BD™ Phosflow Perm Buffer (for 1-10×10^6 cells) and incubating for 30 minutes on ice. Depending on the surface markers and phospho specific antibody conjugates you are using, either BD Phosflow Perm Buffer II (Cat. No. 558052) or III (Cat. No. 558050) is recommended. Please refer to our phospho antibody and CD marker reference charts (pages 26–30) for details.

   Note: Longer incubation times in this permeabilization buffer may decrease the signal intensity of surface marker staining.

14. Wash the cells twice with BD Pharmingen™ Stain Buffer (Cat. No. 554656). Centrifuge the cells at 300 × g for 5-10 minutes and remove supernatant.
15. Resuspend the cells in BD Pharmingen™ Stain Buffer at 1×10^7 cells/ml.
16. Add 10µg of normal mouse Ig for each 100µl of cells. Incubate the cells at room temperature for 10 minutes.
17. Aliquot optimal concentrations of fluorochrome-conjugated antibodies to each tube, and add 100µl of the fixed and permeabilized cells.
18. Incubate at room temperature for 30 minutes in the dark.
19. Wash once with 2ml of BD Pharmingen™ Stain Buffer. Centrifuge at 300 × g for 5-10 minutes and remove the supernatant. Resuspend the cells in 500µl of the same buffer prior to flow cytometric analysis.

BD™ Phosflow Protocols for Human Whole Blood Samples

Protocol I (Detergent Method)

Lyse/Fix Buffer used:

BD™ Phosflow Lyse/Fix Buffer (Cat. No. 558049)

Perm Buffer used:

BD™ Phosflow Perm/Wash Buffer I (Cat. No. 557885)

1. Collect normal human blood in the presence of anticoagulant.
2. Dilute 5× BD™ Phosflow Lyse/Fix Buffer to 1× with distilled water. Pre-warm the 1× BD Phosflow Lyse/Fix Buffer in a 37°C water bath for 5-10 minutes before use.
3. Treat the blood cells with appropriate stimulators.

   Note: Methods of activation vary and should be determined by the researcher. Some examples tested in house are given on page 30. Visit **www.bdphosflow.com** for updates.
4. Fix the cells immediately in order to maintain phosphorylation state. Rather than spinning down the cells, we recommend fixing the cells by mixing one volume of blood with 20 volumes of the pre-warmed 1× BD Phosflow Lyse/Fix Buffer.
5. Incubate the cells at 37°C for 10 minutes.
6. (The fixed cells can be frozen in –80°C directly at this step for later usage. The cells should be thawed at 37°C and washed immediately, to continue with the following procedure).
7. Pellet the cells by centrifugation (300 × g) for 5–10 minutes and remove the supernatant.
8. Permeabilize the cells by adding 1ml of BD™ Phosflow Perm/Wash Buffer I (for 1-10×10^6 cells) and incubating for 10 minutes at room temperature.
9. Pellet the cells by centrifugation (300 × g) for 5–10 minutes and remove the supernatant.
10. Wash the cells once with BD Phosflow Perm/Wash Buffer I. Centrifuge the cells at 300 × g for 5-10 minutes and remove the supernatant.
11. Resuspend the cells derived from 100-300ml of blood in 100ml of BD Phosflow Perm/Wash Buffer I.
12. Aliquot optimal concentrations of fluorochrome-conjugated antibodies to each tube, and add 100µl of cells.
13. Incubate the cells at room temperature for 30 minutes in the dark.
14. Wash once with 2ml of BD Phosflow Perm/Wash Buffer I. Centrifuge at 300 × g for 5-10 minutes and remove supernatant. Resuspend the cells in 500µl of BD Pharmingen™ Stain Buffer (Cat. No. 554656) prior to flow cytometric analysis.

BD™ Phosflow Protocols for Human Whole Blood Samples

Protocol II and III (Mild or Harsh Alcohol Method)

Lyse/Fix Buffer used:

BD™ Phosflow Lyse/Fix Buffer (Cat. No. 558049)

Perm Buffer used:

Depending on the surface markers and phospho antibody conjugates you are using, either BD™ Phosflow Perm Buffer II (Cat. No. 558052) or III (Cat. No. 558050) is recommended. Please refer to our phospho antibody and CD marker reference charts (pages 26–30) for details.

1. Collect normal human blood in the presence of anticoagulant.
2. Dilute 5× BD™ Phosflow Lyse/Fix Buffer to 1× with distilled water. Pre-warm the 1× BD Phosflow Lyse/Fix Buffer in a 37°C water bath for 5-10 minutes before use.
3. Treat the blood cells with appropriate stimulators.

   Note: Methods of activation vary and should be determined by the researcher. Some examples tested in house are given on page 30. Visit **www.bdphosflow.com** for updates.
4. Fix the cells immediately in order to maintain phosphorylation state. Rather than spinning down the cells, we recommend fixing the cells by mixing one volume of blood with 20 volumes of the pre-warmed 1× BD Phosflow Lyse/Fix Buffer.
5. Incubate the cells at 37°C for 10 minutes.
6. (The fixed cells can be frozen in –80°C directly at this step for later usage. The cells should be thawed at 37°C and washed immediately to continue with the following procedure).
7. Pellet the cells by centrifugation (300 × g) for 5–10 minutes and remove the supernatant.
8. Wash the cells once with PBS, and pellet by centrifugation (300 × g) for 5–10 minutes and remove the supernatant.
9. Vortex or mix to disrupt the pellet. Permeabilize the cells by adding 1ml of BD™ Phosflow Perm Buffer (for 1-10×10^6 cells) and incubating for 30 minutes on ice. Depending on the surface markers and phospho specific antibody conjugates you are using, either BD Phosflow Perm Buffer II (Cat. No. 558052) or III (Cat. No. 558050) is recommended. Please refer to our phospho antibody and CD marker reference charts (pages 26–30) for details.

   Note: Longer incubation times in this permeabilization buffer may decrease the signal intensity of surface marker staining.
10. Wash the cells twice with BD Pharmingen™ Stain Buffer (Cat. No. 554656). Centrifuge at 300 × g for 5–10 minutes and remove supernatant.

11. Resuspend the cells derived from 100-300ml of blood in 100ml of BD Pharmingen™ Stain Buffer.
12. Aliquot optimal concentrations of fluorochrome-conjugated antibodies to each tube, and add 100µl of cells.
13. Incubate at room temperature for 30 minutes in the dark.
14. Wash once with 2ml of BD Pharmingen™ Stain Buffer. Centrifuge at 300 × g for 5-10 minutes and remove supernatant. Resuspend the cells in the same buffer prior to flow cytometric analysis.

BD™ Phosflow Protocols for CD3 Activation of Human Whole Blood

Protocol I (Detergent Method)

Lyse/Fix Buffer used:

BD™ Phosflow Lyse/Fix Buffer (Cat. No. 558049)

Perm Buffer used:

BD™ Phosflow Perm/Wash Buffer I (Cat. No. 557885)

1. Collect normal human blood in the presence of heparin.
2. Dilute 5x BD™ Phosflow Lyse/Fix Buffer to 1× with distilled water. Pre-warm the 1× BD Phosflow Lyse/Fix Buffer in a 37°C water bath for 5-10 minutes before use.
3. Add anti-CD3 (Cat. No. 555329), 1µg for 100µl whole blood, incubate on ice for 20 minutes.
4. Wash the cells with cold PBS with 1%FCS, and spin at 4°C (250 × g) for 5 minutes.
5. Carefully remove the supernatant.
6. Add goat anti-mouse Ig (1ug of Cat. No 553998 for 100µl whole blood), and incubate the cells on ice for 20 minutes.
7. Transfer the tube to 37°C water bath for 2-5 minutes. (The incubation time depends on the phospho protein tested and should be determined by the researcher).
8. Fix the cells immediately in order to maintain phosphorylation state. Rather than spinning down the cells, we recommend fixing the cells by mixing one volume of blood with 20 volumes of the pre-warmed 1× BD Phosflow Lyse/Fix Buffer.
9. Incubate the cells at 37°C for 10 minutes.

10. (The fixed cells can be frozen in –80°C directly at this step for later usage. The cells should be thawed at 37°C, washed immediately, and continue with the following procedure).
11. Pellet the cells by centrifugation (300 × g) for 5–10 minutes and remove the supernatant.
12. Permeabilize the cells by adding 1ml of BD™ Phosflow Perm/Wash Buffer I (for 1-10×10^6 cells) and incubating for 10 minutes at room temperature.
13. Pellet the by centrifugation (300 × g) for 5–10 minutes and remove the supernatant.
14. Wash the cells once with BD Phosflow Perm/Wash Buffer I. Centrifuge at 300 × g for 5-10 minutes and remove supernatant.
15. Resuspend the cells derived from 100-300ml of blood in 100ml of BD Phosflow Perm/Wash Buffer I.
16. Add 10µg of normal mouse Ig for each 100µl of cells. Incubate at room temperature for 10 minutes.
17. Aliquot optimal concentrations of fluorochrome-conjugated antibodies to each tube, and add 100µl of cells from step 16.
18. Incubate at room temperature for 30 minutes in the dark.
19. Wash once with 2ml of BD Phosflow Perm/Wash Buffer I. Centrifuge at 300 × g for 5-10 minutes and remove supernatant. Resuspend the cells in 500µl of BD Pharmingen™ Stain Buffer (Cat. No. 554656) prior to flow cytometric analysis.

BD Phosflow protocols for CD3 Activation of Human Whole Blood

Protocol II and III (Mild or Harsh Alcohol Method)

Lyse/Fix Buffer used:

BD™ Phosflow Lyse/Fix Buffer (Cat. No. 558049)

Perm Buffer used:

Depending on the surface markers and phospho specific antibody conjugates you are using, either BD™ Phosflow Perm Buffer II (Cat. No. 558052) or III (Cat. No. 558050) is recommended. Please refer to our phospho antibody and CD marker reference charts (pages 26–30) for details.

1. Collect normal human blood in the presence of heparin.
2. Dilute 5× BD™ Phosflow Lyse/Fix Buffer to 1× with distilled water. Pre-warm the 1× BD Phosflow Lyse/Fix Buffer in a 37°C water bath for 5-10 minutes before use.
3. Add anti-CD3 (Cat. No. 555329), 1µg for 100µl whole blood, and incubate on ice for 20 minutes.

4. Wash the cells with cold PBS, and spin at 4°C (250 × g) for 5 minutes.
5. Carefully remove the supernatant.
6. Add goat anti-mouse Ig (1µg for Cat. No. 553998 for 100µl whole blood), and incubate on ice for 20 minutes.
7. Transfer the tube to 37°C water bath for 2-5 minutes. (The incubation time depends on the phospho protein tested and should be determined by the researcher).
8. Fix the cells immediately in order to maintain phosphorylation state. Rather than spinning down the cells, we recommend fixing the cells by mixing one volume of blood with 20 volumes of the pre-warmed 1× BD Phosflow Lyse/Fix Buffer.
9. Incubate the cells at 37°C for 10 minutes.
10. (The fixed cells can be frozen in –80°C directly at this step for later usage. The cells should be thawed at 37°C and washed immediately, to continue with the following procedure).
11. Pellet the cells by centrifugation (300 × g) for 5–10 minutes and remove supernatant.
12. Wash the cells once with PBS, and pellet by centrifugation (300 ×g) for 5–10 minutes and remove the supernatant.
13. Vortex or mix to disrupt the pellet. Permeabilize the cells by adding 1ml of BD™ Phosflow Perm Buffer (for 1-10×10^6 cells) and incubating for 30 minutes on ice. Depending on the surface markers and phospho specific antibody conjugates you are using, either BD Phosflow Perm Buffer II (Cat. No. 558052) or III (Cat. No. 558050) is recommended. Please refer to our phospho antibody and CD marker reference charts (pages 26–30) for details.

    Note: Longer incubation times in this permeabilization buffer may decrease the signal intensity of surface marker staining.
14. Pellet the by centrifugation (300 × g) for 5–10 minutes and remove the supernatant.
15. Wash the cells twice with BD Pharmingen™ Stain Buffer (Cat. No. 554656). Centrifuge at 300 × g for 5-10 minutes and remove supernatant.
16. Resuspend the cells derived from 100-300ml of blood in 100ml of BD Pharmingen Stain Buffer.
17. Add 10µg of normal mouse Ig for each 100µl of cells. Incubate the cells at room temperature for 10 minutes.
18. Aliquot optimal concentrations of fluorochrome-conjugated antibodies to each tube, and add 100µl of fixed and permeabilized cells from step 17.
19. Incubate the cells at room temperature for 30 minutes in the dark.
20. Wash once with 2ml of BD Pharmingen Stain Buffer. Centrifuge at 300 × g for 5-10 minutes and remove supernatant. Resuspend the cells in 500µl of the same buffer prior to flow cytometric analysis.

Recommended Protocols for Phospho Protein Detection in Human Cells

Depending on subcellular localization of the phospho protein of interest, as well as epitope susceptibility to cell fixing and permeabilizing agents, we recommend different BD™ Phosflow protocols for our antibodies.

For phenotyping of cell populations in multi color experiments, please use the table on pages 27–30, showing our human cell surface markers tested under different fixation and permeabilization conditions.

Protocol			I	II	III
Fix buffers used for PBMCs:			BD Phosflow Fix Buffer I (557870)	BD Cytofix Buffer (554655)	BD Cytofix Buffer (554655)
Fix buffers used for Whole Blood:			BD Phosflow Lyse/Fix Buffer (558049)	BD Phosflow Lyse/Fix Buffer (558049)	BD Phosflow Lyse/Fix Buffer (558049)
Perm buffers used:			BD Phosflow Perm/Wash I Buffer (557885)	BD Phosflow Perm II Buffer (558052)	BD Phosflow Perm III Buffer (558050)
Specificity	**Clone**	**Fluorochrome**			
ERK 1/2 (pT202/pY204)	20a	Alexa 488	+	+	+
		Alexa 647	+	+	+
		PE	+	+	+
LCK (pY505)	4/Lck-Y505	Alexa 488	+	+	+
		PE	+	+	+
p38 MAPK (pT180/pY182)	36	Alexa 488	+	+	+
		Alexa 647	+	+	+
		PE	+	+	+
ZAP70 (pY319)/SYK (pY352)	17a	Alexa 488		+	+
		Alexa 647		+	+
		PE	+	+	+
C-Cbl (pY700)	47/c-Cbl (Y700)	Alexa 488		+	+
		Alexa 647			+
		PE	+	+	+
Stat1 (pY701)	4a	Alexa 488	-	+/-	+
		Alexa 647	-	+/-	+
		PE	+/-	+	+
Stat3 (pY705)	4	Alexa 488	-	+/-	+
		Alexa 647	-	+/-	+
		PE	-	+	+
Stat3 (pY727)	49	Alexa 488	?	?	?
		Alexa 647	?	?	?
Stat4 (pY693)	38/p-Stat4	Alexa 488	-	-	+
		Alexa 647			
		PE	-	-	+
Stat5 (pY694)	47	Alexa 488	-	+/-	+
		Alexa 647	-	+/-	+
		PE	-	+	+
Stat6 (pY641)	18	Alexa 488	-	+/-	+
		Alexa 647			+/-
		PE	-	+	+

Validated Human Cell Surface Markers for BD™ Phosflow Analysis

Many cell surface markers are sensitive to fixation/permeabilization procedures and lose recognition by relevant antibodies afterwards. For your convenience, many antibodies conjugated to various fluorochromes have been tested under several conditions. The results are summarized below.

Protocol			I	II	III
Fix buffers used for PBMCs:			BD Phosflow Fix Buffer I (557870)	BD Cytofix Buffer (554655)	BD Cytofix Buffer (554655)
Fix buffers used for Whole Blood:			BD Phosflow Lyse/Fix Buffer (558049)	BD Phosflow Lyse/Fix Buffer (558049)	BD Phosflow Lyse/Fix Buffer (558049)
Perm buffers used:			BD Phosflow Perm/Wash I Buffer (557885)	BD Phosflow Perm II Buffer (558052)	BD Phosflow Perm III Buffer (558050)
Specificity	**Clone**	**Fluorochrome**			
CD3	SK7	PE	+	+	+
		PerCP	+	+	+/-
		PerCP-Cy5.5	+	+	+
		PE-Cy7	+	+	+/-
		APC-Cy7		+	
CD3	UCHT1	FITC	+	+	+
		PE	+	+	+
		APC	+	+	+
		PerCP-Cy5.5	+	+	+
		Alexa 488	+	+	+
		Alexa 647	+	+	+
		Alexa 700		+	
		Pacific Blue		+	+
CD3	HIT3a	FITC		-	-
		PE		-	-
		APC		-	-
CD4	L200	FITC	+	+	+
		PE	+	+	+
		PerCP	+	+	+
		PerCPCy5.5	+	+	+
CD4	RPA-T4	PE	+	+	+
		APC	+	+	+
		FITC	+	+	+
		Alexa 488	+	+	+
		Alexa 647	+	+	+
		Pacific Blue		+	+
CD4	SK3	PerCP	+	+	+
		PerCP-Cy5.5	+	+	+
		PE-Cy7	+	+	+
CD5	UCHT2	PE	+	+	+
		APC	+	+/-	-
CD8	HIT8a	FITC	+	+	+
		PE	+	+	+

Specificity	Clone	Fluorochrome	I	II	III
CD8	RPA-T8	FITC	+	+	+
		PE	+	+	+
		APC	+	+	+
		PE-Cy7	+	+	-
		APC-Cy7		+	
		Alexa 488	+	+	+
		Alexa 647	+	+	+
		Alexa 700		+	
		Pacific Blue		+	+
CD8	SK1	PE	+	+	+
		PerCP	+	+/-	
		PerCP-Cy5.5	+	+	+
CD11a/LFA-1	G43-25B	PE	+	+/-	-
CD11a/LFA-1	Hi111	PE	+	+/-	-
		APC	+	-	-
CD11b	ICRF44	PE	+	+/-	+/-
		APC	+	-	-
		PE-Cy7	+	-	
		Alexa 488	+		
		Alexa 647	+		
CD11c	BLy6	PE	+	+/-	+/-
		APC	+	+/-	+/-
CD13	WM-15	PE		+/-	+/-
CD14	M5E2	FITC	+	+/-	-
		PE***	+	+/-	-
		APC	+	+/-	-
		PerCP-Cy5.5	+		
		PE-Cy7	+	-	
		Alexa 488***	+	+/-	
CD14	MoP9	FITC***			-
		PE***	+	-	-
CD15	W6D3	PE		+	+
CD15	H198	PE		+	+
CD16	3G8	PE	+	+/-	-
		FITC	+/-	+/-	
		PE-Cy7	+	-	-
		Alexa 488	+/-		
		Alexa 647	+		
CD16	B73.1	PE***	+	+/-	-
CD16	NKP15	FITC		-	-
CD18	6.7	PE	+	+	+
		APC	+	+	+
CD19*	HIB19	PE***	+	+/-	-
		APC	+	+/-	-
		FITC	+/-	+/-	
		PerCP-Cy5.5	+/-		
		PE-Cy7	-		
		Alexa 488***	-		
		Alexa 647***	+/-		
CD19	SJ25C1	PE***	+	+/-	-
		PE-Cy7	+/-	-	-
CD20	2H7	FITC	+/-	-	-
		PE	+	-	-
		APC	+		
CD20	L27	PE	-	-	-

Specificity	Clone	Fluorochrome	I	II	III
CD20 (I/C)	H1(FB1)	PerCP-Cy5.5	+	+	+
		Alexa 488	+	+	+
		Alexa 647	+	+	+
CD23	M-L233	PE	+	+/-	-
CD25	M-A251	FITC		+	+
		PE	+	+	+
		APC	+	+	+
CD27	L128	FITC	+	+	+
		PE	+	+	+
		APC	+	+	+
CD27	MT271	PE	-	-	-
CD28	CD28.2	FITC		-	-
		PE		-	-
		APC		-	-
CD31	WM59	PE	+	+/-	+/-
CD33	WM53	PE	+	+/-	+/-
CD33	p67.6	PE		+/-	+/-
CD34	8G12	PE	+	+/-	-
CD34	581	PE	+	+	+
		APC	+	+/-	-
CD38	HB7	FITC	-	-	-
		PE	-	-	-
CD38	HIT2	PE	-	-	-
		APC	-		
CD40	5C3	FITC		-	-
		PE	-	-	-
		APC	-	-	-
CD44	515	PE		+	+
CD44	G44-26	PE		+	+
		APC		+	+
CD45	HI30	PE	+	+	+
CD45RA	5H9	PE	+	+	+
		FITC	+	+	
CD45RA	HI100	FITC	+	+	+
		PE	+	+	+
		APC	+	+	+
CD45RO	UCHL1	FITC	+	+	+
		PE	+	+	+
		APC	-		
CD54	HA58	PE	+/-	+/-	+/-
CD56	B159	PE	-	-	-
		FITC	-		
		PE-Cy7	-	-	
		APC	-		
		Alexa 488	-		
		Alexa 647	-		
CD56	MY31	PE	+/-	+/-	
CD56	NCAM16.2	PE	-	-	-
CD69	FN50	FITC		-	-
		PE	+/-	-	-
		APC	-	-	-
CD79a (I/C)	HM47	PE	+	+	+
		APC	+	+	+
CD80	L307.4	PE		DC+	DC+

Specificity	Clone	Fluorochrome	I	II	III
CD83	HB15e	PE	+	+/-	+/-
		APC	+		
CD86	2331	PE	+	+	+
		APC	+		
CD86	IT2.2	PE	+	+	+
CD94	HP-3D9	FITC	+	+	+
		PE	+	+	+
		APC	+	+	+
CD138	Mi15	PE	+	+	+
CD195	2D7	FITC	-	-	-
		PE		-	-
		APC		-	-
		PE-Cy7	+/-	-	-
CD195	3A9	PE	-	-	-
		APC		-	-
DC-SIGN	DCN46	PE	+	+	+
CCR7		PE	-	-	-
HLA-DR	G46-6	FITC	+	+	+/-
		PE	+	+	
		APC	+	+	+*
HLA-DR	Tu36	PE	+	+/-	-
		APC	+		
IgD	IA6-2	PE	-	-	-
IgM	G20-127	PE	+	+/-	+/-

Legend: DC *Dendritic Cells* ***+*** *Positive* ***-*** *Negative* ***+/-*** *Inconclusive*

Stimulators used for Multi-Color Analysis of Human Whole Blood or PBMCs

PMA	IFNα	IL-2	IL-4	IL-6	CD3/CD28 Activation
pERK 1/2					
pp38 MAPK					
	pStat1			pStat1 (+/-)	
	pStat3			pStat3	
	pStat5	pStat5			
			pStat6		
					pZAP70
					pLck

	Bulk Conc.	Final Conc. For PBMC	Final Conc. For Whole Blood
PMA	1mg/ml	40nM	400nM
IFNα	0.2mg/ml (3.8×10^8 U/ml)	10,000U/ml	40,000U/ml
IL-2	0.03µg/µl	0.1µg/ml	
IL-4	0.1µg/µl	0.1µg/ml	
IL-6	0.2µg/µl	0.1µg/ml	

BD™ Phosflow Protocols for Mouse Cells

BD Phosflow Protocols using Mouse Splenocytes or Thymocytes

Protocol I (Detergent Method)

Fix buffer used:

BD™ Phosflow Lyse/Fix Buffer (Cat. No. 558049)

Perm buffer used:

BD™ Phosflow Perm/Wash Buffer I (Cat. No. 557885)

1. Suspend thymocytes or splenocytes from whole thymus or spleen cells.
2. Treat the cells with appropriate stimulators.

   Note: Methods of activation vary and should be determined by researcher.
3. Fix the cells immediately in order to maintain phosphorylation state. Rather than spinning down the cells, we recommend fixing the cells by adding 20 volumes of pre-warmed 1× BD™ Phosflow Lyse/Fix Buffer to the cell suspension.
4. Incubate the cells at 37°C for 10 minutes. Pellet by centrifugation (300 × g) for 5–10 min and remove supernatant.
5. Vortex or mix to disrupt the pellet. Permeabilize the cells by adding 1ml of BD™ Phosflow Perm/Wash Buffer I for 1-10×10^6 cells (Cat. No. 557885) and incubate for 10 minutes at room temperature.
6. Pellet the cells by centrifugation (300 × g) for 5–10 minutes and remove the supernatant.
7. Wash the cells once with BD Phosflow Perm/Wash Buffer I. Centrifuge at 300 × g for 5–10 min and remove the supernatant.
8. Resuspend the cells in BD Pharmingen™ Stain Buffer (Cat. No. 554656) at 10×10^6 cells/ml.
9. Add 0.06µg of BD™ FcBlock antibody (Cat. No. 553141 or 553142) for each 1×10^6 cells. Incubate on ice for 15 minutes.
10. Aliquot 100 µl of the cell suspension (1×10^6 cells) to each tube and add the recommended volumes of BD™ Phosflow antibodies.
11. Incubate the cells at room temperature for 30 min in the dark.
12. Wash the cells once with 2mL of BD Pharmingen Stain Buffer and resuspend the cells in the same buffer prior to flow cytometric analysis.

BD™ Phosflow Protocols using Mouse Splenocytes or Thymocytes

Protocol II and III (Mild or Harsh Alcohol Method)

Lyse/Fix Buffer used:

BD™ Phosflow Lyse/Fix Buffer (Cat. No. 558049)

Perm Buffer used:

Depending on the surface markers and phospho specific antibody conjugates you are using, either BD™ Phosflow Perm Buffer II (Cat. No. 558052) or III (Cat. No. 558050) is recommended. Please refer to our phospho antibody and CD marker reference charts (pages 33–35) for details.

1. Prepare single cell suspensions from mouse thymus or spleen in PBS with 5% FBS (no azide). Wash once with PBS - 5% FBS. Keep the cells at 4°C before stimulation.
2. Treat cells with appropriate stimulators.

   Note: Methods of activation vary and should be determined by researcher.
3. Fix the cells immediately to maintain phosphorylation state by mixing one volume of single cell suspension with 20 volumes of prewarmed 1 × BD™ Phosflow Lyse/Fix buffer (Cat. No. 558049)
4. Incubate the cells at 37°C for 10 minutes. Pellet by centrifugation (300 × g) for 5–10 minutes and remove supernatant.
5. Wash cells once with PBS, and pellet by centrifugation (300 × g) for 5–10 minutes and remove supernatant.
6. Vortex or mix to disrupt the pellet Permeabilize the cells by adding 1ml of BD™ Phosflow Perm Buffer II or III (Cat. No. 558052 or 558050) (for $1–10\times10^6$ cells) and incubating for 30 minutes on ice.

   Note: Longer incubation times may decrease the signal intensity of surface marker staining.
7. Wash the cells twice with BD Pharmingen™ Stain Buffer (Cat. No. 554656) and pellet cells at 300 × g for five minutes. Remove the supernatant.
8. Resuspend the cells in BD Pharmingen Stain Buffer at $1–10\times10^6$ cells/ml.
9. Add 0.06µg BD FcBlock™ antibody (Cat. No. 553141 or 553142) for each 1×10^6 cells. Incubate on ice for 15 minutes.
10. Aliquot optimal concentration of fluorochrome-conjugated antibodies to each tube and add 100 µl of cells.
11. Incubate the cells at room temperature for 30 minutes in the dark.
12. Wash the cells once with 2 ml of BD Pharmingen Stain Buffer, and resuspend in the same buffer prior to flow cytometric analysis.

Recommended Protocols for Phospho Protein Detection in Mouse Cells

Depending on subcellular localization of the phospho protein of interest, as well as epitope susceptibility to cell fixing and permeabilizing agents, we recommend different BD™ Phosflow protocols for our antibodies.

For phenotyping of cell populations in multi color experiments, please use the table on pages 34–35, showing our mouse cell surface markers tested under different fixation and permeabilization conditions.

Protocol			I	II	III
Fix buffers used:			BD Phosflow Lyse/Fix Buffer (558049)	BD Phosflow Lyse/Fix Buffer (558049)	BD Phosflow Lyse/Fix Buffer (558049)
Perm buffers used:			BD Phosflow Perm/Wash I Buffer (557885)	BD Phosflow Perm II Buffer (558052)	BD Phosflow Perm III Buffer (558050)
Specificity	**Clone**	**Fluorochrome**			
ERK 1/2 (pT202/pY204)	20a	Alexa 488	+	+	+
		Alexa 647	+	+	+
		PE	+	+	+
p38 MAPK (pT180/pY182)	36	Alexa 488	+	+	+
		Alexa 647	+	+	+
		PE	+	+	+
ZAP70 (pY319)/SYK (pY352)	17a	Alexa 488		+	+
		Alexa 647		+	+
		PE	+	+	+
C-Cbl (pY700)	47/c-Cbl (Y700)	Alexa 488		+	+
		Alexa 647			+
		PE	+	+	+
Stat1 (pY701)	4a	Alexa 488	-	+/-	+
		Alexa 647	-	+/-	+
		PE	+/-	+	+
Stat3 (pY705)	4	Alexa 488	-	+/-	+
		Alexa 647	-	+/-	+
		PE	-	+	+
Stat4 (pY693)	38/p-Stat4	Alexa 488	-	-	+
		Alexa 647			
		PE	-	-	+
Stat5 (pY694)	47	Alexa 488	-	+/-	+
		Alexa 647	-	+/-	+
		PE	-	+	+
Stat6 (pY641)	S71-773.58.11	Alexa 488			+
		Alexa 647			+
		PE			+

Validated Mouse Cell Surface Markers for BD Phosflow Analysis

Many cell surface markers are sensitive to fixation/permeabilization procedures and lose recognition by relevant antibodies afterwards. For your convenience, many antibodies conjugated to various fluorochromes have been tested under several conditions. The results are summarized below.

Protocol			I	II	III
Fix buffers used:			BD Phosflow Lyse/Fix Buffer (558049)	BD Phosflow Lyse/Fix Buffer (558049)	BD Phosflow Lyse/Fix Buffer (558049)
Perm buffers used:			BD Phosflow Perm/Wash I Buffer (557885)	BD Phosflow Perm II Buffer (558052)	BD Phosflow Perm III Buffer (558050)
Specificity	**Clone**	**Fluorochrome**			
CD3	145-2C11	FITC	+	+/-	-
		PE	+	+	+
		PerCP		+/-	+/-
		PerCP-Cy5.5		+/-	+/-
		APC	+		
		Alexa 488		+	+/-
		Alexa 647		-	-
CD3e	500A2	PE		+	+
CD3 Molecular Complex	17A2	PE		+	+
		Alexa 647		+	+
CD4	GK1.5	FITC		+	+
		PE	+	+	+
CD4	RM4-5	FITC		+	+
		PE		+	+
		PerCP		+	+
		PerCP-Cy5.5		+	+
		Alexa 488		+	+
		Alexa 647		+	+
CD8	53-6.7	FITC		-	-
		PE	+	+	+
		PerCP		+	+
		APC	+		
		PerCP-Cy5.5		+	+
		Alexa 488		+	+
		Alexa 647		+	+
CD11b	M1/70	FITC	+	+/-	+/-
		PE	+	+	+
		PerCP-Cy5.5		+	+
		Alexa 488		+	+
CD11c	HL3	PE	+		+/-
CD19	1D3	PE	-	-	-
		PerCP-Cy5.5		-	-
		Alexa 488		-	-
CD21/35	7G6	FITC		-	-
		PE	+/-		-
CD23	B3B4	FITC	+/-		-
		PE	+	-	-
CD24	M1/69	FITC	+	-	-
		PE	+	-	-

Specificity	Clone	Fluorochrome	I	II	III
CD25	7D4	FITC		+	+
CD25	3C7	PE		+	+
CD25	PC61	PE		+	+
		PerCP-Cy5.5		+	+
		APC		+	+
CD28	37.51	PE		-	-
CD43	S7	PE	+		+
CD44	IM7	FITC		+	+
		PE		+	+
		APC		+	+
CD45	30-F11	PerCP-Cy5.5	+		+
		PE		+	+
CD45R/B220	RA3-6B2	FITC	+	+	+
		PE	+	+	+
		PerCP		+	+
		APC	+		
		PerCP-Cy5.5	+		+
		Alexa 488		+	+
		Alexa 647		+	+
CD49b	DX5	FITC		-	-
CD49b	HMα2	Alexa 488		+	+
CD69	H1.2F3	FITC		-	-
		PE		-	-
Ly-6G and Ly6C (GR-1)	RB6-8C5	FITC		+	+
		PE	+	+	+
I-A/I-E	M5/114.15.2	PE		+	+
I-A/I-E	2G9	FITC		+	+
IgD	11-26c2.a	FITC	+		-
IgM	II/41	FITC	+		-
		APC	+		
IgM	R6-60.2	PerCP-Cy5.5	+		-
Ly-6C	AL-21	FITC	+	-	-
$I\text{-}A_b$	AF6-120.1	FITC	+/-	-	-
		PE	+	-	-
NK1.1	PK136	FITC	-		-
		PE	+	-	-
TCR β chain	H57-597	FITC		+	+/-
		PE	+	+	+
		APC	+	+	+

Legend: ***+*** *Positive* ***-*** *Negative* ***+/-*** *Inconclusive*

Product List

BD™ Phosflow Reagents

Description	React	Clone	Isotype	Format	Size	Cat. No.
Akt (pY308)	Hu, Ms	J1-223.371	Ms IgG_1	PE	50 tests	inquire
Akt (pY473)	Hu, Ms	F29-763	Rabbit IgG	Alexa Fluor® 647	50 tests	inquire
Btk (pY551) & Itk (pY511)	Hu	24a	Ms IgG_1	Alexa Fluor® 488	50 tests	558138
				Alexa Fluor® 647	50 tests	558134
				PE	50 tests	558129
Caveolin-1 (pY14)	Hu, Ms, Rat	56	Ms IgG_1	PE	50 tests	612568
c-Cbl (pY700)	Hu	47	Ms IgG_1	Alexa Fluor® 488	50 tests	558101
				Alexa Fluor® 647	50 tests	558100
				PE	50 tests	558057
c-Cbl (pY774)	Hu	29	Ms IgG_1	Alexa Fluor® 647	50 tests	558103
				PE	50 tests	558102
ERK1/2 (p44/42 MAPK) (pT202/pY204)	Hu, Ms, Rat	20a	Ms IgG_1	Alexa Fluor® 488	50 tests	612592
				Alexa Fluor® 647	50 tests	612593
				PE	50 tests	612566
JNK (pT183/pY185)	Hu, Rat	Polyclonal	Rabbit IgG	Purified	50 tests	inquire
Lck (Y505)	Hu	4	Ms IgG_1	Alexa Fluor® 488	50 tests	557879
				PE	50 tests	558552
MEK (pS222)	Hu, Ms, Rat	Polyclonal	Rabbit IgG	Purified	50 tests	inquire
p38 MAPK (pT180/pY182)	Hu, Ms, Rat	36	Ms IgG_1	Alexa Fluor® 488	50 tests	612594
				Alexa Fluor® 647	50 tests	612595
				PE	50 tests	612565
Pan/Phospho-SYK (pY352)	Hu	4D10/17a	Ms IgG_{2a}/IgG_1	FITC/PE Set	50 tests	557952
Phosphotyrisine	Hu	P-Tyr-E12	Human Fab	PE	50 tests	558285
Phosphotyrosine	Hu, Ms, Rat	PY20	Ms IgG_{2b}	PE	50 tests	558008
PLCγ1 (pY783)	Hu	27		Alexa Fluor® 488	50 tests	557884
				Alexa Fluor® 647	50 tests	557883
Stat1 (pY701)	Hu, Ms	4a	Ms IgG_{2a}	Alexa Fluor® 488	50 tests	612596
				Alexa Fluor® 647	50 tests	612597
				PE	50 tests	612564
Stat3 (pY705)	Hu, Ms	4	Ms IgG2a	Alexa Fluor® 488	50 tests	557814
				Alexa Fluor® 647	50 tests	557815
				PE	50 tests	612569
Stat3 (pS727)	Hu	49	Ms IgG_1	Alexa Fluor® 488	50 tests	558085
				Alexa Fluor® 647	50 tests	558099
Stat4 (pY693)	Hu	38	Ms IgG_{2b}	Alexa Fluor® 488	50 tests	558136
				Alexa Fluor® 647	50 tests	558137
				PE	50 tests	558249
Stat5 (pY694)	Hu, Ms	47	Ms IgG_1	Alexa Fluor® 488	50 tests	612598
				Alexa Fluor® 647	50 tests	612599
				PE	50 tests	612567
Stat6 (pY641)	Hu	18	Ms IgG_{2a}	Alexa Fluor® 488	50 tests	612600
				Alexa Fluor® 647	50 tests	612601
				PE	50 tests	612701
STAT6 (pY641)	Ms	J71-773.58.11	Ms IgG_1	Alexa Fluor® 488	50 tests	inquire
				Alexa Fluor® 647	50 tests	inquire
				PE	50 tests	inquire
ZAP70 (pY319)/SYK (pY352)	Hu	17a	Ms IgG_1	Alexa Fluor® 488	50 tests	557818
				Alexa Fluor® 647	50 tests	557817
				PE	50 tests	557881

Product List

BD FcBlock™ Antibody and Antibodies for T Cell Activation

Description	React	Clone	Isotype	Format	Size	Cat. No.
CD16/CD32 (Fcγ III/II Receptor)	Ms	2.4G2	Rat IgG_{2b}, κ	Purified	0.1 mg	553141
CD3	Hu	UCHT1	Ms IgG_1, κ	NA/LE	0.5 mg	555329
CD3e (CD3 ε chain)	Ms	145-2C11	Ar Ham IgG1, κ	NA/LE	0.5 mg	553057
CD4 (L3T4)	Ms	GK1.5	Rat IgG_{2b}, κ	NA/LE	0.5 mg	553726
CD28	Hu	CD28.2	Ms IgG_1, κ	NA/LE	0.5 mg	555725
CD28	Ms	37.51	Sy Ham IgG2, λ	NA/LE	0.5 mg	553294
Goat Anti-Mouse Ig (multiple adsorption)	Ms	Goat Ig	Polyclonal	Purified	0.5 mg	553998

Supporting Reagents

Description	Clone	Isotype	Format	Size	Cat. No.
BD Cytofix Fixation Buffer				100 mls	554655
BD Phosflow Fix Buffer I				250 ml	557870
BD Phosflow Lyse/Fix Buffer (5X)				250 ml	558049
BD Phosflow Perm/Wash Buffer I				125 ml	557885
BD Phosflow Perm Buffer II				125 ml	558052
BD Phosflow Perm Buffer III				125 ml	558050
BD Pharmingen Stain Buffer (FBS)				500 ml	554656
Mouse IgG1 Isotype Control	MOPC-21	Ms IgG_1	Alexa Fluor® 488	50 tests	557782
			Alexa Fluor® 647	50 tests	557783
			PE	50 tests	551436
Mouse IgG2a Isotype Control	MOPC-173	Ms IgG_{2a}	PerCP-Cy5.5	50 tests	558020

Notes

Chapter 2

BD™ Cytometric Bead Array (CBA) Multiplexed Quantification of Phospho Proteins

Introduction

The BD™ Cytometric Bead Array (CBA) uses a series of particles with discrete fluorescent intensities to simultaneously detect multiple analytes from a single cell lysate. The specific capture beads are mixed with the phycoerythrin (PE)-conjugated detection antibodies and then incubated with recombinant protein standards or test samples to form sandwich complexes. Following acquisition of sample data using the flow cytometer, the sample results are generated in graphical and tabular format using the BD™ CBA Analysis Software or FCAP Array™ Software. Multiple specificities can be analyzed simultaneously from the same sample in a flexible format. The assays can be run on any BD dual-laser cytometer. However, the BD FACSArray™ Bioanalyzer, which is a plate based system, is particularly well suited to this task.

Unlike conventional assays used to detect or measure phosphorylated proteins like immunoprecipitation and Western blotting, the phospho specific BD™ CBA Kits can provide sensitive, quantitative measurements of protein phosphorylation in considerably less time (approximately 5 hours). In addition, the phosphorylation status of multiple proteins can be measured on the same sample in a quantitative fashion. A list of the kits available for phospho protein quantification can be seen in the product list on page 55. The assays are designed to measure either phosphorylated or total specificities from a cell lysate. The specificity of the assays has been validated by immunoprecipitation and Western blotting to ensure that only the protein of interest is detected. Test samples are run against a standard curve which is generated using dilutions of a phosphorylated, recombinant standard. We have assessed the linearity of the assays by making dilutions of activated cell lysates and comparing them against the standard curve. In all cases the dilution curve of the sample is parallel to the standard curve. Reproducibility assays, both intra-assay and inter-assay demonstrated a low %CV. The sensitivity of the assay is comparable to or better than a conventional Western blot.

BD™ Cytometric Bead Array	BD™ Phosflow
Phospho-Protein detection in cell lysates Uses antibody pairs in a sandwich approach to detect phospho-proteins	**Intracellular staining of phospho-proteins** Uses directly conjugated antibodies to detect phospho-proteins in fixed and permeabilized cells
Population analysis Provides an average value for multiple cells	**Single cell analysis** Provides data for each individual cell
Highly multiplexed analysis With up to 72 answers per sample	**Phenotyping and multiplexed activation state analysis** Using BD Phosflow in combination with cell surface markers in heterogeneous cell populations
Quantitative	**Semi-quantitative**
Time to results 6 hours	**Time to results** 3 hours
Sensitivity Similar to Western Blot Analysis (ECL)	**Sensitivity** Detects phosphorylation events in single cells
Typical sample Cell lines, tissues, purified primary cells, PBMCs	**Typical Sample** Heterogeneous samples like whole blood or PBMCs, cell lines
96-well compatible Using the BD FACSArray Bioanalyzer	**96-well compatible** Using the BD FACSArray Bioanalyzer

Table 1. Comparison of BD™ Phosflow and BD™ Cytometric Bead Array Technology

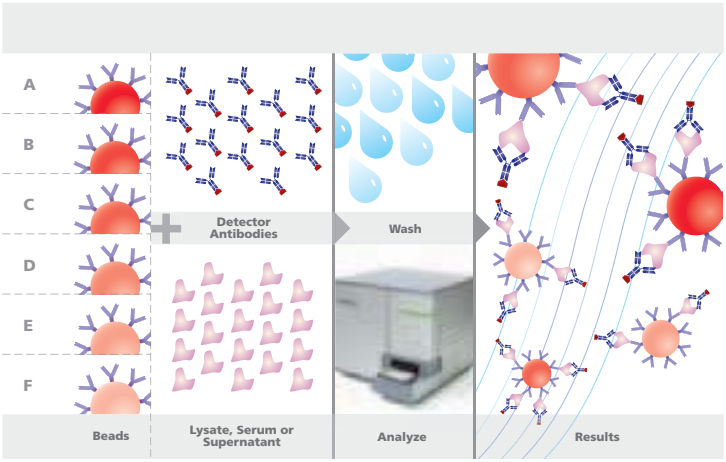


Figure 1. BD™ CBA Flex Set Assay Protocol Schematic. Only a small representative sample of the available beads is shown.

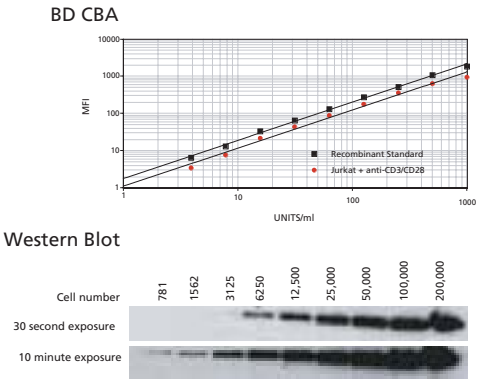


Figure 2. Jurkat cells were activated by adding anti-CD3 and anti-CD28 which were crosslinked by anti-mouse Ig for 2 minutes. The reaction was stopped by the addition of SDS (1% final) whereupon the samples were placed in a boiling water bath. A phosphorylated recombinant standard was used to generate a standard curve. The upper panel shows the standard curve versus a titration of the activated Jurkat lysate. An important characteristic of the assay is that these two curves are parallel to each other. This ensures linearity of the readings so that twice as much lysate will have twice as many units/ml. The same concentrations of lysates were also run on an SDS-PAGE gel followed by immunoblotting with an anti-phospho-ZAP70 antibody. Both a 30 second and a 10 minute exposure are shown. The BD™ CBA assay is at least as sensitive as a Western blot. In this particular example, phosphorylation of ZAP70 can be detected in a CBA assay using lysate from less than 1000 cells.

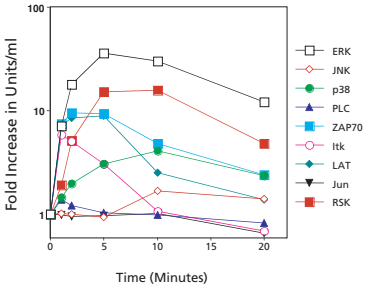
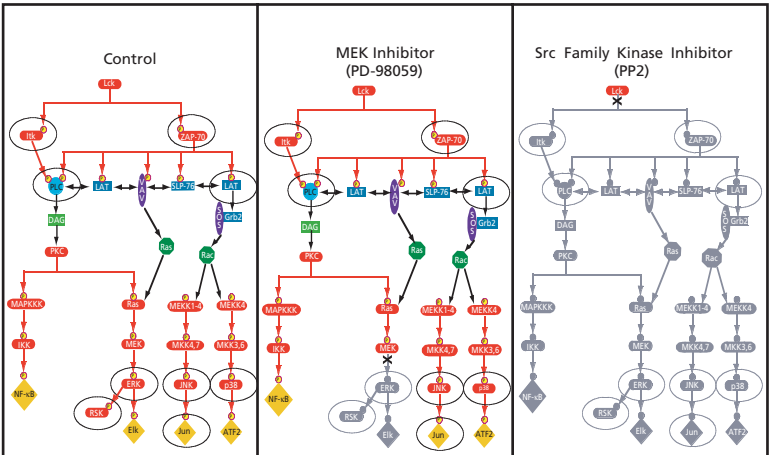


Figure 3. Kinetic analysis of T cell activation by anti-CD3/CD28. Jurkat cells were activated with anti-CD3 and anti-CD28 for different lengths of time. Lysates were prepared as outlined in Figure 5. A 9-plex BD™ CBA Flex Set assay using 10 µg of lysate was run measuring phosphorylated ERK, JNK, p38, PLCg, ZAP70, Itk, LAT, c-Jun, and RSK. Using standard curves, units/ml were determined for each specificity and the fold increase in activity was plotted.



Specificity	Change	% of Control
ZAP-70	655	100
Itk	77	100
LAT	170	100
PLCγ	272	100
ERK	2946	100
JNK	141	100
p38	2398	100
c-Jun	142	100
RSK	385	100

Specificity	Change	% of Control
ZAP-70	970	148
Itk	61	79
LAT	157	92
PLCγ	125	46
ERK	372	13
JNK	92	65
p38	1692	71
c-Jun	195	137
RSK	9	2

Specificity	Change	% of Control
ZAP-70	-10	-2
Itk	-8	-10
LAT	-2	-1
PLCγ	19	7
ERK	-2	0
JNK	15	11
p38	475	20
c-Jun	32	23
RSK	22	6

Figure 4. Effect of inhibitors on T cell signaling. Jurkat cells were pre-incubated with either buffer, 200 µM PD-98059 (MEK inhibitor), or 10 µm PP2 (Src family tyrosine kinase inhibitor) for 20 minutes before being activated with anti-CD3/CD28 for two minutes. In the left panel, a T cell signaling pathway is shown. The nine phospho specificities that were tested simultaneously using a 9-plex BD™ CBA Flex Set assay are circled. The table at the bottom of the panel shows the units/ml for each specificity. The middle panel shows the effects of pre-incubation with PD-98059. Since this compound inhibits MEK, only ERK and RSK should be affected. This is shown in gray on the pathway. However, although ERK and RSK are almost completely inhibited, this compound does have effects on other signaling molecules. In the right panel, Jurkat cells were pre-incubated with PP2 which inhibits Src family kinases such as Lck. This should shut down all of the signaling which is what is seen in the table.

BD™ CBA Cell Signaling Flex Set System

The BD™ CBA Cell Signaling Flex Set system uses the sensitivity of amplified fluorescence detection by flow cytometry to measure a soluble analyte. Each bead in a BD CBA provides a capture surface for a specific protein and is analogous to an individually coated well in an ELISA plate. The BD CBA Cell Signaling Flex Set capture bead is in suspension to allow for the detection of an analyte in a small sample volume. The combined advantages of the broad dynamic range of fluorescent detection via flow cytometry and the efficient capturing of analytes via suspended particles enable the BD CBA Flex Set system to use fewer sample dilutions and to obtain the value of an unknown in substantially less time (compared to conventional ELISA and Western blot techniques).

The BD CBA Cell Signaling Master Buffer Kit contains all of the supporting reagents necessary to perform an assay using a BD CBA Cell Signaling Flex Set. The buffers and instrument setup reagents provided in this kit have been optimized for use on BD flow cytometers.

Note: The BD CBA Cell Signaling Master Buffer Kit should not be used with any non-Cell Signaling BD CBA Flex Sets.

The Easiest Way to Multiplex with Beads

BD Biosciences has always provided the most reliable and complete multiplex bead array system. Now we've combined the performance you trust and the ease-of-use you rely on for the ultimate in flexibility. In addition to your need for a flexible assay system, we also recognize the value of your precious samples and the importance of data reproducibility. We've engineered the new BD™ Cytometric Bead Array (CBA) Flex Set system to maximize both.

The new BD™ CBA Flex Set system is an open and configurable system designed to be the easiest method of creating multiplex assays yielding the most reproducible data. With our proprietary conjugation chemistry, pair optimization strategies, and direct PE detection reagents, you can be assured consistent and superior assay performance in complex biological samples. Each antibody pair we develop is evaluated for dynamic range, sensitivity, and parallel titration to native biological samples. We have also specifically formulated the assay diluent and wash buffers for each assay type to reduce potentially detrimental effects on assay performance when using serum and plasma samples. By avoiding a streptavidin-biotin-PE detection method employed by other assays, our direct PE detection reagents minimize the risk of increased background often caused by endogenous biotin in serum and lysate samples. Thus, BD™ CBA Flex Sets provide a reliable, flexible, and reproducible method for quantitative detection of multiple analytes in a single serum, plasma, tissue culture supernatant, or cell lysate sample.

Principle of the Test

A BD CBA Cell Signaling Flex Set capture bead is a single bead population with a distinct fluorescence intensity and is coated with a capture antibody specific for an intracellular protein. The bead population is resolvable in the FL3 and FL4 channels of a BD FACSCalibur™ flow cytometer or the NIR and Red channels of a BD FACSArray™ bioanalyzer. There are approximately 72 bead positions available on the BD FACSArray bioanalyzer (and other dual-laser, digital flow cytometers), and approximately 30 bead positions available on the BD FACSCalibur flow cytometer.

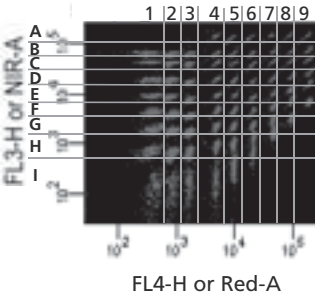


Figure 5. BD™ CBA Flex Set Bead Positions.

Each bead population is given an alpha-numeric position designation indicating its position relative to other beads in the BD CBA Flex Set system. Beads with different positions can be combined in assays to create a multiplex assay.

In a BD CBA Flex Set assay the capture bead, PE-conjugated detection reagent, and standard or test samples are incubated together to form sandwich complexes. Following acquisition of sample data using the flow cytometer, the sample results are generated in a graphical and tabular format using the FCAP Array™ software. The BD™ CBA Cell Signaling Master Buffer Kit provides sufficient reagents for the analysis of 100 samples and 5 instrument setup procedures (Cat. No. 558223) or 500 samples and 10 instrument setup procedures (Cat. No. 558224) of any size multiplex.

Advantages

The BD™ CBA Cell Signaling Flex Set system provides several advantages when compared with conventional ELISA and Western blot methodologies:

- A BD CBA Cell Signaling Flex Set assay experiment takes significantly less time than a Western blot assay and provides quantitative results.
- The BD CBA Cell Signaling Flex Set assays have a wider dynamic range than conventional ELISAs.

Limitations

The BD CBA Cell Signaling Flex Set System is not recommended for use on stream-in-air instruments where signal intensities may be reduced, adversely affecting assay sensitivity. Stream-in-air instruments include the BD FACStar™ Plus and BD FACSVantage™ flow cytometers.

Reagents Provided

Bead Reagents

Instrument Setup Bead A1: A single vial of concentrated beads, position A1, that is used for instrument setup. Store at 4°C. Do not freeze.

Instrument Setup Bead A9: A single vial of concentrated beads, position A9, that is used for instrument setup. Store at 4°C. Do not freeze.

Instrument Setup Bead F1: A single vial of concentrated beads, position F1, that is used for instrument setup. Store at 4°C. Do not freeze.

Instrument Setup Bead F9: A single vial of concentrated beads, position F9, that is used for instrument setup. Store at 4°C. Do not freeze.

PE Instrument Setup Bead F1: A single vial of concentrated beads, position F1, that are used for generating a PE positive bead population for instrument setup. Store at 4°C. Do not freeze.

Antibody Reagents

PE Positive Control Detector: A single vial of PE-conjugated antibody that is formulated for use at 50 µl/test. This reagent is used with the PE Instrument Setup Bead F1 to set instrument compensation settings. Store at 4°C. Do not freeze.

Buffer Reagents

Wash Buffer: A single bottle of phosphate buffered saline (PBS) solution (1×), containing protein* and detergent, used for wash steps and to resuspend beads for analysis. Store at 4°C.

Assay Diluent: A single bottle of a buffered solution (1×) used to dilute the BD™ CBA Cell Signaling Flex Set Standards and to dilute test samples. Store at 4°C.

5× Denaturation Buffer: A single bottle of a sodium dodecyl sulfate (SDS) solution (5×) used to denature test samples. Store at RT or 4°C.

Capture Bead Diluent: A single bottle of phosphate buffered saline (PBS) solution (1×), containing protein*, used to dilute BD CBA Cell Signaling Flex Set Capture Beads prior to each experiment. Store at 4°C.

Detection Reagent Diluent: A single bottle of phosphate buffered saline (PBS) solution (1×), containing protein*, used to dilute BD CBA Cell Signaling Flex Set Detection Reagents prior to each experiment. Store at 4°C.

** Source of all serum proteins is from USDA inspected abattoirs in the United States.*

Materials Required but not Provided

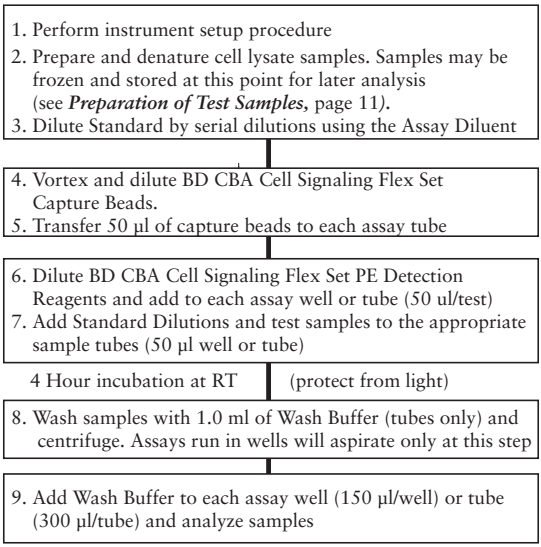
In addition to the reagents provided in the BD™ CBA Cell Signaling Master Buffer Kit and the BD™ CBA Cell Signaling Flex Sets, the following items are also required:

- A flow cytometer equipped with a 488 nm and/or a 532 nm laser and a 635 nm laser capable of detecting and distinguishing fluorescence emission at 576 nm and 670 nm (off the 488 nm laser) and 660 nm (off the 635 nm laser) such as a BD FACSCalibur™ or 576 nm (off the 532 nm laser) and 660 nm and >680 nm (off the 635 nm laser) such as a BD FACSArray™ bioanalyzer.
- 12 × 75 mm sample acquisition tubes for a flow cytometer (eg, BD Falcon™ Cat. No. 352008).
- FCAP Array™ software (Cat. No. 338621).
- BD CaliBRITE™ 3 Beads, (Cat. No. 340486) to run experiments on a BD FACSCalibur flow cytometer.
- BD CaliBRITE™ APC Beads, (Cat. No. 340487) to run experiments on a BD FACSCalibur flow cytometer.
- BD FACSComp™ software to run experiments on a BD FACSCalibur.
- Microcentrifuge

Optional for plate-loader-equipped flow cytometers:

- Millipore MultiScreen® BV 1.2 µm Clear non-sterile filter plates, (Cat. No. MABVN12), **www.millipore.com/catalogue.nsf/docs/MABVN1250** (50 pack), **www.millipore.com/catalogue.nsf/docs/MABVN1210** (10 pack)
- Millipore MultiScreen® Vacuum Manifold, (Cat. No. MAVM0960R), www.millipore.com/catalogue.nsf/docs/MAVM0960R
- MTS 2/4 Digital Stirrer, IKA Works, VWR, (Cat. No. 82006-096)
- Vacuum source

Overview: BD™ CBA Cell Signaling Flex Set Assay Procedure



Preparation of BD™ CBA Cell Signaling Flex Set Standards

The standard provided with each BD™ CBA Cell Signaling Flex Set is provided as a 50× bulk recombinant protein (50,000 Units/ml) and should be serially diluted before mixing with the Capture Beads and the PE Detection Reagent for a given assay. The protocol below indicates how standards should be mixed and diluted for use in a BD CBA Cell Signaling Flex Set assay. Each assay (single bead or multiplex) performed in a given experiment will need to have a standard curve prepared.

It is recommended that the Instrument Setup procedure be performed before starting your first experiment using the BD CBA Cell Signaling Flex Set assays.

Each Cell Signaling BD CBA Flex Set Standard was assigned an arbitrary unit value. In each case, the unit potency of the BD CBA Flex Set Standard will be kept consistent from lot to lot.

1. Warm Standard vial to 37°C and vortex to mix thoroughly.
2. Label 12 × 75 mm tubes (BD Falcon™, Cat. No. 352008) and arrange them in the following order: Top Standard, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256.
3. Add 20 µl of each BD CBA Cell Signaling Standard to be run in the experiment to the Top Standard tube.

4. Add Assay Diluent to the Top Standard tube to bring the final volume to 1 ml.

   Example: If 5 BD™ CBA Cell Signaling Flex Sets are being multiplexed for a given experiment, you will add 20 µl of each standard to the Top Standard tube (5 × 20 µl = 100 µl total volume) and will then add 900 µl of Assay Diluent (1 ml Assay Diluent - 100 µl [volume of standards added] = 900 µl Assay Diluent).

5. Add 500 µl of Assay Diluent to each of the remaining tubes.

6. Perform a serial dilution by transferring 500 µl from the Top Standard to the 1:2 dilution tube and mix thoroughly. Continue making serial dilutions by transferring 500 µl from the 1:2 tube to the 1:4 tube and so on to the 1:256 tube and mix thoroughly (see Figure 6). The Assay Diluent serves as the negative control.

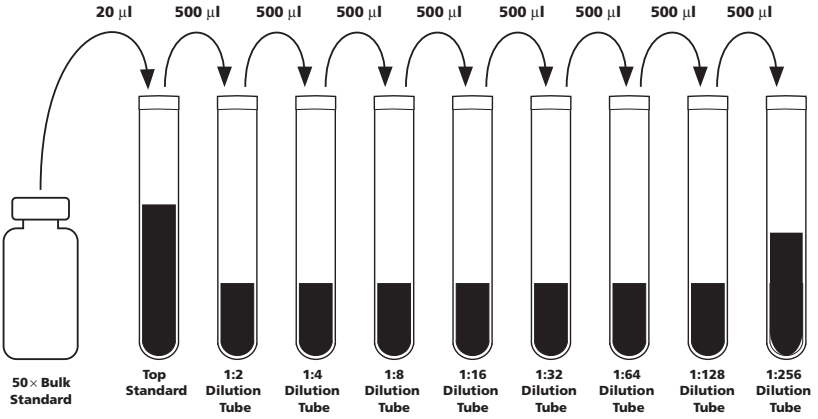


Figure 6. Preparation of BD™ CBA Cell Signaling Flex Set Standard Dilutions

The approximate concentration (Units/ml) of each BD™ CBA Cell Signaling Flex Set Standard in each dilution tube is shown in **Table 2.**

Protein (Units/ml)	Top Standard	1:2 Dilution Tube	1:4 Dilution Tube	1:8 Dilution Tube	1:16 Dilution Tube	1:32 Dilution Tube	1:64 Dilution Tube	1:128 Dilution Tube	1:256 Dilution Tube
BD CBA Cell Signaling Flex Set Standard	1000	500	250	125	62.5	31.25	15.6	7.8	3.9

Table 2. BD™ CBA Cell Signaling Flex Set Standard concentrations after dilution

Preparation of Test Samples

BD™ CBA Cell Signaling Flex Sets are designed to measure total or phosphorylated proteins from denatured cell lysate samples. It is necessary to lyse and denature cell samples using the 5× Denaturation Buffer provided in the BD CBA Cell Signaling Master Buffer Kit before use in a BD CBA Cell Signaling Flex Set assay.

The standard curve for each BD CBA Cell Signaling Flex Set covers a defined set of concentrations from 3.9 – 1000 Units/ml. It may be necessary to dilute test samples to ensure that their mean fluorescence values fall within the limits or range of the generated standard curve. For best results, samples that are known or assumed to contain high levels of a given protein should be diluted as described below. It is important that the cell number or the total protein concentration of the cell lysate sample is known so that results determined using the BD CBA Cell Signaling Flex Sets can be normalized (eg, Units/ml/10^6 cells or Units/ml/µg of cell lysate).

It is necessary to heat the 5× Denaturation Buffer to 37°C before use (shake or vortex until all precipitates have gone back into solution). To denature the cell lysate, it is important that the final concentration of the Denaturation Buffer is 1× once mixed with cells.

Cells in Suspension

1. Count cells in sample. This is to give an approximate idea of protein concentration, which should be greater than 1 mg/ml (protein concentration is dependent on cell type, eg, Jurkat = 100 – 200 µg/10^6 cells while peripheral blood lymphocytes [PBL] = 25 – 50 µg/10^6 cells).
2. Treat cells to induce or inhibit protein phosphorylation as required for your experiment.
3. Add appropriate amount of 5× Denaturation Buffer so that the final concentration is 1×. Alternatively, ice-cold PBS can be added to the tube and the cells pelleted. Add an appropriate amount of 1× Denaturation Buffer (prepared by diluting the 5× Denaturation Buffer with water) to resuspend the cell pellet.
4. Immediately place in a boiling water bath for 5 minutes.
5. Determine protein concentration.
6. Cell lysates may be stored at –70°C for up to 6 months at this point. If samples are stored frozen, thaw sample before proceeding to Step 7. Avoid multiple freeze/thaw treatments of sample.
7. Dilute cell lysate sample by the desired dilution factor (ie, 1:2, 1:10, or 1:20) using the appropriate volume of Assay Diluent. Sample must be diluted at least 1:2 to reduce the percentage of SDS.
8. Mix sample dilutions thoroughly before transferring samples to the appropriate assay tubes containing Capture Beads.

Adherent Cells

1. Count cells before plating. This is to give an approximate idea of protein concentration, which should be greater than 1 mg/ml.
2. Treat cells to induce or inhibit protein phosphorylation as required for your experiment.
3. Add the appropriate amount of 5× Denaturation Buffer so that the final concentration is 1×. Alternatively, aspirate off all liquid and add Denaturation Buffer which has been diluted to 1× with water. Scrape or agitate cells to dislodge from plate.
4. Immediately place in a boiling water bath for 5 minutes.
5. Determine protein concentration.
6. Cell lysates may be stored at –70°C for up to 6 months at this point. If samples are stored frozen, thaw sample before proceeding to Step 8. Avoid multiple freeze/thaw treatments of sample.
7. Dilute cell lysate sample by the desired dilution factor (ie, 1:2, 1:10, or 1:20) using the appropriate volume of Assay Diluent. Sample must be diluted at least 1:2.
8. Mix sample dilutions thoroughly before transferring samples to the appropriate assay tubes containing Capture Beads.

Preparation of BD™ CBA Cell Signaling Flex Set Capture Beads

The Capture Beads provided with each BD™ CBA Cell Signaling Flex Set are a 50× bulk (1 µl/test) and should be mixed with other BD CBA Cell Signaling Flex Set Capture Beads and diluted to their optimal volume per test (50 µl/test) before adding the beads to a given tube or assay well. The protocol that follows, indicates how Capture Beads should be mixed and diluted for use in a BD CBA Cell Signaling Flex Set assay. It is recommended that the Instrument Setup procedure be performed before starting your first experiment using the BD CBA Cell Signaling Flex Set assays.

1. Determine the number of BD CBA Cell Signaling Flex Sets to be used in each tube or assay well in the experiment (size of the multiplex).
2. Determine the number of assay of tubes or wells to be run in the experiment.
3. Vortex each BD CBA Cell Signaling Flex Set Capture Bead and then transfer 1 µl/test of each Capture Bead to a conical tube labeled “mixed Capture Beads”.
4. Add Capture Bead Diluent to the mixed Capture Beads tube to bring the final volume to 50 µl/test.

Example: If 5 BD™ CBA Cell Signaling Flex Sets are being multiplexed for a given 20 test experiment, you would add 1 µl/test of each BD CBA Cell Signaling Flex Set Capture Bead to the mixed Capture Bead tube (1 µl/test × 20 tests = 20 µl total volume of each Capture Bead) and then add Capture Bead Diluent to bring the final volume to 50 µl/test by determining the remaining volume to add (the final volume of mixed Capture Beads is 20 tests × 50 µl/test = 1,000 µl). A total of 100 µl of Capture Beads were added to the mixed Capture Beads tube above when 20 µl total volume of each Capture Bead was added from the 5 BD CBA Cell Signaling Flex Sets. The amount of Capture Bead Diluent to add is 1,000 µl total volume - 100 µl of Capture Beads = 900 µl). This calculation is also described in **Table 3**.

Note: It is recommended that a few more tests are prepared during this step than you plan to use in the experiment to ensure that there is sufficient material to preform the experiment.

5. Vortex the beads to mix thoroughly. Mixed Capture Beads are now ready to be used in the experiment.

No of Flex Sets to be used	Volume of each Capture Bead or PE Detection Reagent/test*	Total Capture Bead volume/test*	Volume of Capture Bead or Detection Reagent Diluent/test*	Total volume of mixed Capture Beads or PE Detection Reagents/test*
1	1 µl	1 µl	49 µl	50 µl
2	1 µl	2 µl	48 µl	50 µl
3	1 µl	3 µl	47 µl	50 µl
4	1 µl	4 µl	46 µl	50 µl
5	1 µl	5 µl	45 µl	50 µl
6	1 µl	6 µl	44 µl	50 µl
7	1 µl	7 µl	43 µl	50 µl
8	1 µl	8 µl	42 µl	50 µl
9	1 µl	9 µl	41 µl	50 µl
10	1 µl	10 µl	40 µl	50 µl
11	1 µl	11 µl	39 µl	50 µl
12	1 µl	12 µl	38 µl	50 µl
13	1 µl	13 µl	37 µl	50 µl
14	1 µl	14 µl	36 µl	50 µl
15	1 µl	15 µl	35 µl	50 µl
16	1 µl	16 µl	34 µl	50 µl
17	1 µl	17 µl	33 µl	50 µl
18	1 µl	18 µl	32 µl	50 µl
19	1 µl	19 µl	31 µl	50 µl
20	1 µl	20 µl	30 µl	50 µl
21	1 µl	21 µl	29 µl	50 µl
22	1 µl	22 µl	28 µl	50 µl
23	1 µl	23 µl	27 µl	50 µl
24	1 µl	24 µl	26 µl	50 µl
25	1 µl	25 µl	25 µl	50 µl
26	1 µl	26 µl	24 µl	50 µl
27	1 µl	27 µl	23 µl	50 µl
28	1 µl	28 µl	22 µl	50 µl
29	1 µl	29 µl	21 µl	50 µl
30	1 µl	30 µl	20 µl	50 µl
31	1 µl	31 µl	19 µl	50 µl
32	1 µl	32 µl	18 µl	50 µl
33	1 µl	33 µl	17 µl	50 µl
34	1 µl	34 µl	16 µl	50 µl
35	1 µl	35 µl	15 µl	50 µl
36	1 µl	36 µl	14 µl	50 µl
37	1 µl	37 µl	13 µl	50 µl
38	1 µl	38 µl	12 µl	50 µl
39	1 µl	39 µl	11 µl	50 µl
40	1 µl	40 µl	10 µl	50 µl
41	1 µl	41 µl	9 µl	50 µl
42	1 µl	42 µl	8 µl	50 µl
43	1 µl	43 µl	7 µl	50 µl
44	1 µl	44 µl	6 µl	50 µl
45	1 µl	45 µl	5 µl	50 µl
46	1 µl	46 µl	4 µl	50 µl
47	1 µl	47 µl	3 µl	50 µl
48	1 µl	48 µl	2 µl	50 µl
49	1 µl	49 µl	1 µl	50 µl
50	1 µl	50 µl	0 µl	50 µl

Table 3. Capture Bead Positions.

Preparation of BD™ CBA Cell Signaling Flex Set PE Detection Reagents

The PE Detection Reagent provided with each BD™ CBA Cell Signaling Flex Set is a 50× bulk (1 µl/test) and should be mixed with other BD CBA Cell Signaling Flex Set PE Detection Reagent and diluted to their optimal volume per test (50 µl/test) before adding the PE Detection Reagents to a given tube or assay well. The protocol below indicates how PE Detection Reagents should be mixed and diluted for use in a BD CBA Cell Signaling Flex Set assay. It is recommended that the Instrument Setup procedure be performed before starting your first experiment using the BD CBA Cell Signaling Flex Set assays.

1. Determine the number of BD CBA Cell Signaling Flex Sets to be used in each tube or assay well in the experiment (size of the multiplex).
2. Determine the number of assay tubes or wells to be run in the experiment.
3. Transfer 1 µl/test of each PE Detection Reagent to a conical tube labeled "mixed PE Detection Reagent".

   Note: For BD CBA Cell Signaling Flex Sets containing two PE detection reagent parts, transfer 0.5 µl/test of each PE detection reagent part to a "mixed PE detection reagent" tube.

4. Add Detection Reagent Diluent to the mixed PE Detection Reagent tube to bring the final volume to 50 µl/test.

   Example: If 5 BD CBA Cell Signaling Flex Sets are being multiplexed for a given 20 test experiment, you would add 1 µl/test of each PE Detection Reagent to the mixed PE Detection Reagent tube (1 µl/test × 20 tests = 20 µl total volume of each PE Detection Reagent) and then add Detection Reagent Diluent to bring the final volume to 50 µl/test by determining the remaining volume to add (the final volume of mixed PE Detection Reagent is 20 tests × 50 µl/test = 1,000 µl). A total of 100 µl of PE Detection Reagent was added to the mixed PE Detection Reagent tube above when 20 µl total volume of each Detection Reagent was added from the 5 BD CBA Cell Signaling Flex Sets. The amount of Detection Reagent Diluent to add is 1,000 µl total volume - 100 µl of PE Detection Reagents = 900 µl). This calculation is also described in Table 3.

   Note: It is recommended that more tests are prepared during this step than you plan to use in the experiment to ensure that there is sufficient material to preform the experiment.

5. Vortex mixed PE Detection Reagent briefly. Mixed PE Detection Reagent is now ready to be used in the experiment.

BD™ CBA Cell Signaling Flex Set Assay Procedure

Following the preparation and dilution of the Capture Beads, PE Detection Reagent, and Standards, transfer the Standards, Capture Beads, PE Detection Reagent, and test samples to the appropriate assay tubes or wells for incubation and analysis. In order to calibrate the flow cytometer, it is necessary to perform the Instrument Setup procedure every day on each flow cytometer before analyzing experiments. In order to quantitate test samples, it is necessary to run the BD™ CBA Cell Signaling Flex Set Standards in each experiment. (see ***Table 4*** for a detailed description of the materials to add to these assay control tubes or wells)

1. Add 50 µl of the mixed Capture Beads (see ***Preparation of BD™ CBA Cell Signaling Flex Set Capture Beads,*** page 50) to the appropriate assay tubes or wells. Vortex the mixed Capture Beads before adding them to the assay tubes or wells. Save unused mixed Capture Beads for instrument setup.
2. Add 50 µl of the mixed PE Detection Reagent to the assay tubes or wells.
3. Add 50 µl of the Standard dilutions to the control assay tubes or wells.
4. Add 50 µl of each denatured cell lysate test sample to the test assay tubes or wells.
5. For assays performed in tubes, mix assay tubes gently and incubate for 4 hours at RT and protect from direct exposure to light. For assays performed in filter plate wells, mix the microwell plate for 15 minutes using a digital shaker at 500 RPM (do not exceed 600 RPM) and incubate plate for 4 hours at RT and protect from direct exposure to light.
6. For assays run in tubes, add 1.0 ml of Wash Buffer to each assay tube and centrifuge at 200 × g for 5 minutes. For assays run in filter plate wells, apply the plate to the vacuum manifold and vacuum aspirate (do not exceed 10" Hg of vacuum) until wells are drained (2 – 10 seconds).
7. For assays run in tubes, carefully aspirate and discard the supernatant from each assay tube. For assays run in filter plate wells, proceed to step 8.
8. Add 300 µl of Wash Buffer to each assay tube or 150 µl of Wash Buffer to each assay well. Vortex assay tubes briefly or shake microwell plate on a digital shaker at 500 RPM for 5 minutes to resuspend beads.
9. Begin analyzing samples on a flow cytometer. For assays run in tubes, it is recommended that each tube be vortexed briefly before analyzing on the flow cytometer.*

**Note:* It is best to analyze CBA samples on the day of the experiment. Samples analyzed using the BD CBA Cell Signaling Flex Set assays may be analyzed up to 24 hours after completion of the assay. Prolonged storage of samples, once the assay is complete, can lead to increased background and reduced sensitivity.

Tube No.	Reagents (All reagent volumes are 50 µl)
1 (Negative Control 0 Units/ml Standard)	Capture Beads, Assay Diluent, PE Detection Reagent
2 (3.9 Units/ml Standard)	Capture Beads, Standard 1:256 Dilution, PE Detection Reagent
3 (7.8 Units/ml Standard)	Capture Beads, Standard 1:128 Dilution, PE Detection Reagent
4 (15.6 Units/ml Standard)	Capture Beads, Standard 1:64 Dilution, PE Detection Reagent
5 (31.25 Units/ml Standard)	Capture Beads, Standard 1:32 Dilution, PE Detection Reagent
6 (62.5 Units/ml Standard)	Capture Beads, Standard 1:16 Dilution, PE Detection Reagent
7 (125 Units/ml Standard)	Capture Beads, Standard 1:8 Dilution, PE Detection Reagent
8 (250 Units/ml Standard)	Capture Beads, Standard 1:4 Dilution, PE Detection Reagent
9 (500 Units/ml Standard)	Capture Beads, Standard 1:2 Dilution, PE Detection Reagent
10 (1000 Units/ml Standard)	Capture Beads, Standard "Top Standard", PE Detection Reagent

Table 4. Essential control assay tubes

Product List

Instrumentation

Description	Cat. No.
BD FACSArray Bioanalyzer	340128

BD™ Cytometric Bead Array Flex Sets

Software

Description	Contents	Size	Cat. No.
FCAP Array Software	PC Compatible Software and User's Guide	CD	338621

Phospho specific and Total Signaling Proteins Supporting Reagents

Description	Contents	Size	Cat. No.
Cell Signaling Proteins Master Buffer Kit	Buffers and setup reagents required for use with BD CBA Cell Signaling Proteins Flex Sets	Sufficient for 100 tests of any size BD CBA Flex Set	558223
Cell Signaling Proteins Master Buffer Kit	Buffers and setup reagents required for use with BD CBA Cell Signaling Proteins Flex Sets	Sufficient for 500 tests of any size BD CBA Flex Set	558224

Phospho specific Flex Sets*

Description	Contents	Bead Location	Size	Cat. No.
Btk (Y551)	Capture Bead and Detection Reagents plus 1 standard	D5	100 tests	558236
eNOS	Capture Bead and Detection Reagents plus 1 standard	C7	100 tests	558239
ERK1/2 (T202/Y204)	Capture Bead and Detection Reagents plus 1 standard	C4	100 tests	558234
Itk (Y511)	Capture Bead and Detection Reagents plus 1 standard	C6	100 tests	558230
JNK1/2 (T183/Y185)	Capture Bead and Detection Reagents plus 1 standard	B5	100 tests	558235
p38/MAPKinase (T180/Y182)	Capture Bead and Detection Reagents plus 1 standard	B6	100 tests	558233
PLCγ (Y783)	Capture Bead and Detection Reagents plus 1 standard	B7	100 tests	558228
RSK (T573)	Capture Bead and Detection Reagents plus 1 standard	D7	100 tests	558240
Stat1 (Y701)	Capture Bead and Detection Reagents plus 1 standard	C5	100 tests	558222
Syk (Y352)	Capture Bead and Detection Reagents plus 1 standard	B9	100 tests	558237
ZAP70 (Y319)	Capture Bead and Detection Reagents plus 1 standard	B8	100 tests	558229

Total Signaling Protein Flex Sets*

Description	Contents	Bead Location	Size	Cat. No.
Stat1	Capture Bead and Detection Reagents plus 1 standard	D4	100 tests	558227
Syk	Capture Bead and Detection Reagents plus 1 standard	B9	100 tests	558238
ZAP-70	Capture Bead and Detection Reagents plus 1 standard	B8	100 tests	558232

* BD™ CBA Total Signaling Protein Flex Sets may not be run in the same assay well as Phospho specific Flex Sets.

Notes

Chapter 3

Non-Flow Cytometry Based Applications for Phospho Antibodies

Immunofluorescent Microscopy and High Content Analysis using phospho-specific antibodies

Introduction

Immunofluorescent microscopy (IF) is the most widely used microscopy technique enabling the visualization of cellular components through the use of i.e. fluorochrome conjugated antibodies. IF further allows researchers to study the temporal and spatial localization of protein-phosphorylation events in their natural microenvironment.

A large selection of BD Transduction Laboratories™ and BD Pharmingen™ high quality monoclonal antibodies has been evaluated for IF. ***Figures 1 – 5*** give examples how our phospho-antibodies enable you to visualize site-specific phosphorylation events within cells.

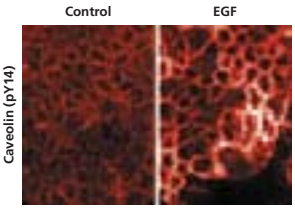


Figure 1. Immunofluorescent staining using anti-Caveolin (pY14) (Cat. No. 611338) in A431 cells either untreated or treated with EGF.

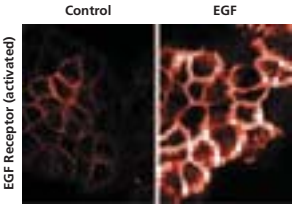


Figure 2. Immunofluorescent staining using anti-Activated EGFR (Cat. No. 610025) in A431 cells either untreated or treated with EGF.

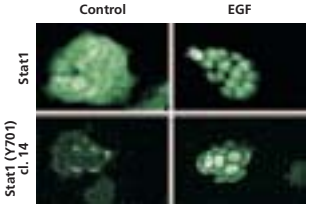


Figure 3. Immunofluorescent staining using anti-Stat1 (Cat. No. 610115) and anti-Stat1 (pY701) (Cat. No. 612132) in A431 cells either untreated or treated with EGF.

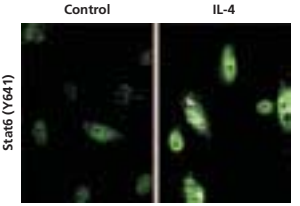


Figure 4. Immunofluorescent staining using anti-Stat6 (pY641) (Cat. No. 611566) in human endothelial cells either untreated or treated with IL-4.

Figure 5. Immunofluorescent staining using anti-IkBa (Cat. No. 610690) shows transient degradation of IkBa in HeLa cells during treatment with TNF.

With the addition of the instrument platforms BD™ Pathway and BD™ CARV II for automated life cell confocal imaging, BD Biosciences now offers complete solutions for high content analysis (see ***Appendix A,*** page 93 for instrument details).

Using directly fluorochrome conjugated phospho-antibodies on a BD Pathway imaging system enables you to analyze multiple phospho-proteins within single cells in a high throughput set up. The example presented (***Figures 6,7***) show A431 (epidermal carcinoma) cells in 96-well plates, tested for their activation status upon exposure to IL-4 and/or IFN-γ.

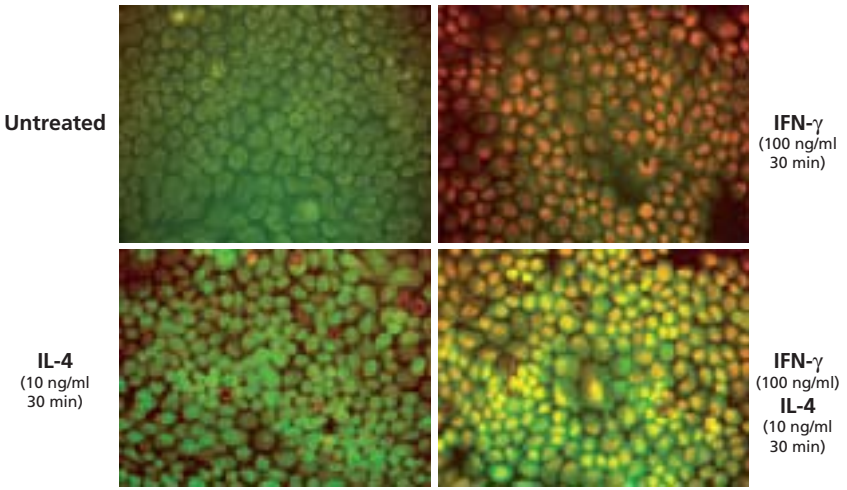


Figure 6. A431 cells in 96-well plates were either untreated or treated with IL-4 and/or IFN-γ. Cells were then fixed, permeabilized and stained with AlexaFluor® 647 –anti Stat1 (pY701) (Red), and AlexaFluor® 488 –anti Stat6 (pY641) (Green). Please inquire for the availability of directly conjugated phospho-antibodies for imaging.

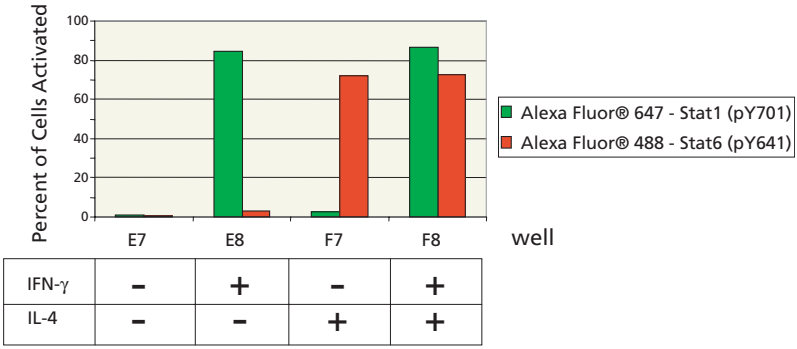


Figure 7. Pathway Specific Activation. While cells stimulated with IFN-γ show mainly Stat1 phosphorylation, stimulation with IL-4 leads mainly to Stat6 phosphorylation. Activation with both cytokines results in Stat1 and Stat6 phosphorylation. No phosphorylation is detected in unstimulated cells.

Alexa Fluor® is a registered trademark of Molecular Probes, Inc., Eugene, Or.

Protocols

Cell Staining for Immunofluorescence Microscopy

Preparation of Cells

Chamber Slide or 96 well Plate Preparation for Adherent Cells

1. Grow cells on 96-well plate or chamber slide.
2. Remove the medium from each well or chamber.

Coverslip Preparation for Adherent Cells

1. Grow cells on glass coverslips.
2. Rinse briefly in phosphate-buffered saline (PBS).

Coverslip Preparation for Non-Adherent Cells

1. Coat coverslips with an excess of poly-L-lysine (0.01% solution, Sigma, Cat. No. P4707) for 10 minutes at room temperature.
2. Aspirate the poly-L-lysine solution and allow coverslips to dry completely.
3. Transfer cells in medium to 50ml tubes.
4. Centrifuge (400 × g, 15°C) for 5 minutes.
5. Aspirate the medium and resuspend the cells in 30ml phosphate-buffered saline (PBS).
6. Cover the dried, treated coverslips with the cell suspension.
7. Incubate at room temperature for 30-60 minutes.
8. Aspirate excess cell suspension.
9. Rinse briefly in PBS.

Fixing the Cells

Paraformaldehyde Fixation

1. Dump the medium and cover cells with 3.7% paraformaldehyde solution at 37°C (for 100ml: 10ml 10× PBS, 33.4ml of 11.1% formaldehyde, 0.6ml 30% Triton-X, 56ml distilled water).

   Note: The solution is stable for 1 week at 4°C. Paraformaldehyde should be prepared fresh. It is toxic and should be handled appropriately in a fume hood.

2. Incubate at room temperature for 10 minutes.
3. Dump the paraformaldehyde solution solution and rinse 3× with PBS.

Methanol/Acetone Fixation

1. Dump the medium and a 1:1 mixture of methanol and acetone (prepared at –20°C) to each well, chamber slide or coversip.
2. Incubate the plate at –20°C for 10 minutes.
3. Dump the methanol/acetone solution and rinse 3× with PBS (100 ul/well).

Blocking

Place the coverslips cells-side-up in a petri dish. Rinse the cells with PBS, then cover the cells with blocking buffer (1% BSA in PBS) for 30 minutes at 37°C to minimize non-specific adsorption of the antibodies to the surface.

Incubation with Primary Antibody

1. Remove the blocking buffer.
2. Dilute primary antibody to 1.0–10 µg/ml in blocking buffer (optimal concentration will depend on several variables, such as the affinity of the antibody and the abundance of the antigen).
3. Distribute sufficient primary antibody solution to cover all cells in each well, chamber slide, or coverslip and incubate for 1 hour at room temperature.
4. Decant the antibody solution or remove by aspiration.
5. Wash 3× in PBS, 5 minutes each wash.

Incubation with more than one Primary Antibody

If it is desirable to examine the co-distribution of two different antigens in the same cell, a double immunofluorescence procedure may be used. Cells may be incubated simultaneously with two primary antibodies, provided they are monospecific and can be distinguished with secondary antibodies conjugated to different fluorochromes (or with primary antibodies directly conjugated to different fluorochromes).

1. Incubate cells with the mixture of diluted primary antibodies for 1 hour at room temperature.
2. Decant the antibody solution or remove by aspiration.
3. Wash 3× in PBS, 5 minutes each wash.

Incubation with Secondary Antibodies

Note: If the primary antibodies are already conjugated to a fluorochrome, incubation with secondary antibody is unnecessary.

The cells are now incubated with secondary antibodies conjugated to a fluorochrome; eg, anti-mouse IgG:FITC or Cy3, depending on the donor species of the primary antibody and the desired fluorochrome. We recommend the use of cross-adsorbed and affinity-purified secondary antibodies to minimize background and non-specific reactivity from the secondary antibody. High-quality conjugated antibodies are essential for the avoidance of cross-reactivity between two different antibodies in double immunofluorescence protocols.

1. Dilute the secondary antibody to the appropriate concentration in blocking buffer. Add enough secondary antibody solution to cover the surface of each well, chamber slide, or coverslip.
2. Incubate for 1 hour at room temperature.
3. Remove the secondary antibody.
4. Wash three times in PBS, 5 minutes each wash.

Immunohistochemistry using Phospho Specific Antibodies

Introduction

Using Immunohistochemistry (IHC), protein phosphorylation now can also be studied *In Situ*. At BD Biosciences we have validated our phosphorylation site specific antibodies across different applications. The same monoclonal antibody that detects total phospho-protein concentration in lysates, now can also be used to study protein localization within cells and tissues. IHC allows researchers to visualize protein-phosphorylation in its micro-environment and to study protein protein interaction and translocation events simultaneously.

We have used different *In Vivo* stimulation rat models and an *Ex Vivo* stimulation model using human tonsils to study protein activation *In Situ*. In addition, we also have looked at a variety of tumor tissues to detect the up and down regulation of phospho specific markers. For each model, the specificity of antibodies has been validated by Western blotting. As a control, we have used phosphatase-treated tissue sections to verify phospho-specific binding. Please refer to our ***Antibodies for Phospho Protein Analysis*** product list (pages 79–87) for antibodies tested by IHC.

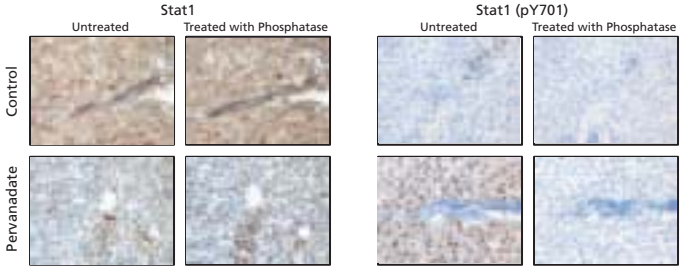


Figure 8. Stat1 staining on rat liver. The solutions of pervanadate or PBS were injected intraperitoneally into rats at a dose of 10 µl/g of body weight. The liver was removed and fixed in formalin, processed, and sectioned. The liver sections were then either left untreated or treated with a phosphatase to eliminate all phosphorylation. The tissue sections were stained with purified Stat1 antibody (Cat. No. 610185) or purified Stat1 (pY701) (Cat. No. 612232).

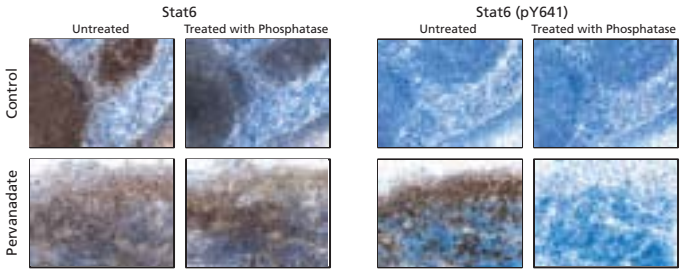


Figure 9. Stat6 staining on human tonsil. Fresh human tonsil was either incubated in PBS (Control) or 5 mM Pervanadate solution for 2 hours. Following stimulation the tonsil was fixed in formalin and processed. The tonsil sections were then either left untreated or treated with a phosphatase to eliminate all phosphorylation. The tissue sections were stained with purified Stat6 antibody (Cat. No. 611290) or purified Stat6 (pY641) (Cat. No. 611820).

Preparation and Staining of Paraffin Sections

I. Fixation and Processing of Tissue for Paraffin Sections

A. Fixation of Tissues in 10% Neutral Buffered Formalin

1. Sacrifice animal by prescribed and approved euthanasia techniques. Tissues to be fixed and processed should be cut to a size no larger than 3mm thick. Let tissues fix in 10% formalin at room temperature for 8 hours but not to exceed 24 hours. For small rodent tissue, it is recommended to fix tissues for 4-8 hours prior to processing the tissue.
2. Follow processing schedule recommended in section C.

B. Fixation of Tissues in Zinc Fixative:

Many antigenic epitopes are masked or even destroyed by 10% formalin fixation. In some cases fixation in a milder fixative such as Zinc fixative for IHC (Cat. No. 550523) is helpful to preserve the antigenic epitopes.

1. Place freshly dissected tissues trimmed 3mm thick into Zinc Fixative and allow tissues to fix for 24-48 hours at room temperature.
2. Follow processing schedule recommended in section C.

C. Processing Schedule:

Note: The processing, embedding and sectioning of paraffin blocks requires specialized equipment and expertise and is usually performed by a histology or pathology laboratory. While hand processing can be performed according to the following protocol the results may show marked variation in histology quality and antigenicity.

Station Time Solution

1. - Original fixative
2. 45 minutes 70% Alcohol
3. 45 minutes 80% Alcohol
4. 45 minutes 95% Alcohol
5. 45 minutes 100% Alcohol
6. 60 minutes 100% Alcohol
7. 60 minutes 100% Alcohol
8. 60 minutes Clearing Reagent (xylene or substitute)
9. 60 minutes Clearing Reagent (xylene or substitute)
10. 60 minutes Paraffin 1
11. 60 minutes Paraffin 2
12. 60 minutes Paraffin 3

II. Preparation of Paraffin Sections for Immunohistochemistry

A. Sectioning Protocol:

1. Section paraffin blocks at the desired thickness (usually 4-5 µm) on a microtome and float on a 40°C water bath containing distilled water.
2. Transfer the sections onto a Superfrost Plus slide. Allow the slides to dry overnight and store slides at room temperature until ready for use.

B. Deparaffinization and Rehydration of Tissue Slide:

1. Before deparaffinization, place the slides in a 55°C oven for ten minutes to melt the paraffin. Deparaffinize slides in 2 changes of xylene or xylene substitute for 5 minutes each.
2. Transfer slides to 100% alcohol, 2 changes for 3 minutes each and transfer once through 95% alcohol for 3 minutes.
3. Block endogenous peroxidase activity by incubating sections in 3% H_2O_2 solution in methanol for 10 minutes.
4. Rinse twice in PBS for 5 minutes each time.
5. If the antibody staining requires antigen retrieval to unmask the antigenic epitope refer below to section C. If antigen retrieval is not required proceed to section D.

C. Pretreatment of Paraffin Sections with BD Retrievagen A* (pH 6.0):

1. Make a working solution of Retrievagen A (Cat. No. 550524) by mixing 18 ml of Retrievagen A solution 1 and 82 ml of Retrievagen A solution 2 and bring the final volume to 1 liter in distilled water.
2. Place slides in a plastic coplin jar filled with the working Retrievagen solution and heat in a microwave oven to 193°F (89°C) (microwave oven ** or other heating sources such as pressure cooker (see alternate protocol), water bath can be used).
3. Mix the working Retrievagen A solution in the coplin jar with a disposable pipet and incubate the slides at 193°F for 10 minutes.
4. Remove the coplin jar with the slides, cover the jar tightly, and allow the solution to slowly cool to room temperature for 20 minutes.

   Note: It is important to let the temperature ramp down slowly to allow the protein molecules to fold properly.

5. Rinse slides in PBS 3×, 5 minutes each time.

   Note: *For methodology on other antigen retrieval systems, refer to the instructions in technical data sheets.

   Note: **Heating by use of microwave oven may require a license under US patent No. 5244787.

Alternate Protocol

1. For antigen retrieval using a pressure cooker or an autoclave, pretreat slides with BD Retrievagen A solution in a coplin jar as outlined in step C1 above.
2. Heat coplin jar containing slides with BD Retrievagen A solution in a pressure cooker or autoclave at 120-125°C and 17-25 psi for 5 minutes.
3. When completed, open pressure cooker or autoclave and allow slides to cool to room temperature (approximately 20-30 minutes) prior to removing them from the coplin jar.
4. Wash slides as indicated in step C5 above.

D. Immunohistochemical Staining of Paraffin Embedded Tissues:

Refer to ***Standard Immunohistochemical Staining Procedure*** (Section III of Immunohistochemical Staining of Frozen Sections).

Begin at step 5 and proceed through coverslipping.

Preparation and Staining of Frozen Tissue Sections

I. Preparation of Frozen Tissue for Sectioning

Materials neeed:

- 2-methylbutane (isopentane)
- Liquid Nitrogen
- Dry ice
- Peel-Away® base molds
- Frozen tissue matrix (OCT® or Cryomatrix®)
- Long forceps
- Necropsy tools
- Superfrost Plus slides

1. Label base mold and partially fill the mold with frozen tissue matrix.
2. Sacrifice animal by prescribed and approved euthanasia techniques.
3. Remove desired tissues, trim and cut tissue no more than 5 mm thick. Place in pre-labeled base molds filled with frozen tissue matrix. Arrange tissue in the matrix near the bottom so tissue is easily exposed when sections are cut.
4. Place a stainless steel beaker of 2-methylbutane in liquid nitrogen and allow to cool adequately. Place base mold with tissue into the beaker of cold 2-methylbutane and quickly immerse the block. Allow the tissue matrix to solidify completely and remove block from 2-methylbutane and place on dry ice or in the -20°C cryostat.

   Note: If block is left in 2-methylbutane too long, the block may crack.
5. Store blocks in the -80°C freezer until ready for sectioning.

II. Sectioning of Frozen Tissues

1. Before cutting sections, allow the temperature of the block to equilibrate to the temperature of the cryostat (-20°C).
2. Place the tissue block on the cryostat specimen disk. Adjust the positioning of the block to align the block with the knife blade. Cut tissue block until the desired tissue is exposed.
3. Cut sections of the desired thickness (usually 5 µm), place the sections on a Superfrost slide and dry overnight at room temperature.
4. Fix slides by immersion in cold acetone (-20°C) for 2 minutes or other suitable fixative (e.g. alcohol, formal alcohol, formalin, etc.), air dry at room temperature and proceed to staining (***Section III***).
5. Alternatively, the frozen section slides can be stored for a short period of time at -70°C in a sealed slide box. When ready to stain, remove slides from freezer and warm to -20°C in the cryostat or -20° freezer, fix for 2 minutes in cold fixative (acetone or other suitable fixative) and allow to come to room temperature to continue with the staining.

III. Standard Immunohistochemical Staining Procedure for Frozen Sections

Please read entire procedure before staining sections. Perform all incubations in a humid chamber and do not allow sections to dry out. Isotype and system controls should also be run and must be matched to the isotype of each primary antibody to be tested.

Materials needed:

- Phosphate Buffered Saline (PBS)
- H_2O_2 Solution
- Antibody Diluent for IHC (Cat. No. 559148)
- Streptravidin-Horseradish Peroxidase (Cat. No. 550946)
- DAB Substrate Kit (Cat. No. 550880)
- Hematoxylin
- Bluing Reagent
- Graded alcohols
- Xylene

More conveniently, our Ig HRP Detection Kits can be used to perform the immunohistochemical staining (Cat. Nos. 551011, 551012, and 551013).

1. Label slides with a solvent resistant pen and demarcate the tissue if required.
2. Rinse slides 3× in PBS, to remove the tissue-freezing matrix.
3. Block endogenous peroxidase activity by incubating the slides in 0.3% H_2O_2 solution in PBS for 10 minutes.

4. Rinse slides 3× in PBS, 2 minutes each time.
5. Block non-specific binding by incubating with blocking buffer (10% serum from host species of secondary antibody diluted in PBS or 10% FBS in PBS) for 30-60 min at room temperature in a humidified chamber.
6. Dilute the primary antibody in the Antibody Diluent for IHC. Alternatively, a buffered solution with a source of protein can be used as antibody diluent. Apply the diluted antibody to the tissue sections on the slide. Incubate for 1 hour at room temperature in a humidified chamber.
7. Rinse slides 3× in PBS, 2 minutes each time.
8. Dilute the biotinylated secondary antibody in the Antibody Diluent for IHC. Alternatively, a buffered solution with a source of protein can be used as antibody diluent. Apply to the tissue sections on the slide and incubate for 30 minutes at room temperature.
9. Rinse slides 3× in PBS, 2 minutes each time.
10. Apply the Streptravidin-Horseradish Peroxidase pre-diluted to the tissue sections on the slide and incubate for 30 minutes at room temperature.
11. Rinse slides 3× in PBS, 2 minutes each time.
12. Prepare DAB substrate solution by adding 1 drop of DAB chromagen to every 1 ml of DAB buffer. (When using other substrates follow manufacturers recommendations.)

    Safety Note: DAB is a suspect carcinogen. Handle with care. Wear gloves, lab coat and eye protection.
13. Drain PBS from slides and apply the DAB substrate solution. Allow slides to incubate for 5 minutes or until the desired color intensity is reached.
14. Wash 3× in water, 2 minutes each time.
15. Counterstain slides:
    a. Dip twice in Hematoxylin.
    b. Rinse thoroughly in water.
    c. Dip twice in Bluing Reagent or dilute ammonia water.
    d. Rinse thoroughly in water.
16. Dehydrate through 4 changes of alcohol (95%, 95%, 100% and 100%). Clear in 3 changes of xylene (or xylene substitute) and coverslip.

Immunopurification and immunoprecipitation using Phospho Specific Monoclonal Antibodies

Introduction

Immunoprecipitation (IP) is a procedure by which proteins that react specifically with an antibody are removed from solutions like whole cell lysates or culture supernatants for subsequent further analysis including SDS PAGE and Western blot.

Typically the target specific antibody is immobilized on beads that allow the precipitation of antibody-antigen complexes by centrifugation.

The antibodies can be directly linked to the matrix covalently or indirectly by immunoglobulin binding proteins like Protein A, Protein G or secondary antibody.

The choice of immobilized antibody binding protein depends upon the species that the antibody was raised in. ***Table 1*** provides relative affinities of immunoglobulin binding proteins to the different immunoglobulin isotypes of various species.

BD Biosciences also offers anti-phospho tyrosine directly conjugated to agarose (Cat. No. 610015), which enables the purification of tyrosine phosphorylated proteins from whole cell lysates. In subsequent Western blot analysis the purified activated proteins can be identified using the broad range of BD Transduction Laboratories™ antibodies.

Species/ Subclass	Protein A	Protein G
Monoclonal		
Human		
IgG_1	+++	+++
IgG_2	+++	+++
IgG_3	-	+++
IgG_4	+++	+++
Mouse		
IgG_1	+	++
IgG_{2a}	+++	+++
IgG_{2b}	++	++
IgG_3	+	+++
IgM	-	-
IgA	-	-
IgE	-	-
Rat		
IgG_1	+	+
IgG_{2a}	-	+++
IgG_{2b}	-	++
IgG_{2c}	+	++
Polyclonal		
Rabbit	+++	++
Goat	-	++

Table 1. Relative Affinity of Immobilized Protein A and Protein G for Various Antibody Species and Subclasses of Polyclonal and Monoclonal Immunoglobulins

Protocols

Immunoprecipitation With Soluble Antibodies

Note: Please read the entire protocol before starting your experiment.

Preparation of the Cell Lysate

Denaturing Conditions

1. Rinse a 60mm culture dish of confluent cells with 1× phosphate-buffered saline (PBS).
2. Lyse the cells with 0.5ml boiling Lysis Buffer (1% SDS, 1.0mM sodium ortho-vanadate, 10mM Tris pH 7.4).
3. Scrape the cells from the dish, transfer lysate to a 1.5 ml microcentrifuge tube, and boil for an additional 5 minutes.
4. Pass several times through a 26 gauge needle; centrifuge (16,000 × g) for 15 minutes.

Native Conditions

1. Rinse a 60mm culture dish of confluent cells with PBS.
2. Lyse the cells with 0.5ml cold Immunoprecipitation (IP) Buffer (1% Triton X-100, 150mM NaCl, 10mM Tris pH 7.4, 1mM EDTA, 1mM EGTA pH 8.0, 0.2mM sodium ortho-vanadate, protease inhibitor cocktail (Roche Applied Science), 0.5% IGEPAL CA-630).
3. Maintain constant agitation for 30 minutes at 4°C.
4. Scrape the cells from the dish and pass several times through a 26 gauge needle to disperse any large aggregates. Centrifuge (16,000 × g, 4°C) for 15 minutes; keep on ice.

Pre-clearing

It is important to pre-clear the lysate immediately before immunoprecipitation.

1. Centrifuge the cell lysates (16,000 × g, 4°C) for 15 minutes. Remove the supernatant.
2. For the denatured lysates, boil the supernatant for 5 minutes.
3. To 750-1000µl of supernatant, add 5µg of rabbit anti-mouse IgG antibody, vortex, and then add 75-100µl of Protein A:Agarose (Cat. No. 610437). Incubate at 4°C for 30 minutes with agitation.
4. Centrifuge lysate (9000 × g, 4°C) for 2 minutes to pellet the agarose beads. The supernatant is the "total cell lysate".

Immunoprecipitation

1. To a microcentrifuge tube, add 1–5µg of antibody, 400ml of water, 200-500µg of total lysate and 500µl of 2× Immunoprecipitation (IP) Buffer (2% Triton X-100, 300mM NaCl, 20mM Tris pH 7.4, 2mM EDTA, 2mM EGTA pH 8.0, 0.4mM sodium ortho-vanadate, protease inhibitor cocktail (Roche Applied Science), 1.0% IGEPAL CA-630).
2. Vortex and incubate for 1 hour with agitation at 4°C. If monoclonal antibodies are used, add 5µg rabbit anti-mouse IgG antibody, vortex, and continue the incubation for an additional 30 minutes at 4°C.
3. Add 50µl of 50% Protein A:Agarose (Cat. No. 610437). Vortex and incubate for 30 minutes with agitation at 4°C.
4. Wash with cold 1× IP Buffer (1% Triton X-100, 150mM NaCl, 10mM Tris pH 7.4, 1mM EDTA, 1mM EGTA pH 8.0, 0.2mM sodium ortho-vanadate, protease inhibitor cocktail, 0.5% IGEPAL CA-630) by centrifuging 2 minutes (8000 × g, 4°C). Decant supernatant and repeat wash twice.
5. Resuspend pellet in 50µl of 0.1 Glycine pH 2.5 vortex and incubate with agitation for 10 minutes at 4°C.
6. Centrifuge (9000 × g, 4°C) for 2 minutes. Remove supernatant, this is your IP sample.
7. Add 5µl of 1M Tris pH 8.0 to each tube to neutralize the pH. Add approximately 10µl of 5× concentrated electrophoresis sample buffer (125mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% ß-mercaptoethanol) to each sample, and boil for 5 minutes.
8. Load the supernatant onto an SDS-PAGE gel and electrophorese.
9. Transfer to PVDF and probe with appropriate antibodies (Refer to Western blot protocols for monoclonal or polyclonal antibodies).

Immunoprecipitation with Antibody:Agarose Conjugates

Preparation of the Cell Lysate

Please follow the same protocol as described for ***Immunoprecipitation with Soluble Antibodies*** (page 69).

Immunoprecipitation

1. To a microcentrifuge tube, add 25µl of a 50% suspension of antibody:agarose conjugate, 400ml of water, 200-500mg of total lysate and 500ml of 2× IP Buffer (2% Triton X-100, 300mM NaCl, 20mM Tris pH 7.4, 2mM EDTA, 2mM EGTA pH 8.0, 0.4mM sodium ortho-vanadate, protease inhibitor cocktail (Roche Applied Bioscience), 1.0% IGEPAL CA-630).
2. Vortex and incubate for 1 hour with agitation at 4°C.

3. Wash with cold 1× IP Buffer (1% Triton X-100, 150mM NaCl, 10mM Tris pH 7.4, 1mM EDTA, 1mM EGTA pH 8.0, 0.2mM sodium ortho-vanadate, protease inhibitor cocktail, 0.5% IGEPAL CA-630) by centrifuging 2 minutes (8000 × g, 4°C). Decant supernatant and repeat wash twice.
4. Resuspend pellet in 50µl of 0.1M Glycine pH 2.5 vortex and incubate with agitation for 10 minutes at 4°C.
5. Centrifuge (9000 × g, 4°C) for 2 minutes. Remove supernatant, this is your IP sample.
6. Add 5µl of 1M Tris pH 8.0 to each tube to neutralize the pH. Add approximately 10µl of 5× Concentrated Electrophoresis Sample Buffer (125mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% ß-mercaptoethanol) to each sample, and boil for 5 minutes.
7. Load the supernatant onto an SDS-PAGE gel and electrophorese.
8. Transfer to PVDF and probe with appropriate antibodies (Refer to Western blot protocols for monoclonal or polyclonal antibodies).

Immunoprecipitation with anti-Phosphotyrosine:Biotin Conjugates

Preparation of the Cell Lysate

Please follow the same protocol as described for ***Immunoprecipitation with Soluble Antibodies*** (page 69).

Immunoprecipitation

1. To a microcentrifuge tube, add 2–5µg of anti-phosphotyroscine: biotin, 400ul of water, 200-50µg of total lysate and 500µl of 2× IP Buffer (2% Triton X-100, 300mM NaCl, 20mM Tris pH 7.4, 2mM EDTA, 2mM EGTA pH 8.0, 0.4mM sodium ortho-vanadate, protease inhibitor cocktail (Roche Applied Bioscience), 1.0% IGEPAL CA-630).
2. Vortex and incubate for 1 hour with agitation at 4°C.
3. Add 20µl of Streptavidin immobilized on agarose (Pierce). Vortex and incubate for 30 minutes with agitation at 4°C.
4. Wash with cold 1× IP Buffer (1% Triton X-100, 150mM NaCl, 10mM Tris pH 7.4, 1mM EDTA, 1mM EGTA pH 8.0, 0.2mM sodium ortho-vanadate, protease inhibitor cocktail, 0.5% IGEPAL CA-630) by centrifuging 2 minutes (8000 × g, 4°C). Decant supernatant and repeat wash twice.
5. Resuspend pellet in 50µl of 0.1M Glycine pH 2.5 vortex and incubate with agitation for 10 minutes at 4°C.
6. Centrifuge (9000 × g, 4°C) for 2 minutes. Remove supernatant, this is your IP sample.

7. Add 5µl of 1M Tris pH 8.0 to each tube to neutralize the pH. Add approximately 10µl of 5X Concentrated Electrophoresis Sample Buffer (125mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% ß-mercaptoethanol) to each sample, and boil for 5 minutes.
8. Load the supernatant onto an SDS-PAGE gel and electrophorese.
9. Transfer to PVDF and probe with appropriate antibodies (Refer to Western blotting Protocol).

Note: Phosphotyrosine containing proteins can be selectively purified by loading a Streptavidin:agarose column and eluting the proteins with 10mM phenyl phosphate.

Immunopurification of Tyrosine Phosphorylated Proteins

Preparation of the cell lysate

Non-Denaturing Conditions

1. Rinse adherent or non-adherent cells from a 15cm dish with an excess of phosphate-buffered saline (PBS); repeat wash.
2. Lyse cells with 6ml of ice-cold lysis buffer (10mM imidazole pH 7.3, 0.5 M NaCl, 1% Triton X-100, 0.2mM sodium ortho-vanadate, 0.2mM PMSF, 2mM sodium azide) with gentle rotation for 30 minutes at 4°C.
3. Remove the lysate from the plate and centrifuge at 40,000rpm for 1.5 hour at 4°C.

Denaturing Conditions

1. Rinse adherent or non-adherent cells from a 15cm dish with an excess of phosphate-buffered saline (PBS); repeat wash.
2. Lyse cells with 3ml of boiling lysis buffer (1% SDS, 10mM Tris pH 7.4, 0.2mM sodium ortho-vanadate).
3. Microwave the cells for 5 seconds to assure complete lysis and denaturation.
4. Scrape the lysate from plates and centrifuge at 40,000rpm for 1.5 hour at 15°C.
5. Dilute the lysate 5-fold to a final concentration of 0.2% SDS, adjusted to 10mM imidazole pH 7.3, 50mM NaCl, 1% Triton X-100, 0.2mM sodium ortho-vanadate, 1mM EDTA.

Immunoprecipitation

1. Equilibrate anti-phosphotyroscine (PY20) coupled Affi-Gel 10 (Bio-Rad) in cold Resin Wash Buffer (10mM imidazole pH 7.3, 50mM NaCl, 1% Triton X-100, 0.1%SDS, 1mM EDTA) by repeated centrifugation and resuspension in resin wash buffer.
2. Incubate the lysate (up to 250 mg total protein) with 3ml of anti-phosphotyrosine (PY20) coupled to Affi-Gel 10 overnight at 4°C with constant rotation.

3. Collect the Affi-Gel by low speed centrifugation, resuspend in resin wash buffer, and load into a small column.
4. Wash the column with 20 volumes of resin wash buffer.
5. Wash the column with three volumes of low-detergent resin wash buffer (10mM imidazole pH 7.3, 50mM NaCl, 0.1% Triton X-100, 0.05% SDS, 1mM EDTA).
6. Elute the tyrosine-phosphorylated proteins with 5mM phenyl phosphate in the low-detergent buffer. The eluted proteins can be monitored by protein determination or Western blotting with anti-phosphotyrosine antibodies.
7. The column should be regenerated by extensive washing with a neutral pH buffer (like PBS) containing 1M NaCl, followed by several washes with PBS alone. Store the column at 4°C in PBS + 1.5mM sodium azide between uses.

Western Blot Analysis using Phospho Antibodies

Introduction

Western blotting is a technique used to electrophoretically transfer proteins from polyacrylamide gels onto a more stable membrane substrate such as nitrocellulose.

The resulting nitrocellulose membrane (Western blot) contains a replica of the protein pattern found on the polyacrylamide gel. An additional advantage is that proteins adsorbed onto nitrocellulose are more accessible to i.e. antibodies than are proteins within polyacrylamide gels.

BD Transduction Laboratories™ monoclonal antibodies have been developed to meet the highest quality standards. Screening for highest specificity and affinity, the goal always has been to provide single specific bands with a minimum of background for your Western blot analysis. BD Transduction Laboratories high quality monoclonal antibodies have enabled us to develop our patented BD PowerBlot℠ Western Array Screening Service (see page 88). As shown in figure 8, multiple high quality monoclonal antibodies can be mixed together in cocktails and allow the detection of up to 10 different proteins within one lane of a Western blot. Any lower quality antibodies wouldn't provide the specific and significant results you get, represented in this experiment.

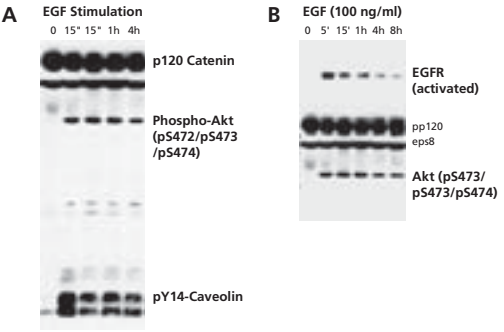


Figure 8. Multiple antibody probing using optimized antibody mixtures. Chosen Antigens vary by at least 10-20kD in size. ***A.*** Western blot analysis using anti-Akt (pS472/ pS473/pS474) (Cat. No. 559029) and anti-Caveolin (pY14) (Cat. No. 611338) in A431 cells treated with EGF. ***B.*** Western blot analysis using anti-Activated EGFR (Cat. No. 610025) and anti-Akt (pS472/pS473/ pS474) (Cat. No. 559029) in A431 cells treated with EGF.

Protocols

Western Blotting with Monoclonal Antibodies

Sample Preparation

For Protein Concentration Determination of Cell Culture

1. Decant medium from 10cm dish of adherent cells and rinse plate rapidly with phosphate-buffered saline (PBS).
2. Aspirate excess PBS.
3. Add 1ml boiling Lysis Buffer (1% SDS, 1.0mM sodium ortho-vanadate, 10mM Tris pH7.4).
4. Scrape cells from dish, transfer to a microcentrifuge tube, and boil for an additional 5 minutes. To reduce viscosity, the sample may be sonicated briefly or passed several times through a 26-gauge needle.
5. Centrifuge the sample for 5 minutes to pellet insoluble material, then discard pellet.
6. Dilute an aliquot of the cell lysate sample at least 10-fold for the BCA (Pierce) protein concentration assay (SDS concentration must be below 0.1% to avoid interference with the colorimetric reading).

For Protein Gel Electrophoresis of Cell Culture (without determining protein concentration)

1. Decant medium from 10cm dish of adherent cells and rinse plate rapidly with phosphate-buffered saline (PBS).
2. Aspirate excess PBS.
3. Add 1ml boiling 2× Concentrated Electrophoresis Sample Buffer (125mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 1.8% beta-mercaptoethanol).
4. Scrape cells from dish, transfer to a microcentrifuge tube, and boil for an additional 5 minutes. To reduce viscosity, the sample may be sonicated briefly or passed several times through a 26-gauge needle. Centrifuge the sample for 10 minutes to pellet insoluble material. Discard pellet.
5. The cell lysate sample (supernatant) is now ready for loading onto your gel.

For Protein Concentration Determination of Whole Tissue

1. Rapidly homogenize every 0.25g tissue in 3.5ml of boiling Lysis Buffer (1% SDS, 1.0mM sodium ortho-vanadate, 10mM Tris pH 7.4).
2. Microwave for 10–15 seconds.
3. Centrifuge the homogenate (16,000 × g, 15°C) for 5 minutes to pellet insoluble material, then discard pellet.
4. Dilute an aliquot of the tissue lysate sample at least 10-fold for the BCA (Pierce) protein concentration assay.

Polyacrylamide Gel Electrophoresis

Guidelines for choosing the percent gel to be used for certain molecular weight proteins (based on 37:1 acrylamide: bis acrylamide ratio)

4-5% gels: > 250 kDa

7.5% gels: 250-120 kDa

10% gels: 120-40 kDa

13% gels: 40-15 kDa

15% gels: < 20 kDa

Gel Electrophoresis

1. If not already in electrophoresis sample buffer, add an equal volume of 2× Sample Buffer (125mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 1.8% b-mercaptoethanol) to all samples and boil for 3–5 minutes.
2. Apply 5-20µg total protein of cell or tissue lysate to each well of a 0.75–1.0mm thick gel. For thicker gels (1.5mm thick), apply up to 25-40µg in each well.
3. Electrophorese until the bromophenol blue in the samples reaches the bottom of the gel. Turn off power supply. Keep gels in running buffer until ready to transfer.

Protein Blotting

Wet Transfer

Note: Since extra negative charges are needed to reach 1Amp in a wet transfer system, adjust the pH of the transfer buffer to approximately pH 8.0 using NaOH.

1. For transfer of proteins smaller than 20 kDa, transfer proteins from gel to PVDF (polyvinylidene difluoride) membrane at 1Amp constant current for 45 minutes or equivalent (250mAmp for 3 hours or 500mAmp for 90 minutes) in Transfer Buffer (25mM Tris, 190mM glycine, 20% MeOH).
2. For transfer of proteins smaller than 120 kDa, transfer proteins from gel to PVDF membrane at 1Amp constant current for 1 hour or equivalent (250mAmp for 4 hours or 500mAmp for 2 hours) in Transfer Buffer (25mM Tris, 190mM glycine, 20% MeOH).
3. For proteins larger than 120kDa, transfer to PVDF membrane at 1Amp constant current for 90 minutes or equivalent (250mAmp for 6 hours or 500mAmp for 3 hours) in Transfer Buffer + SDS (25mM Tris, 190mM glycine, 20% MeOH, 0.05% SDS).
4. For Proteins larger than 250kDa, transfer to PVDF membrane at 1Amp constant current for 1 hour and 45 minutes or equivalent (500mAmp for 3.5 hours) in Transfer Buffer + SDS (25mM Tris, 190mM glycine, 20% MeOH, 0.05% SDS).

Semi-Dry Transfer

For transfer of proteins from 10% or 13% gels to PVDF membranes semi-dry transfer can also be used. Transfer proteins to PVDF membrane at 1.2 mAmp/cm2 for 1 hour and 45 minutes in Transfer Buffer (25mM Tris, 190mM glycine, 20% MeOH).

Optional

If blots are not to be used for colorimetric detection, visualize the transferred proteins by staining the membrane for 15 minutes with India ink (Higgins black India ink, Eberhard Faber) diluted 1:1000 in Wash Buffer (10mM Tris pH 7.5, 100mM NaCl, 0.1% Tween 20). Rinse excess stain with wash buffer before blocking.

Blocking

For All Antibodies Except Phosphotyrosine

1. Remove the blot from the transfer apparatus or staining tray and immediately place into Blocking Buffer (5% non-fat dry milk, 10mM Tris pH 7.5, 100mM NaCl, 0.1% Tween 20).
2. Incubate the blot for 30 minutes at 37°C, 1 hour at room temperature, or overnight at 4°C.

For Phosphotyrosine Antibodies

1. Remove the blot from the transfer apparatus or staining tray and immediately place into Blocking Buffer (1% BSA, 10mM Tris pH 7.5, 100mM NaCl, 0.1% Tween 20).
2. Incubate the blot for 30 minutes at 37°C, 1 hour at room temperature, or overnight at 4°C.

Incubation with Primary and Secondary Antibodies

Primary antibody

1. Dilute the antibody in the corresponding blocking buffer.
2. Decant the blocking buffer from the blot, add the antibody solution, and incubate with agitation for 30 minutes at 37°C, 1 hour at room temperature, or overnight at 4°C.

Enzyme conjugated secondary antibody

Note: The inclusion of sodium azide is to be avoided in all steps using HRPO (horseradish peroxidase) conjugates.

1. Decant the primary antibody solution, add Wash Buffer (10mM Tris pH 7.5, 100mM NaCl, 0.1% Tween 20), and wash for 30 minutes with agitation, changing the wash buffer every 3–5 minutes.
2. Dilute the enzyme conjugate anti-mouse Ig HRP (Cat. No. 554002) 1:2000 in wash buffer containing 5% non-fat dry milk (or 1% BSA for phosphotyrosine antibodies).
3. Decant the wash buffer, add the diluted enzyme conjugate and incubate with agitation for 30 minutes at 37°C or 1 hour at room temperature.

Substrate Incubation

1. Decant the secondary antibody solution, add Wash Buffer (10mM Tris pH 7.5, 100mM NaCl, 0.1% Tween 20), and wash for 30 minutes with agitation, changing the wash buffer every 3–5 minutes.
2. Decant wash buffer and place the blot in a plastic bag or clean tray containing chemiluminescent working solution (0.125 ml/cm2). Rotate the bag or tray to allow the solution to cover the surface of the membrane for 1–5 minutes.
3. Remove blot from the bag or tray and place it between two pieces of write-on acetate transparency film. Smooth over covered blot to remove air bubbles and excess substrate.

   Expose to X-ray film or any sensitive screen. An initial exposure of 10-60 seconds is rcommended for film.

Antibodies for Phospho Protein Analysis

Detection	React	Clone	Apps	Format	Size	Cat. No.
Serine, Therine, and Tyrosine Phosphorylation Detection						
Phosphoserine	Hu, Rat	19	IF, WB	Purified	50 µg/150 µg	612546/47
Phosphoserine/Threonine	Hu, Rat	22a	IF, WB	Purified	50 µg/150 µg	612548/49
Phosphotyrosine	C, D, F, Hu, Ms, Rat	PY20	FCM, IF, IHC, IP, WB	Purified	1 mg	610000
Phosphotyrosine	C, D, F, Hu, Ms, Rat	PY20	IP, WB	Biotin	50 µg/150 µg	610007/08
Phosphotyrosine	C, D, F, Hu, Ms, Rat	PY20	WB	HRP	50 µg/150 µg	610011/12
Phosphotyrosine	C, D, F, Hu, Ms, Rat	PY69	FCM, IF, IHC, IP, WB	Purified	1 mg	610430
Phosphotyrosine	C, D, F, Hu, Ms, Rat	RC20	WB	AKP	50 µg/150 µg	610019/20
Phosphotyrosine	C, D, F, Hu, Ms, Rat	RC20	IP, WB	Biotin	50 µg/150 µg	610021/22
Phosphotyrosine	C, D, F, Hu, Ms, Rat	RC20	WB	HRP	50 µg/150 µg	610023/24
Phosphotyrosine	C, D, F, Hu, Ms, Rat	Polyclonal	FCM, IF, IHC, IP, WB	Purified	50 µg/150 µg	610009/10
Phosphotyrosine	C, D, F, Hu, Ms, Rat	PY69	IP	Agarose	500 µl	610015
Akt (PKBα)						
Akt (PKBα)	Hu, Ms, Rat	7	IF, WB	Purified	50 µg/150 µg	610836/37
Akt (PKBα)	Hu, Ms, Rat	55	IF, WB	Purified	50 µg/150 µg	610860/61
Akt (PKBα)	D, Hu, Ms, Rat	2	IF, WB	Purified	50 µg/150 µg	610876/77
Akt (pS472/pS473), Phospho-Specific	Hu, Ms, Rat	104A282	IP, WB	Purified	50 µg	550747
Akt (PKBα) Sampler Kit			WB	Kit	10 µg each	611437
Btk/Itk						
Btk	Hu	53	IF, IHC, WB	Purified	50 µg/150 µg	611116/17
Btk	Hu	G149-11	WB	Purified	0.1 mg	554239
c-cbl (pY774)	Hu	29	WB	Purified	0.1 mg	558035
Caveolin						
Caveolin	C, D, Hu, Ms, Rat	Polyclonal	FCM, IF, IHC, IP, WB	Purified	50 µg/150 µg	610059/60
Caveolin (pY14), Phospho-Specific	Hu, Ms, Rat	56	FCM, IF, IHC, WB	Purified	50 µg/150 µg	611338/39
Caveolin (pY14) Peptide				Peptide	100 µg	611582
Caveolin 1	C, Hu	2234	FCM, IF, IP, WB	Purified	50 µg/150 µg	610493/94
Caveolin 1	C, D, Hu, Ms, Rat	2297	FCM, IF, IHC, IP, WB	Purified	50 µg/150 µg	610406/07
Caveolin 1	D, Hu, Ms, Rab, Rat	C060	FCM, IF, IHC, IP, WB	Purified	50 µg/150 µg	610057/58
Caveolin 1	C	C20B	IF, WB	Purified	50 µg/150 µg	610387/88
Caveolin 2	Hu, Ms, Rat	65	WB	Purified	50 µg/150 µg	610684/85
Caveolin 2 (pY19), Phospho-Specific	Hu, Ms, Rat	PAb	WB	Purified	0.1 mg	557859
Caveolin 3	Ms, Rab, Rat	26	IHC, WB	Purified	50 µg/150 µg	610420/21
Caveolin Sampler Kit			WB	Kit	10 µg each	611766
c-Cbl						
c-Cbl	C, D, Hu, Ms, Rat	17	IF, IHC, IP, WB	Purified	50 µg/150 µg	610441/42
c-Cbl (pY700), Phospho-Specific	Hu	47	IF, IHC, WB	Purified	50 µg/150 µg	612304/05
B Cell Signaling Sampler Kit			IF, WB	Kit	10 µg each	611661
CD22						
CD22	Hu	48	IHC, WB	Purified	50 µg/150 µg	612462/63
CD22 (pY828)	Hu	46	IHC, WB	Purified	0.1 mg	558029
CD22 (pY843)	Hu	12a	WB	Purified	0.1 mg	558030

Antibodies for Phospho Protein Analysis *(continued)*

Detection	React	Clone	Apps	Format	Size	Cat. No.
Cdk1/Cdc2						
Cdk1/Cdc2	Hu, Ms, Rat	1	IF, IHC, IP, WB	Purified	50 µg/150 µg	610037/38
Cdk1/Cdc2		Polyclonal	IP, WB	Serum	0.1 ml	558900
Cdk1/Cdk2	Hu	AN21.2	WB	Purified	50 µg/150 µg	551526/25
Cdk1/Cdc2 (pY15), Phospho-Specific	Hu	44	IHC, WB	Purified	50 µg/150 µg	612306/07
Cell Cycle I Sampler Kit			IF, WB	Kit	10 µg each	611423
Cell Cycle II Sampler Kit			IF, WB	Kit	10 µg each	612744
β-Dystroglycan						
β-Dystroglycan	Hu	56	IHC, WB	Purified	50 µg/150 µg	612090/91
β-Dystroglycan (pY892), Phospho-Specific	Hu, Ms	27.1	FCM, IF, IHC, WB	Purified	50 µg/150 µg	612524/25
EGF Receptor						
EGF Receptor	Hu, Ms	13	IF, IP, IHC, WB	Purified	50 µg/150 µg	610016/17
EGF Receptor	Hu, Ms	13	IF	FITC	50 µg/150 µg	612554/55
EGF Receptor	Hu	EGFR1	FCM, IF	Purified	100 µg	555996
EGF Receptor	Hu	EGFR1	FCM	PE	100 tests	555997
EGF Receptor (Activated Form)	Hu	74	IF, IP, IHC, WB	Purified	50 µg/150 µg	610025/26
EGFR Activation Sampler Kit			WB	Kit	10 µg each	612476
ERK1/2						
ERK (pan ERK)	C, D, F, Hu, Ms, Rat	16	IF, IHC, IP, WB	Purified	50 µg/150 µg	610123/24
ERK1	Hu, Ms	G262-118	WB	Purified	100 µg	554100
ERK1	B, C, D, F, Hu, Ms, Rat	MK1	FCM, IF, IHC, IP, WB	Purified	50 µg/150 µg	610408/09
ERK1	B, C, D, F, Hu, Ms, Rat	MK12	FCM, IF, IHC, IP, WB	Purified	50 µg/150 µg	610030/31
ERK1	B, C, D, F, Hu, Ms, Rat	MK12	IHC, WB	HRP	50 µg/150 µg	610032/33
ERK1/2 (pT202/pY204), Phospho-Specific	B, C, D, F, Hu, Ms, Rat	20A	FCM, IF, IHC, WB	Purified	50 µg/150 µg	612358/59
ERK2	C, D, F, Hu, Ms, Rat	33	IF, IHC, IP, WB	Purified	50 µg/150 µg	610103/04
	Hu, Ms	I	IP, WB	Purified	0.1 mg	554095
MAP Kinase Sampler Kit			WB	Kit	10 µg each	611419
MAP Kinase Activation Sampler Kit			WB	Kit	10 µg each	612544
FAK						
FAK	C, D, Hu, Ms, Rat	77	IF, IHC, IP, WB	Purified	50 µg/150 µg	610087/88
FAK	F, Hu, Ms	Polyclonal	IP, WB	Serum	100 µg	556368
FAK (pY397), Phospho-Specific	Hu	14	IF, IHC, WB	Purified	50 µg/150 µg	611722/23
FAK (pY397), Phospho-Specific	Hu, Ms, Rat	18	IF, WB	Purified	50 µg/150 µg	611806/07
Focal Adhesion Sampler Kit			IF, WB	Kit	10 µg each	611433
Fyn						
Fyn	Hu, Rat	25	IF, IHC, WB	Purified	50 µg/150 µg	610163/64
Fyn (pY528)/c-Src (pY530)	Hu	31	IHC, WB	Purified	50 µg/150 µg	612668/69

Antibodies for Phospho Protein Analysis *(continued)*

Detection	React	Clone	Apps	Format	Size	Cat. No.
GSK-3β						
GSK-3β	C, D, Hu, Ms, Rat	7	IF, IHC, IP, WB	Purified	50 µg/150 µg	610201/02
GSK-3β (pY216), Phospho-Specific	B, D, Hu, Ms, Rat	13a	IHC, WB	Purified	50 µg/150 µg	612312/13
PKB/Akt Sampler Kit			IF, WB	Kit	10 µg each	611437
IκBα						
IκBα	Hu	25	IF, IP, WB	Purified	50 µg/150 µg	610690/91
IκBα	Hu	6A920	IP, WB	Purified	50 µg	551819
IκBα	Hu, Ms	Polyclonal	WB	Serum	.01 ml	554135
IκBα (pS32/pS36), Phospho-Specific	Hu	39A1431	WB	Purified	50 µg	551818
NF-κB Sampler Kit			IF, WB	Kit	10 µg each	611665
Integrin β3						
Integrin β3	Hu	1	IF, IHC, WB	Purified	50 µg/150 µg	611140/41
Integrin β3 (pY759), Phospho-Specific	Hu, Ms, Rat	7a	IHC, WB	Purified	50 µg/150 µg	612528/29
Integrin Sampler Kit			IF, WB	Kit	10 µg each	611435
JNK/SAPK						
pan-JNK/SAPK1 (p49 MAPK)	C, D, F, Hu, Ms, Rat	37	IF, IHC, WB	Purified	50 µg/150 µg	610627/28
JNK (pT183/pY185), Phospho-Specific	Hu, Ms, Rat	41	FCM, IHC, WB	Purified	50 µg/150 µg	612540/41
JNK1	Hu	G151-333	IP, IVK, WB	Purified	50 µg/150 µg	551196/97
JNK1/JNK2	Hu	G151-666	IHC, IP, WB	Purified	100 µg	554285
Stress Response Sampler Kit			WB	Kit	10 µg each	611442
Jun						
c-Jun (pS63)	Hu	2	WB	Purified	0.1 mg	558036
Jun	Hu, Ms, Rat	G56-206	WB	Purified	0.1 mg	554083
Jun	B, C, D, Hu, Ms, Rat	3	IF, IHC, IP, WB	Purified	50 µg/150 µg	610326/27
Lck						
Lck	Hu, Ms, Rat	28	IF, IHC, WB	Purified	50 µg/150 ug	610097/98
Lck (pY505), Phospho-Specific	Hu, Ms, Rat	4	FCM, IHC, WB	Purified	50 µg/150 ug	612390/91
T Cell Signaling I Sampler Kit			IF, WB	Kit	10 µg each	611662
Neurofilament Proteins						
NF-H	Rat	RFN402	WB	Purified	50 µg	551349
NF-H, Phospho-Specific	Rat	RNF404	WB	Purified	50 µg	551348
NF-H, Phospho-Specific	Rat	RNF405	WB	Purified	50 µg	551958
NF-M, Phospho-Specific	Rat	RNF403	WB	Purified	50 µg	551957
NF-M, Phospho-Specific	Rat	RNF406	WB	Purified	50 µg	551962

Antibodies for Phospho Protein Analysis *(continued)*

Detection	React	Clone	Apps	Format	Size	Cat. No.
eNOS						
eNOS/NOS Type III	Hu, Ms, Rat	3	IF, IHC, IP, WB	Purified	50 µg/150 µg	610296/97
eNOS/NOS Type III	Hu	33	IF, IP, WB	Purified	50 µg/150 µg	610427/28
eNOS/NOS Type III	Hu, Ms, Rat	Polyclonal	IF, IHC, IP, WB	Purified	50 µg/150 µg	610298/99
eNOS (pS1177), Phospho-Specific	Hu	19	FCM, IHC, WB	Purified	50 µg/150 µg	612392/93
eNOS (pT495), Phospho-Specific	B, D, Hu, Ms, Rat	31	IHC, WB	Purified	50 µg/150 µg	612706/07
eNOS (pS633), Phospho-Specific	Hu	37	WB	Purified	50 µg/150 µg	612664/65
NOS Sampler Kit			WB	Kit	10 µg each	611426
p38 MAPK						
p38α (SAPK2α)	D, Hu, Ms, Rat	27	FCM, IF, IHC, WB	Purified	50 µg/150 µg	612168/69
p38 MAPK (pT180/pY182), Phospho-Specific	Hu, Ms, Rat	30	FCM, IF, IHC, WB	Purified	50 µg/150 µg	612280/81
p38 MAPK (pT180/pY182), Phospho-Specific	Hu, Ms, Rat	36	FCM, IF, IHC, WB	Purified	50 µg/150 µg	612288/89
p90 RSK						
Rsk	C, D, Hu, Ms, Rat	78	IF, IHC, WB	Purified	150 µg	610226
p90 RSK1 (pS380)	Hu	20a	WB	Purified	50 µg/150 µg	612692/93
p120 Catenin (pp120)						
p120 Catenin (pp120)	C, D, Hu, Ms, Rat	98	IF, IHC, IP, WB	Purified	50 µg/150 µg	610133/34
p120 Catenin (pp120)	C, D, Hu, Ms, Rat	98	IF	FITC	50 µg/150 µg	610135/36
p120 Catenin (pp120)	C, D, Hu, Ms, Rat	98	IF	TRITC	50 µg/150 µg	610137/38
p120 Catenin (pY96), Phospho-Specific	Hu, Ms, Rat	25a	IHC, WB	Purified	50 µg/150 µg	612534/35
p120 Catenin (pY228), Phospho-Specific	Hu, Ms, Rat	21a	FCM, IF, WB	Purified	50 µg/150 µg	612536/37
p120 Catenin (pY280), Phospho-Specific	Hu, Ms, Rat	18	IF, IHC, WB	Purified	50 µg/150 µg	612538/39
p120 Catenin (CpY291), Phospho-Specific	Hu, Ms, Rat	15a	IF, WB	Purified	50 µg/150 µg	612690/91
Paxillin						
Paxillin	B, C, D, Hu, Ms, Rat	165	IF, IP, WB	Purified	50 µg/150 µg	610619/20
Paxillin	B, C, D, Hu, Ms, Rat	177	IF, WB	Purified	50 µg/150 µg	610568/69
Paxillin	C, D, Hu, Ms, Rat	349	IF, IHC, IP, WB	Purified	50 µg/150 µg	610051/52
Paxillin	C, D, Hu, Ms, Rat	349	IF	FITC	50 µg/150 µg	610053/54
Paxillin	C, D, Hu, Ms, Rat	349	IF	TRITC	50 µg/150 µg	610055/56
Paxillin (pY118), Phospho-Specific	Hu, Ms	30	IHC, WB	Purified	50 µg/150 µg	611724/25
Focal Adhesion Sampler Kit			IF, WB	Kit	10 µg each	611433
Phospholipase Cγ1						
Phospholipase Cγ1	C, D, Hu, Ms, Rat	10	FCM, IF, IHC, IP, WB	Purified	50 µg/150 µg	610027/28
Phospholipase Cγ1 (pY783), Phospho-Specific	B, Hu, Ms, Rat	27	IHC, WB	Purified	50 µg/150 µg	612464/65

Antibodies for Phospho Protein Analysis *(continued)*

Detection	React	Clone	Apps	Format	Size	Cat. No.
$PKA_{RII\beta}$						
$PKA_{RII\beta}$	C, D, Hu, Ms, Rat	45	IF, IHC, WB	Purified	50 µg/150 µg	610625/26
$PKA_{RII\beta}$ (pS114), Phospho-Specific	Hu, Ms, Rat	24	FCM, WB	Purified	50 µg/150 µg	612572/73
$PKA_{RII\beta}$ (pS114), Phospho-Specific	Hu, Ms, Rat	47	FCM, IHC, WB	Purified	50 µg/150 µg	612550/51
PKA Sampler Kit			IF, WB	Kit	10 µg each	611420
PKC						
PKC	Hu, Ms, Rat, B, C, Rab	MC5	IF, IHC(Fr), IP, WB	Purified	0.1 mg	554207
PKCα	C, D, F, Hu, Ms, Rat	3	IF, IHC, IP, WB	Purified	50 µg/150 µg	610107/08
PKCα (pT638), Phospho-Specific	Hu	35	IHC, WB	Purified	50 µg/150 µg	612698/99
PKCβ	C, Hu, Ms, Rat	36	IP, WB	Purified	50 µg/150 µg	610127/28
PKCδ	Hu, Ms, Rat	14	IF, IHC, WB	Purified	50 µg/150 µg	610397/98
PKCε	C, D, Hu, Ms, Rat	21	IF, IHC, IP, WB	Purified	50 µg/150 µg	610085/86
PKCγ	Ms, Rat	20	WB	Purified	50 µg/150 µg	611158/59
PKCη	Hu, Ms	31	IF, WB	Purified	50 µg/150 µg	610814/15
PKCι	C, D, Hu, Ms, Rat	23	IF, IHC, IP, WB	Purified	50 µg/150 µg	610175/76
PKCι	Dros	23	IF, IP, WB	Purified	50 µg	612645
PKCλ	C, D, Hu, Ms, Rat	41	IF, IHC, IP, WB	Purified	50 µg/150 µg	610207/08
PKCθ	Hu	27	IF, IP, IHC, WB	Purified	50 µg/150 µg	610089/90
PKCθ	Hu	27	WB	HRP	50 µg/150 µg	610091/92
PKCθ (pT538)	Hu	19	IHC, WB	Purified	50 µg/150 µg	612734/35
PKC Sampler Kit			IF, WB	Purified	10 µg each	611421
Ras-GAP						
Ras-GAP	C, D, Hu, Ms, Rat	Polyclonal	IF, IHC, IP, WB	Purified	50 µg/150 µg	610043/44
Ras-GAP	C, D, F,Hu, Ms, Rat	13	IF, IHC, WB	Purified	50 µg/150 µg	610040/41
Ras-GAP (pY460), Phospho-Specific	Hu	19A	IHC, WB	Purified	50 µg/150 µg	612736/37

Antibodies for Phospho Protein Analysis *(continued)*

Detection	React	Clone	Apps	Format	Size	Cat. No.
Rb						
Rb (a.a. 1-240)	Hu	G99-2005	IP, WB	Purified	100 µg	554162
Rb (a.a. 300-380)	C, Hu	C36	GS, IHC, IP, WB	Purified	100 µg	554142
Rb (a.a. 300-380)	Hu	G3-349	IP, WB	Purified	100 µg	554140
Rb (a.a. 300-380)	Hu	G4-340	IP, WB	Purified	100 µg	554141
Rb (a.a. 300-508)	C, Hu	2	IF, IHC, WB	Purified	50 µg/150 µg	610884/85
Rb (a.a. 332-344)	Hu, Ms, Rat	G3-245	FCM, GS, IF, IHC, IP, WB	Purified	100 µg	554136
Rb (specially formatted for IHC)	Hu, Ms, Rat	G3-245	IHC	Purified	1 ml	550830
Rb mAb / Isotype Reagent Set : FITC	Hu, Ms, Rat	G3-245	FCM	FITC Set	100 tests	556538
Rb mAb / Isotype Reagent Set : PE	Hu, Ms, Rat	G3-245	FCM	PE Set	100 tests	556539
Rb (a.a. 393-572)	C, Hu, Ms	XZ104	IP	Purified	100 µg	554143
Rb (a.a. 443-622)	C, F, Hu, Ms	XZ55	GS, IP, WB	Purified	100 µg	554144
Rb (a.a. 444-535)	C, Hu	XZ91	IF, IP, WB	Purified	100 µg	554145
Rb (a.a. 622-665)	C, Hu, Ms	XZ133	IP	Purified	100 µg	554146
Underphosphorylated Rb (a.a. 514-610)	Hu, Ms	G99-549	IHC, IP, WB	Purified	100 µg	554164
Underphosphorylated Rb mAb / Isotype Reagent Set: FITC	Hu	G99-549	FCM	FITC Set	100 Tests	550501
Underphosphorylated Rb mAb / Isotype Reagent Set: FITC	Hu	G99-549	FCM	PE Set	100 Tests	550502
RNA Polymerase II						
RNA Polymerase II	Hu	CTD4H8	WB	Purified	150 µg	552042
RNA Polymerase II, Phospho-Specific	Hu	CTD8A7	WB	Purified	150 µg	552041
Stats						
Stat1 (C-terminus)	C, D, Hu, Ms, Rat	42	IF, IP, IHC, WB	Purified	50 µg/150 µg	610185/86
Stat1 (N-terminus)	C, D, F, Hu, Ms, Rat	1	IF, IHC, IP, WB	Purified	50 µg/150 µg	610115/16
Stat1 (N-terminus)	C, D, F, Hu, Ms, Rat	1	WB	HRP	50 µg/150 µg	610117/18
Stat1 (N-terminus)	C, D, F, Hu, Ms, Rat	Polyclonal	IF, IP, WB	Purified	50 µg/150 µg	610119/20
Stat1 (pY701), Phospho-Specific	Hu, Ms, Rat	4a	FCM, IF, IHC, IP, WB	Purified	50 µg/150 µg	612232/33
Stat1 (pY701), Phospho-Specific	Hu, Ms, Rat	14	FCM, IF, IP, WB	Purified	50 µg/150 µg	612132/33
Stat3	C, D, F, Hu, Ms, Rat	84	IHC, IP, WB	Purified	50 µg/150 µg	610189/90
Stat3 (pY705), Phospho-Specific	Hu, Ms, Rat	4	FCM, IHC, WB	Purified	50 µg/150 µg	612356/57
Stat3 (pS727), Phospho-Specific	Hu, Ms, Rat	49	IF, IHC, WB	Purified	50 µg/150 µg	612542/43
Stat4	Ms, Rat	8	IHC, WB	Purified	50 µg/150 µg	610926/27
Stat4 (pY693), Phospho-Specific	Hu	38	FCM, IF, IHC, WB	Purified	50 µg/150 µg	612738/39
Stat5	D, Hu, Ms, Rat	89	FCM, IF, IHC, WB	Purified	50 µg/150 µg	610191/92
Stat5 (pY694), Phospho-Specific	Hu	47	FCM, IHC, WB	Purified	50 µg/150 µg	611964/65
Stat5 (pY694), Phospho-Specific	Hu	Polyclonal	FCM, IF, WB	Purified	50 µg/150 µg	611818/19
Stat5A	D, Hu, Ms, Rat	51	IF, WB	Purified	50 µg/150 µg	611834/35
Stat5A	Hu	Polyclonal	IP, WB	Purified	50 µg	556516
Stat5B	Hu, Ms, Rat	Polyclonal	IP, WB	Purified	50 µg	556517
Stat6	Hu, Ms, Rat	23	IF, IHC, WB	Purified	50 µg/150 µg	611290/91
Stat6 (pY641), Phospho-Specific	Hu	18	FCM, IF, IHC, WB	Purified	50 µg/150 µg	611566/67
Stat6 (pY641), Phospho-Specific	Hu	Polyclonal	IF, WB	Purified	50 µg/150 µg	611820/21
Stat Sampler Kit			WB	Kit	10 µg each	611422
Stat Activation Sampler Kit			IF, WB	Kit	10 µg each	612477

Antibodies for Phospho Protein Analysis *(continued)*

Detection	React	Clone	Apps	Format	Size	Cat. No.
ZAP-70/Syk						
ZAP-70 Kinase	C, Hu, Ms, Rat	24a	IF, IHC, WB	Purified	50 µg/150 µg	612718/19
ZAP-70 Kinase	C, Hu, Ms, Rat	29	IF, IHC, IHC, WB	Purified	50 µg/150 µg	610239/40
ZAP-70 Kinase	C, Hu, Ms, Rat	29	IF	FITC	50 µg/150 µg	612588/89
ZAP-70 Kinase (pY319)/ Syk(pY352), Phospho-Specific	Hu, Ms, Rat	17a	FCM, IHC, WB	Purified	50 µg/150 µg	612574/75

Activation-State Antibody Sampler Kits

Detection	Clone	Apps	Reactivity	Cat. No.
EGFR Activation Sampler Kit - Cat. No. 612476				
Akt/PKBa	7	IF, WB	Dog, Hu, Ms, Rat	610836/37
Akt/PKBa (pS472/pS473)	104A282	WB	Hu, Ms, Rat	550747
EGF Receptor	13	IF, IP, WB	Hu, Ms	610016/17
EGF Receptor (Activated Form)	74	IF, IP, WB	Hu	610025/26
ERK1	MK12	FCM, IF, IHC, IP, WB	B, C, Dog, Hu, Ms, Rat	610030/31
ERK1/2 (pT202/pY204)	20A	FCM, IF, WB	B, C, D, F, Hu, Ms, Rat	612358/59
Stat1 (N-terminus)	1	IF, IHC, IP, WB	C, Dog, Hu, Ms, Rat	610115/16
Stat1 (pY701)	4a	IF, IP, WB	Hu, Ms, Rat	612232/33
MAP Kinase Activation Sampler Kit - Cat. No. 612544				
ERK1	MK12	FCM, IF, IHC, IP, WB	B, C, Dog, Hu, Ms, Rat	610030/31
ERK1/2 (pT202/pY204)	20A	FCM, IF, WB	B, C, D, F, Hu, Ms, Rat	612358/59
pan-JNK/SAPK1	37	IF, IHC, WB	C, Dog, Hu, Ms, Rat	610627/28
JNK (pT183/pY185)	41	FCM, WB	Hu, Ms, Rat	612540/41
p38-a/SAPK2a	27	IF, WB	Dog, Hu, Ms, Rat	612168/69
p38 MAPK (pT180/pY182)	30	FCM, IF, WB	Hu, Ms, Rat	612280/81
Stat Activation Sampler Kit - Cat. No. 612477				
Stat1 (C-terminus)	42	IF, IP, WB	C, Dog, Hu, Ms, Rat	610185/86
Stat1 (pY701)	14	FCM, IF, IP, WB	Hu, Ms, Rat	612132/33
Stat3	84	IF, IP, WB	C, Dog, Hu, Ms, Rat	610189/90
Stat3 (pY705)	4	FCM, WB	Hu, Ms, Rat	612356/57
Stat5	89	IF, IHC, WB	Dog, Hu, Ms, Rat	610191/92
Stat5 (pY694)	47	FCM, WB	B, Hu, Ms, Rat	611964/65
Stat6	23	IF, WB	Hu, Ms, Rat	611290/91
Stat6 (pY641)	18	FCM, IF, WB	Hu	611566/67

FCM: *Flow Cytometry;* ***GS:*** *Gel Shift;* ***IF:*** *Immunofluorescence Microscopy;* ***IHC:*** *Immunohistochemistry;* ***IP:*** *Immunoprecipitation;* ***WB:*** *Western Blot;* ***B:*** *Bovine;* ***C:*** *Chicken;* ***Hu:*** *Human;* ***Ms:*** *Mouse*

Treated Lysates for Western Blot

Detection	Apps	Tissue Type	Size	Cat. No.
A431 + EGF Cell Lysate	WB	Epidermoid Carcinoma	500 µg	611448
A431 + EGF AP Ctrl Lysate	WB	Epidermoid Carcinoma	500 µg	612190
HE + Pervanadate Cell Lysate	WB	Endothelium	500 µg	611667
HeLa + Anisomycin Lysate	WB	Epitheloid Cervical Carcinoma	500 µg	611692
HeLa Pervanadate Ctrl Lysate	WB	Epitheloid Cervical Carcinoma	500 µg	612014
HeLa + Pervanadate Lysate	WB	Epitheloid Cervical Carcinoma	500 µg	612015
HeLa + Pervanadate AP Ctrl Lysate	WB	Epitheloid Cervical Carcinoma	500 µg	612016
HeLa + Pervanadate + AP Lysate	WB	Epitheloid Cervical Carcinoma	500 µg	612017
HepG2 IL-6 Ctrl Lysate	WB	Hepatocellular Carcinoma	500 µg	612066
HepG2 + IL-6 (15') Lysate	WB	Hepatocellular Carcinoma	500 µg	612067
Hs68 + Pervanadate Lysate	WB	Fibroblast	500 µg	612187
Hs68 + Pervanadate AP Ctrl Lysate	WB	Fibroblast	500 µg	612188
Hs68 + Pervanadate + AP Lysate	WB	Fibroblast	500 µg	612189
Apoptotic Jurkat Lysate Set 1	WB	T Cell Leukemia	500 µg each	550959
Jurkat + Pervanadate Lysate	WB	T Cell Leukemia	500 µg	611755
Jurkat + Pervanadate AP Ctrl Lysate	WB	T Cell Leukemia	500 µg	612192
Jurkat + Pervanadate + AP Lysate	WB	T Cell Leukemia	500 µg	612193
Mouse Macrophage Lysate	WB	Macrophage	500 µg	611479
Mouse Macrophage + IFNγ/LPS	WB	Macrophage	500 µg	611473

BD PowerBlot℠ Western Arrays Screening Service

Introduction

With our patented BD PowerBlot℠ Western Array Screening Service we analyze the expression and phosphorylation levels of 40 to 1000 proteins simultaneously. Our screening results in your hands make targeted follow-up studies easy. All antibodies are available off-the-shelf for multiple applications. Besides Western blot analysis and immunoprecipitation, many of our antibodies have been validated for:

- Immunofluorescence microscopy
- Immunohistochemistry
- Intracellular flow cytometry (BD™ Phosflow)
- Cytometric Bead Array (BD™ CBA)

BD PowerBlot℠ Options

The complete BD PowerBlot lineup includes the full service (including over 1,000 antibodies), plus two custom options in which you select the 40 or 150 antibodies most relevant to your research. In addition, you may select one of our functional screens, each involving 250 – 300 antibodies.

Comprehensive Screens

- Full Service (>1,000 Antibodies)
- Custom 40 Antibody Array
- Custom 150 Antibody Array

Functional Screens

- Apoptosis Array (270 monoclonal antibodies)
- Cancer Array (300 monoclonal antibodies)
- Cell Cycle Array (200 monoclonal antibodies)
- Neuroscience Array (340 monoclonal antibodies)
- Phospho Specific Array (80 monoclonal antibodies)
- Protein Kinase Array (90 monoclonal antibodies)

For more information please visit our website **www.bdpowerblot.com** or contact your local BD office

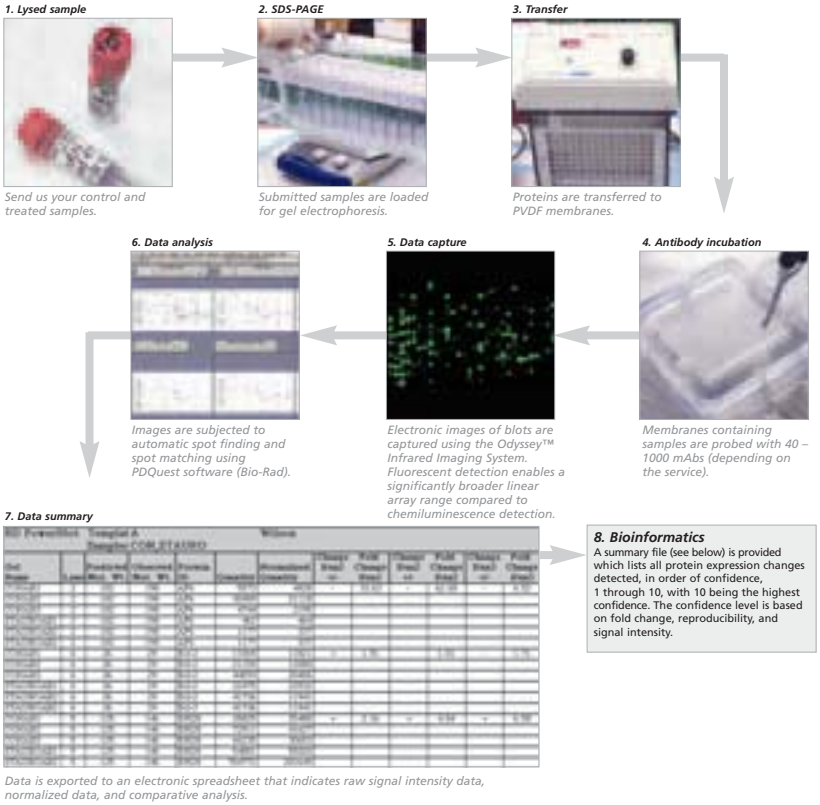


Figure 10. BD PowerBlot[SM] Western Array Screening Workflow Overview

The BD PowerBlot[SM] Phospho Specific Array measures the phosphorylation states of more than 30 signaling proteins, plus total protein expression for each of the phosphospecificities. This screen targets several signaling pathways and can be used to examine intracellular perturbations more subtle than the up- or down-regulation of protein expression.

In ***Table 2*** (next page) we show data from our Phospho specific Array, using A431 cells stimulated with EGF or with calyculin A and okadaic acid. With each service performed we provide you with the raw data, comparative analyses, and a summary of your results (see ***Table 2*** on next page).

Protein	Site	Degree of phosph.	Mode of phosphorylsation	Proteome summary
EGF-induced phosphorylation				
Caveolin 1	Y14	++	Src-dependent	Caveole formation
Caveolin 2	Y27	+	Src-dependent	Forms hetero-oligomeric complex w/ Caveolin 1
EGFR	activated	+++	Ligand-dependent autophosphorylation	Growth factor receptor tyrosine kinase
p-Tyr		+++++	N/A	N/A
p120 Catenin	Y228	++++	Src-dependent	Cell adhesion/signaling
PLCg	Y783	+	EGFR-dependent	Cell motility/mitogenesis
Stat1	Y701	++++	Growth factor/cytokine-dependent	Activator of interferon stimulated genes
Stat3	Y705	++	Growth factor/cytokine-dependent	Binds IL-6-responsive elements/ promotes acute-phase protein expression
Calyculin A / okadaic acid-induced phosphorylation				
ATF2	T71	++	p38 MAPK-dependent	Stimulates CRE-dependent transcription/acetylates histones H2B and H4
p38MAPK	T180/Y182	++++	MKK-dependent phosphorylation	Integration point for multiple biochemical signals
EGF- and Calyculin A / okadaic acid-induced phosphorylation				
Erk1/2	T202/Y204	+/+++	MEK1-dependent	Integration point for multiple biochemical signals
Uninduced				
GSK-3b	Y216	no change	Increased by apoptotic stimuli	Ser/Thr Kinase involved in glycogen metabolism
p53	S392	no change	Casein Kinase II-dependent	Growth control

Table 2. BD PowerBlot℠ Phospho Specific Array Data Summary.

Sample Preparation

Tissue cultured cells

In order to run samples in triplicate, we require ~5mg total protein per sample. Approximately 5-6 confluent 15cm dishes or ~5.5×10^7 – 3.7×10^8 cells are required to yield ~5mg total protein.

1. Prepare 500ml Lysis Buffer:

   10mM Tris, pH7.4

   1mM sodium ortho-vanadate

   1% SDS

2. Heat Lysis Buffer to boiling (hot plate, microwave or hot water bath).
3. Remove media from cells and rinse with 20ml PBS /15cm plate. Completely aspirate PBS from cells.
4. Add 2*ml of boiling lysis solution per 15cm plate. Swirl the solution over the cells to ensure rapid denaturation of cellular proteins. Scrape lysate with a cell scraper. The lysate is usually viscous due to cellular DNA. Transfer the lysate to a 50ml conical polypropylene tube.

   *** The goal is to obtain a sample with a protein concentration of >1mg/ml; thus the less lysis buffer used per plate the better.**

5. Briefly heat lysate (heating block or water bath at 95-100°C for 30 seconds, or microwave 5-10 seconds, uncapped). This step should be carefully monitored, as there is a potential for sample loss due to spills.
6. One of these three methods can be used to shear the cellular DNA:

   a) homogenize lysate with a polytron for 15-30 seconds

   b) sonicate lysate for 10-30 seconds

   c) pass lysate 10× through a 25-gauge needle

7. Estimate protein concentration. Remove a small aliquot(~10-50µl), dilute to 100-500µl with water to reduce SDS to 0.1%. Measure total protein content using the BCA reagent from Pierce. Be sure that protein standards are also made in a 0.1% SDS solution.
8. Freeze samples at -80°C

Tissues:

We require ~0.15g tissue or 5ml of prepared sample to run triplicate analysis.

1. Prepare Lysis Buffer as above.
2. Recently obtained tissues, or frozen tissues can be prepared as follows:

   For 0.15g tissue, add 4.1ml of boiling Lysis Buffer and homogenize using a polytron at full speed for 15-20 seconds. Next, add 0.9ml of 6× electrophoresis Sample Buffer and mix well. Heat again 95°C, 30 seconds in heat block or water bath, or just to boiling in a microwave (5-10 seconds, careful, do not over boil). Yield ~5ml sample.*

   *** For most tissues, this method allows for fair representation of cellular proteins (there is no need to measure protein concentration). If your tissues vary in fluid content, do not add electrophoresis sample buffer, instead send samples in lysis buffer and we will determine protein concentration.**

   6X Sample Buffer

   360mM Tris pH6.8

   600mM DTT

   12% SDS

   60% glycerol

   0.018% bromophenol blue
3. Freeze the samples at -80°C
4. Ship samples.

Please contact us prior to sending samples so we can ensure their safe arrival.

Note: A quote is required before placing an order and sending samples. (Outside the US please contact your local BD office for more information)

Send samples to:

BD Biosciences – Pharmingen
BD PowerBlot Service
Quotation # _____
Attn: Custom Technology Team
11077 N. Torrey Pines Rd.
LaJolla, CA 92037

Please include your quotation number, and return address on the mailing label.
Along with your samples, include your purchase order number, billing and shipping address, telephone number and email address. Also include any special instructions such as to how you would like your samples compared (ie, which sample is control, which is treated).

Appendix A

Flow Cytometry Instrumentation Systems

Introduction

Our flow cytometry systems provide the high performance and quality expected from the leader in cell analysis. Our instruments are designed and optimized as complete systems that include hardware, software, and reagents.

We can also work with you individually to design solutions that meet your specific needs.

The BD FACSAria™, BD FACSArray™, BD FACSCanto™, BD™ LSR II, BD FACSVantage™ SE, and BD FACSCalibur™ flow cytometers provide maximum performance and reliability. They are also modular, for flexibility in the future.

The BD FACStation™ data management system is a key component of our instrument solutions. It combines the most powerful computer hardware available today with unsurpassed software tools for both clinical and research cell analysis.

Flow Cytometry Instrument Systems

Analyzers

- BD FACSArray bioanalyzer
- BD FACSCalibur system
- BD FACSCanto system
- BD LSR II system

Sorters

- BD FACSAria cell sorting system
- BD FACSVantage SE system

BD FACSArray™ Bioanalyzer System

For Your Bead and Cellular Analysis Needs

The BD FACSArray™ bioanalyzer is a new research platform for fast and sensitive high-content analysis in cell biology, immunology, and proteomic applications.

This compact system rapidly detects and quantifies concentrations of secreted proteins, proteins in cell lysates, and cell associated proteins using small sample volumes. It supports your search for answers in apoptosis, cytokine and chemokine profiling, and even phosphorylation of key signal transduction proteins with our multiplexed BD™ Cytometric Bead Array technology. The bioanalyzer system may also be used to assess hybridomas, cell viability, proliferation, and immunotoxicology effects in non-human systems. The possibilities are extensive.

The BD FACSArray bioanalyzer is a fully integrated system composed of instrument, computer station, and an easy-to-use software user interface. It is designed for speed, ease-of-use, and minimal training requirements, to turn your research into immediate success.

BD Biosciences commitment to application development is one of our strongest distinctions in supporting our customers. Numerous carefully optimized applications and over one thousand reagents are available for the BD FACSArray platform.

BD FACSArray Bioanalyzer System

- Fast microtiter plate sampler
- Six-parameter detection (forward scatter, side scatter, four fluorescent channels)
- Intuitive software
- Digital signal processing with up to 15,000 events per second
- Compact benchtop unit
- BD Biosciences reagents and applications
- Interactive training CD-ROM

Class 1 (I) Laser Product

BD FACSCalibur™ System

Multipurpose Flow Cytometer

The BD FACSCalibur™ system is another BD first: the only four-color, dual-laser, benchtop system available today that is capable of both cell analysis and sorting. Designed specifically to support a wide range of applications, the BD FACSCalibur system is a fully integrated multiparameter system.

The BD FACSCalibur system combines unique dual-laser technology, an automated sample loader option, and powerful software to provide the high throughput necessary to meet productivity requirements of today's clinical laboratories. The modularity and innovative technology designed into the BD FACSCalibur system also offer investigators the performance and flexibility required for a variety of research applications.

The built-in versatility of the BD FACSCalibur flow cytometer meets the current demands of both clinical and research environments. Its flexibility will help you meet tomorrow's demands.

BD FACSCalibur System

- Three- or four-color fluorescence capability
- BD FACStation™ data management system
- Flexible and modular for future upgrades
- BD™ High Throughput Sampler Option for 96-well microtiter tray acquisition

BD FACSCanto™ System

Efficient • Easy • Powerful

The BD FACSCanto™ is a new digital benchtop flow cytometer with many optical design elements first shown in the BD™ LSR II analyzer and the BD FACSAria™ cell sorter. It is designed for optimal workflow, highest performance, and flexibility in experimental design. The BD FACSCanto system supports a broad range of research applications and is particularly suited for rare event analysis, employing frontier-breaking speed, highest sensitivity, and minimal sample-to-sample carryover.

BD FACSCanto System

- 10,000 events/sec processing capability
- Best of class fluorescence sensitivity*
- Six-color detection
- <0.1% sample carryover
- Digital electronics
- Fully integrated fluidics cart
- New BD FACSDiva™ software (version 4.0 or higher)

Options

- BD FACS™ Loader for automated sample loading

**<50 MESF PE and <100 MESF FITC, measured with Spherotech Rainbow Beads*
Class I (1) Laser Product

BD™ LSR II System

Four-Laser, 18-color Benchtop Flow Cytometer

The new BD™ LSR II has performance, capabilities, and flexibility never seen before in a benchtop analyzer. Free up your high-performance cell sorter by moving your application(s) that require either a UV or Krypton laser to the BD LSR II.

The BD LSR II can be configured with up to four fixed alignment lasers and the ability to detect up to 20 parameters, utilizing a revolutionary optical design. The BD LSR II can be configured with 488 nm, 405 nm, 633 nm, 532 nm, and UV lasers. At the heart of the BD LSR II are new digital electronics. The electronics digitize signals 10 million times per second in 16,384 discrete levels. This expands channel resolution and increases detection sensitivity. Electronic dead time is eliminated; more precise fluorescent measurements can be made improving linearity, compensation, and quantitation of fluorescence measurements.

BD LSR II System

- Up to 18-color detection
- Digital acquisition and analysis system
- Flexible and modular for future upgrades
- PC workstation
- BD™ High Throughput Sampler Option for 96-well microtiter tray acquisition

Laser Options

- UV, 405 nm, 633 nm, and 532 nm

BD™ High Throughput Sampler Option

BD™ High Throughput Sampler option on the BD FACSCalibur™ flow cytometer

BD™ High Throughput Sampler option on the BD™ LSR II benchtop flow cytometer

The BD™ High Throughput Sampler (HTS) option is a compact, easily installable option for the BD FACSCalibur™ and BD™ LSR II. The HTS introduces samples into the cytometer at high speed, increasing productivity and efficiency. The BD HTS is an option for new instruments and is retrofittable to existing BD FACSCalibur and BD LSR II flow cytometers.

- Compatible with 96- and 384-well microtiter trays
- Under 15-minute acquisition for a 96-well tray*
- Carryover <1% in high throughput mode, <0.5% carryover in standard mode
- User-adjustable mixing, wash, and acquisition parameters
- Adaptable to a wide range of applications
- Robotic-accessible design

** Based on a 3 sec acquisition*

BD FACSAria™ Cell Sorting System

The BD FACSAria™ cell sorter sets a new standard for high-performance flow cytometry. Based on a revolutionary new design in instrumentation, this easy-to-use benchtop system delivers high-speed sorting and multicolor analysis.

The BD FACSAria instrument is the first benchtop sorter that incorporates a fixed-alignment cuvette flow cell. This new flow cell provides superior fluorescence sensitivity. The fixed optical system offers freedom from instrument alignment responsibility for up to three air-cooled lasers at 488-nm, 633-nm, and 407-nm wavelengths. Capable of detecting up to 13 colors and 2 scatter parameters for a total of 15 parameters.

Sort setup and sort monitoring features in the system software make sorting on the BD FACSAria instrument uncomplicated and more efficient. BD FACS™ Accudrop is integrated into the system for quick and accurate drop-delay determination. Stream monitoring of the breakoff point and clog detection are incorporated for walkaway sorting.

BD FACSAria Cell Sorter Base Instrument

- First benchtop high-speed sorter with fixed-alignment cuvette flow cell
- Cuvette flow cell for superior fluorescence sensitivity
- Two air-cooled lasers at 488-nm and 633-nm wavelengths
- Digital acquisition rates of up to 70,000 events/second
- Multicolor analysis of 8 parameters, 7 colors and 2 scatter
- Two- and four-way bulk sorting devices for a variety of tube sizes
- Integrated BD FACS Accudrop drop delay determination system
- Sample input cooling and heating
- Full digital electronics
- PC workstation
- No special facility requirements necessary for water, air, or vacuum

Class 1 (I) laser product.

BD FACSAria™ Options

Violet Laser Upgrade

Upgrade includes an air-cooled violet laser at 407-nm and one trigon with two additional PMTs

Automatic Cell Deposition Unit (ACDU) Upgrade

Allows for multiwell plate and slide sorting

2 Laser PMT Upgrade Kit: Field Upgrades/(for Two-Laser Configuration Only)

For expanded fluorescent detection this option provides two additional PMTs for the 488-nm octagon and one additional PMT for 633-nm trigon field upgrade for instrument installed in field. No additional optical filters provided.

3 Laser PMT Upgrade Kit: Field Upgrade (for Three-Laser Configuration Only)

For expanded fluorescent detection this option provides two additional PMTs for the 488-nm octagon, one additional PMT for 633-nm trigon, and one additional PMT for 407-nm trigon field upgrade for instrument installed in field. No additional optical filters provided.

Aerosol Management Option

For enhanced removal of aerosols in the ACDU chamber

Temperature Control Option

Water recirculator bath for cooling of the sort collection device for tubes and multiwell plates

BD FACSAria Instrument and Computer Table

- Table will support the BD FACSAria cell sorter, computer, and monitors.
- Fluidics cart can be placed under the table.

BD FACSVantage™ SE System

High-speed cell sorting with maximum flexibility

The BD FACSVantage™ SE flow cytometry system is the most flexible high-speed cell sorting instrument for the research laboratory. This instrument platform can accommodate any laser wavelength and a variety of nozzle sizes for maximum performance for any research application.

To meet the demands of today's research, we've improved the BD FACSVantage SE and its many options to allow for customization to meet any possible cell sorting need. All of this technology is accessible through a familiar user interface and efficient ergonomic design. The BD FACSVantage SE flow cytometry system is fully modular, for today's needs and beyond.

BD FACSVantage SE System

- Multicolor analysis and sorting
- BD FACStation™ data management system
- Flexible and modular for future upgrades
- Non-rectangular sort windows

Options

- Digital option
- BD TurboSort™ option
- BD MacroSort™ option
- BD CloneCyt™ Plus
- BD FACS™ Accudrop
- Pulse Processor

Cell Imaging Instrumentation Systems

BD™ Pathway Live Cell Confocal Bioimager

Rapidly develop cell based assays for drug discovery and basic research

- **Proven high-throughput assays for numerous applications including cytotoxicity, apoptosis, translocation, and cell cycle studies**
- **Fully automated sample processing**
- **Robust, integrated software with add-in modules for specific applications**

The BD™ Pathway Bioimager provides realtime confocal kinetic imaging in an easy-to-use compact unit.

Integrated system

The BD Pathway Bioimager enables cell based assays of multiple events within living cells, revealing significantly more information about cellular mechanisms and structure. With the ability to image multiple fluorescent dyes in living cells, you can pursue experiments that were previously not possible. Our Bioimager can be operated in a fully automated mode using imaging software that seamlessly integrates imaging, liquid handling, plate navigation, and image analysis.

Rapidly develop cell based assays

The software included with the BD Pathway Bioimager employs a novel hierarchical data classification algorithm that allows greater flexibility in assay design and implementation. It allows you to configure and analyze many different cell based assays including translocation, ion kinetics, apoptosis, cyctotoxicity, cell proliferation, and cell cycle experiments. Other specific applications such as neurite outgrowth are available separately as add-ins.

To find out how the BD Pathway Bioimager can accelerate your research, visit **www.bdbiosciences.com/pathway**

Figure 1. The BD™ Pathway is a compact, fully automated confocal imaging platform with integrated liquid handling, temperature, and CO_2 regulation.

Features

- True confocal real-time imaging
- Full-spectrum laser-free illumination
- Autofocus, auto-cell identification and tracking
- Confocal and wide field viewing modes
- Integrated temperature and CO_2 control
- Capable of imaging both cells and tissues
- Binocular eyepiece for direct sample viewing
- Kinetic or endpoint measurement assays
- Multiple plate and slide configurations including 96-well, 384-well, chamber slides, and microscope slides
- Versatile liquid handling and mixing
- Motorized high-resolution X, Y, Z
- Proprietary motionless stage with mobile optics

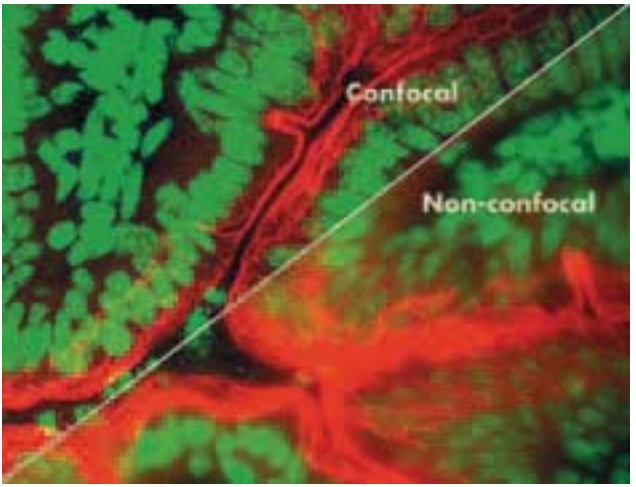


Figure 2. The BD™ Pathway Bioimager employs a unique laser-free confocal device that improves resolution and allows three-dimensional imaging.

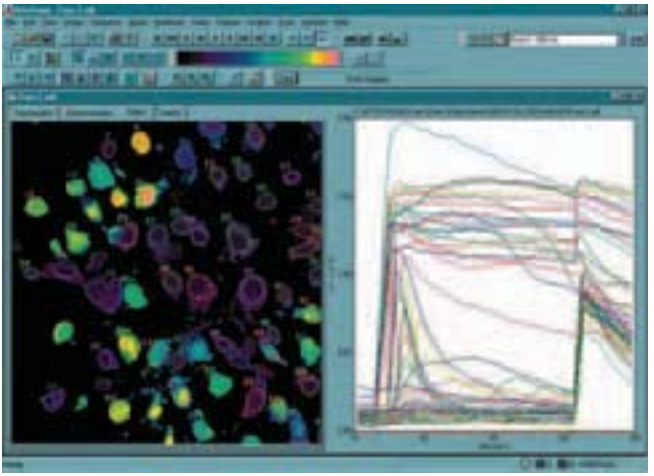


Figure 3. The BD™ Pathway software integrates a novel data classification capability to provide researchers flexibility in designing and implementing cell based assays. After imaging at high resolution, cells are automatically identified and fluorescence intensity is monitored at the cellular or subcellular level. Using selective criteria, each cell can be automatically classified during the experimental run using a number of cell specific features (eg., fluorescence intensity, rate of change, redistribution, etc.). Once classified, cells can be counted and traces color-coded. Additionally, wells can be color-coded based on criteria selected by the user, enabling whole plate maps to be identified by particular responses of interest. The software also incorporates sophisticated 3D rendering capabilities allowing the system to be used as an automated confocal imaging workstation.

BD™ CARV II Confocal Imager

Real-Time High Performance Personal Confocal Imaging

BD™ CARV II Confocal Imager delivers an automated system for high resolution, real-time confocal imaging that can be adapted to existing laboratory microscopes. The proprietary spinning disk optical path simultaneously scans the entire field at a rate of 1,000 times per second. The low intensity, high frequency sampling minimizes photobleaching and phototoxicity, allowing long-term recording of cellular events.

The BD CARV II Confocal Imager uses a 120W Mercury/Metal Halide light source and allows full-spectrum (360nm–700nm) confocal imaging through filter sets matched to the excitation and emission requirements of your fluorescent sample. Automation of internal multiposition excitation, dichroic, and emission filter wheels allows fast multiwavelength confocal imaging. The automated filters reduce reliance on multiband pass filter sets to allow maximum light throughput and fast sequential imaging of up to five or more fluorescent probes in the same sample.

High-speed, full-field confocal scanning allows direct real-time binocular viewing. A variety of high resolution, high quantum efficiency CCD cameras also allow image capture of up to 100 frames per second without compromising confocal resolution. The system can be controlled by software packages for powerful multidimensional data acquisition and analysis including co-localization, 3-D, 4-D & 5-D rendering, and ratio imaging.

The BD CARV II Confocal Imager also offers automation of fluorescence recovery after photobleaching (FRAP). Our FRAP capability allows you to select an area for photobleaching and then record recovery kinetics. Data can be analyzed using a variety of commercially available software packages.

Figure 4. The BD™ CARV II Confocal Imager can be used with your existing microscope set-up.

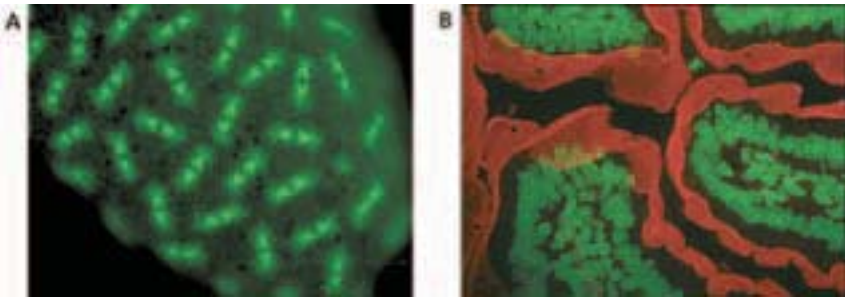


Figure 5. The BD™ CARV II delivers real-time confocal images without lasers and with minimal photobleaching. ***Panel A***. FluoCells® prepared slide #4 from Molecular Probes (Invitrogen) with mouse intestine section stained with Alexa Fluor® 568 Phalloidin and SYTOX® Green. ***Panel B.*** Drosophila embryo cells expressing GFP-tubulin.

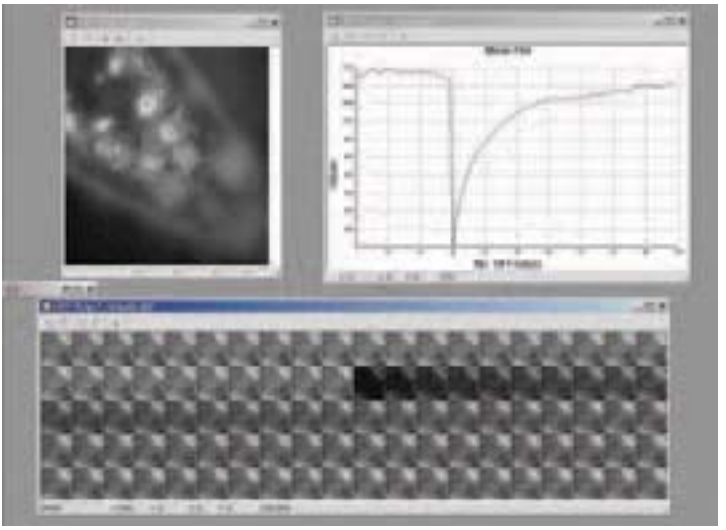


Figure 6. FRAP analysis with the BD™ CARV II Confocal Imager. The FRAP module can be used to select an area for photobleaching. Analysis was performed using IPLab Software (Scanalytics Inc.)

Table I. Specifications of the BD™ CARV II Confocal Imager

Confocal scanner	Nipkow spinning disk (pinholes)
Disk scan rate	1000 scans per second
Pinhole diameter	70 µm
Spectral transmission	360nm–700nm
Z-resolution	0.5 µm (PSF; 100X PlanApo, 1.4 NA)
Illumination source	120W Mercury/Metal Halide (1200 hr)
Internal excitation changer	Automated 8 position wheel (25mm)
Internal dichroic changer	Automated 5 position wheel (25.7 x 36mm)
Internal emission changer	Automated 8 position wheel (25mm)
Filter sets provided	DAPI, EGFP, Texas Red (BrightLine™ Series from Semrock, Inc.)
Operation mode	Automated confocal, wide field, or bright field
Observation	Direct confocal binocular viewing or camera port
Detector Compatibility	CCD camera: SensiCam EM, QE (The Cooke Corporation); CoolSNAP™ HQ, Cascade® 512B (Photometrics, Inc.); ORCA® ER, AG (Hamamatsu Corporation) and more
Microscope Compatibility	Most upright and inverted fluorescence microscopes with a 100% camera port
FRAP	Aperture control, touch pad, or RS232
Control	Touch pad or RS232
Software drivers	IPLab (Scanalytics); RS232 command set also available
Size	11 (w) x 15.5 (l) x 6 (h) inches 27.9 (w) x 39.34 (l) x 15.2 (h) cm
Weight	14.5 lbs/6.6 kg
Power	100–240 VAC/12V DC

Notes

Appendix B

BD Instrument and Multicolor Reagent Solutions

BD Biosciences is committed to providing researchers with the widest range of high-quality fluorochrome-conjugated antibodies to complement our state-of-the-art flow cytometry systems. We are continually adding new colors to our ever-expanding reagent portfolio which now includes PE-Cy7, APC-Cy7, Alexa Fluor®, Pacific Blue®, and AmCyan fluoro-chromes to support an increasing array of multicolor experimentation options.

The future of flow cytometry is here. BD Biosciences is proud to announce our novel instrumentation systems such as the entirely digital BD FACSCanto™ flow cytometer for six-color analysis, the BD FACSAria™ cell sorter for high-performance cell sorting, the BD™ LSR II flow cytometer with up to four lasers to measure up to sixteen colors, and the BD FACSArray™ bioanalyzer for high-content analysis and walkaway simplicity. For detailed information about our new flow cytometry systems, please see ***Appendix A***, orcontact your local BD Biosciences sales representative.

Please visit **www.bdbiosciences.com/colors** for more information about our newest colors and instrumentation.

Typical Instrument Configuration

Instrument	Laser	Exitation Laser Line (nm)	Fluorescence Channel	Fluorochromes provided by BD Biosciences
BD FACScan™	Argon (L1)	488	FL1 Green	FITC, Alexa Fluor® 488
			FL2 Yellow	PE
			FL3 Red	PE-Texas Red®, PE-Cy5*, PerCP, PerCP-Cy5.5, PE-Cy7
BD FACSCalibur™	Argon (L1)	488	FL1 Green	FITC, Alexa Fluor® 488
			FL2 Yellow	PE
			FL3 Red	PE-Cy5*, PerCP, PerCP-Cy5.5, PE-Cy7
	Red Diode (L2)	635	FL4 Red	APC*, Alexa Fluor® 647
BD FACSCanto™	Argon (L1)	488	Green	FITC
			Yellow	PE
			Red	PerCP, PerCP-Cy5.5
			Infra Red	PE-Cy7
	HeNe (L2)	633	Red	APC*
			Infra Red	APC-Cy7
BD FACSVantage™ SE (typical setup)	Argon (L1)	488	FL1 Green	FITC, Alexa Fluor® 488
			FL2 Yellow	PE
			FL3 Red	PE-Texas Red®, PE-Cy5*, PerCP-Cy5.5
			FL3 Infra Red	PE-Cy7
	Krypton (L2)	407	FL4 (1) Blue	Alexa Fluor® 405, Pacific Blue®
	HeNe (L2 or L3)	633	FL4 (2) Red	APC*, Alexa Fluor® 647
			FL5 Infra Red	APC-Cy7
BD™ LSR (typical setup)	Argon (L1)	488	FL1 Green	FITC, Alexa Fluor® 488
			FL2 Yellow	PE
			FL3 Red	PE-Texas Red®, PE-Cy5*, PerCP, PerCP-Cy5.5, PE-Cy7
	HeCd (L2)	325	FL4, FL5 Blue	
	HeNe (L3)	633	FL6 Red	APC*, Alexa Fluor® 647, APC-Cy7

Table 1. Typical Instrument Configuration.

Table 1 (continued). Typical Instrument Configuration.

Typical Instrument Configuration

Instrument	Laser	Exitation Laser Line (nm)	Fluorescence Channel	Fluorochromes provided by BD Biosciences
BD™ LSR II (typical setup)	Argon (L1)	488	Green	FITC, Alexa Fluor® 488
			Yellow	PE
			Red	PE-Texas Red®, PE-Cy5*, PerCP, PerCP-Cy5.5
			Infra Red	PE-Cy7
	HeNe (L2)	633	Red	APC*, Alexa Fluor® 647
			Infra Red	APC-Cy7
	UV (L3)	355	Violet	
			Blue	Alexa Fluor® 405, Pacific Blue®
	Violet (L4)	405	Green	AmCyan
			Blue	Alexa Fluor® 405, Pacific Blue®
BD FACSAria™ (typical setup)	Argon (L1)	488	Green	FITC, Alexa Fluor® 488
			Yellow	PE
			Red	PE-Texas Red®, PE-Cy5*, PerCP
			Far Red	PerCP-Cy5.5
			Infra Red	PE-Cy7
	HeNe (L2)	633	Red	APC*, Alexa Fluor® 647
			Infra Red	APC-Cy7
	Violet (L3)	407	Green	AmCyan
			Blue	Alexa Fluor® 405, Pacific Blue®
BD FACSArray™ (typical setup)	Green Diode (L1)	532	Yellow	PE
			Far Red	PerCP-Cy5.5, PE-Cy7
	Red Diode (L2)	635	Red	APC*, Alexa Fluor® 647
			Infra Red	APC-Cy7

** APC and PE-Cy5 may be used together on instruments with cross-beam compensation.*

Fluorochrome Specifications

Table 2. Fluorochrome Specifications.

Fluorochrome	Fluorescence Emmision Color	Ex-Max (nm)	Excitation Laser Line (nm)	Em-Max (nm)	BD FACScan™	BD FACSCalibur™	BD FACSCanto™	BD FACSVantage™ SE	BD™ LSR	BD™ LSR II	BD FACSAria™	BD FACSArray™
Alexa Fluor® 405	Blue	401	360, 405, 407	421				✓		✓	✓	
Pacific Blue®	Blue	405	360, 405, 407	455				✓		✓	✓	
AmCyan	Green	457	405, 407	491				✓		✓	✓	
Alexa Fluor® 488	Green	495	488	519	✓	✓		✓	✓	✓	✓	
FITC	Green	494	488	519	✓	✓	✓	✓	✓	✓	✓	
PE	Yellow	496, 564	488, 532	578	✓	✓	✓	✓	✓	✓	✓	✓
PE-Texas Red®	Orange	496, 564	488, 532	615	✓			✓	✓	✓	✓	
Texas Red®**	Orange	595	595	615				✓				
APC*	Red	650	595, 633, 635, 647	660		✓	✓	✓	✓	✓	✓	✓
Alexa Fluor® 647	Red	650	595, 633, 635, 647	668		✓		✓	✓	✓	✓	✓
PE-Cy5*	Red	496, 564	488, 532	667	✓	✓		✓	✓	✓	✓	
PerCP	Red	482	488, 532	678	✓	✓	✓		✓	✓	✓	
PerCP-Cy5	Far Red	482	488, 532	695	✓	✓	✓	✓	✓	✓	✓	✓
Alexa Fluor® 700***	Far Red	696	633, 6335	719				✓		✓	✓	
PE-Cy7	InfraRed†	496, 564	488, 532	785	✓	✓	✓	✓	✓	✓	✓	✓
APC-Cy7	InfraRed†	650	595, 633, 635, 647	785			✓	✓	✓	✓	✓	✓

† InfraRed detection requires a Hamamatsu R3896 Photomultiplier Tube (comes with detector option).
** APC and PE-Cy5 may be used together on instruments with cross-beam compensation.*
*** Texas Red® detection requires a dye laser for 595-600 nm excitation.*
**** Alexa Fluor® 700 detection is available through an expanded optical configuration.*

Table 3. Absorption and Emmision Spectra for BD Biosciences Fluorochromes.

Notes

United States
877.232.8995

Canada
888.259.0187

Europe
32.53.720.550

Japan
0120.8555.90

Asia/Pacific
65.6861.0633

Latin America/Caribbean
55.11.5185.9995

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