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<td>Austria</td>
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<td>Israël</td>
<td>Tel.: (972) 1 80 921 6283</td>
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<td>Belgium</td>
<td>Tel.: (32) 2 401 7093</td>
<td>Italy</td>
<td>Tel.: (39) 02 360 036 85</td>
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<tr>
<td>Denmark</td>
<td>Tel.: (45) 80 882 193</td>
<td>Norway</td>
<td>Tel.: (47) 800 18530</td>
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<tr>
<td>Eastern Europe</td>
<td>Tel.: (32) 53 720 740</td>
<td>Portugal</td>
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<td>Middle East &amp; Africa</td>
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<td>South Africa</td>
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<td>Finland</td>
<td>Tel.: (358) 800 11 63 17</td>
<td>Spain</td>
<td>Tel.: (34) 91 414 6250</td>
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<td>France</td>
<td>Tel.: (33) 1 70 70 81 93</td>
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<td>Switzerland</td>
<td>Tel.: (41) 44 580 43 73</td>
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<td>Greece</td>
<td>Tel.: (30) 0 800 12 7506</td>
<td>The Netherlands</td>
<td>Tel.: (31) 10 711 4800</td>
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<tr>
<td>Hungary</td>
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<td>UK</td>
<td>Tel.: (44) 207 075 3226</td>
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Immune Function Technologies to Meet Your Experimental Needs

The immune system is a focus of study for many researchers from diverse scientific disciplines because of its central importance in host defense and its intercommunication with other systems that maintain bodily homeostasis. Having reliable tools and assay systems that can give you the richest information in the least time is critical to all areas of immunology research, whether your focus is on better understanding the cellular and molecular basis of immunological and inflammatory responses, on measuring immunological endpoints in health and disease, or on developing drugs to modulate the immune system.

Committed to providing researchers with flexible and comprehensive solutions for exploring immune function, BD Biosciences offers a comprehensive range of reagents and assay systems for the functional analysis of immune modulators and immune cell populations. Covering the main techniques currently in the “immunologist’s toolbox”, we continue to commercialize state-of-the-art technical advances around cell analysis, helping you to harness the power of flow cytometry for multiplexable, information-rich immune function studies. Understanding the importance of assay standardization and increased throughput in clinical research settings, we incorporate these aspects into our assay and application development, to help you keep up with the demands in clinical research applications.

The technology of choice for your particular experimental needs depends on a number of factors, such as the information needed, sample constraints, and instrumentation available. An overview of the features of each type of assay is shown in Table 1 (page 6), which aims to provide direction in the choice of the appropriate method.

Helping You Keep the Pace

At BD Biosciences we understand that keeping up with technological and methodological advances is key in a laboratory setting. As part of our ongoing commitment to offer you the best service and assistance, we provide you with detailed, carefully optimized protocols for the use of our products. The Techniques for Immune Function Analysis: Application Handbook 2nd Edition is a compilation of protocols and technique-oriented tips and tricks that aim to help you get the most out of your immune function studies.

The original version, published in 1997, included a tremendous amount of input from our customers. Over the years, the collection of protocols and methodological tips has evolved. New additions in this edition include:

- Expanded chapter on BD™ Cytometric Bead Array – now including the newer create-your-own array protocols
- New chapter on Cytotoxicity and Degranulation Assays

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Beyond providing you with optimized protocols, we include training and support as an integral part of our offering.

Our training courses offer the highest quality education in cell analysis using state-of-the-art flow cytometry equipment and methodologies. BD Instructors and Flow Support Specialists blend experience in training with technical expertise to offer you the learning you need to stay current with and take full advantage of this changing technology: from hands-on courses in one of the BD Regional Training Centers to personalized assistance with your instruments and applications, to online learning offerings.

Our Scientific Support team offers personalized phone and e-mail assistance on our products and their applications. All highly qualified Life Scientists, with in-depth understanding of our products and technologies, they are there to provide you expert assistance on-demand. You may contact them at the numbers on the inside cover.

Immune Function Solutions from BD Biosciences

Immune Function Mini-Website: www.bdbiosciences.com/immune_function
Table 1. Tools and Technologies for Immune Function Monitoring

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<thead>
<tr>
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<th>Flow cytometry - Surface</th>
<th>Flow cytometry - Intracellular</th>
<th>ELISpot</th>
<th>MHC mimics</th>
<th>ELISA</th>
<th>In Vivo Capture</th>
<th>Multiple Bead Arrays</th>
</tr>
</thead>
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<tr>
<td>Molecules detected</td>
<td>Surface</td>
<td>Intracellular &amp; surface</td>
<td>Secreted (in-situ)</td>
<td>Surface</td>
<td>Secreted</td>
<td>Secreted</td>
<td>Secreted or intracellular</td>
</tr>
<tr>
<td>Multiparameter?</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Single cell/ cell subset informa-</td>
<td>Yes</td>
<td>Yes</td>
<td>Frequencies, no subset information</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>tion?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Functional testing?</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Antigen-specific?</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Post-assay cell viability?</td>
<td>Yes/possible</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>(N.A.)</td>
<td>Yes, for secreted molecules</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Highly Sensitive</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Sensitive</td>
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### Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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</thead>
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<tr>
<td>7-AAD</td>
<td>7-aminoactinomycin</td>
</tr>
<tr>
<td>ABTS</td>
<td>2', 2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)</td>
</tr>
<tr>
<td>AEC</td>
<td>3-amino-9-ethyl-carbazole</td>
</tr>
<tr>
<td>aka</td>
<td>also known as</td>
</tr>
<tr>
<td>APC</td>
<td>alloglycycyanin or antigen-presenting cell</td>
</tr>
<tr>
<td>APC-H7</td>
<td>alloglycycyanin-H7</td>
</tr>
<tr>
<td>BFA</td>
<td>Brefeldin A</td>
</tr>
<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
</tr>
<tr>
<td>BRM</td>
<td>biological response modifier</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CBA</td>
<td>Cytometric Bead Array</td>
</tr>
<tr>
<td>CFC</td>
<td>cytokine flow cytometry</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CTL</td>
<td>cytolytic T lymphocyte</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole*2HCl</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>distilled deionized water</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethyl formamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ED₅₀</td>
<td>50% effective dose</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ELISpot</td>
<td>enzyme-linked immunospot assay</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescent activated cell sorting</td>
</tr>
<tr>
<td>FcR</td>
<td>immunoglobulin Fc receptors</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
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<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IVC</td>
<td>in vivo capture</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>L</td>
<td>liter</td>
</tr>
<tr>
<td>LAL</td>
<td>limulus amebocyte lysate</td>
</tr>
<tr>
<td>LAMP-1</td>
<td>lysosomal-associated membrane protein 1</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MCP</td>
<td>monocyte chemoattractant protein</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MIP</td>
<td>macrophage inhibitory protein</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NA/LE</td>
<td>no azide/low endotoxin</td>
</tr>
<tr>
<td>ND₅₀</td>
<td>50% neutralizing dose</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>NBCS</td>
<td>newborn calf serum</td>
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<tr>
<td>NK</td>
<td>natural killer</td>
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### Abbreviations (continued)

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<th>Description</th>
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<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-Tween</td>
<td>PBS containing 0.05% Tween-20</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin chlorophyll protein</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
</tr>
<tr>
<td>PMT</td>
<td>photomultiplier tube</td>
</tr>
<tr>
<td>PY</td>
<td>pyronin Y</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon Activation, Normal T Expressed and presumably Secreted</td>
</tr>
<tr>
<td>rhIL</td>
<td>recombinant human interleukin</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPA</td>
<td>ribonuclease protection assay</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SAv-HRP</td>
<td>streptavidin-horseradish peroxidase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEB</td>
<td>Staphylococcal enterotoxin B</td>
</tr>
<tr>
<td>&quot;[^H]-TdR&quot;</td>
<td>tritiated thymidine</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris borate EDTA</td>
</tr>
<tr>
<td>TCC</td>
<td>terminal complement complex</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TDS</td>
<td>Technical Data Sheets</td>
</tr>
<tr>
<td>TE</td>
<td>Tris EDTA</td>
</tr>
<tr>
<td>TMB</td>
<td>tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>U</td>
<td>unit</td>
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<td>V450</td>
<td>Violet 450</td>
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Chapter 1

Immunofluorescent Staining of Cell Surface Molecules for Flow Cytometric Analysis of Immune Function

Introduction

To understand immune responses, it is necessary to identify, isolate, and study a variety of cell types, cell functions, and interactions that constitute those responses. A vast array of different cell surface molecules are involved in mediating immune responses. Methods that determine the types and levels of such membrane molecules (surface markers) that are co-expressed by cells provide important information regarding cell lineage, activation status, adhesion, migration and homing capacity, and ability to respond to stimuli and to interact with other cells. For the purposes of this handbook, this chapter will focus on methods for the detection and measurement of cell surface molecules that mediate cellular functions by virtue of their expression and/or binding of signaling molecules that are critical for cellular intercommunication. Such signaling molecules include cytokines, chemokines, inflammatory mediators, and their receptors, (ie, biological response modifiers [BRMs] of the immune system). Upon interaction with their specific receptors, BRM ligands can influence the physiology of either the producer cell (autocrine action), adjacent target cells (paracrine action) or distant target cells (endocrine action).

In this way, BRMs may influence target cell activation, growth, proliferation, differentiation, migration, and effector function (eg, expression of other BRMs).

Cytokine, Chemokine, and Inflammatory Mediator Receptors

Cytokine receptors are grouped into superfamilies based on the common sequence homologies of their extracellular regions. The main superfamilies recognized today are the Cytokine Receptor (aka, Hematopoietic Receptor), Protein Tyrosine Kinase Receptor, TNF Receptor, Interferon Receptor (aka, Cytokine Receptor Type II), and IL-1/Toll-like Receptor Superfamilies.1-3 Some receptors consist of a single polypeptide chain that is responsible for both cytokine binding and signal transduction. Other receptors consist of two or more chains, one of which is primarily associated with ligand binding while the other chain(s) is associated with changes in the binding affinity or mediates signal transduction. The high affinity IL-2 receptor serves as an example of a complex receptor with α (CD25), β (CD122), and γ (common gamma chain, CD132) subunits that play different roles. Different receptor complexes may also share the same signaling subunit while they consist of different binding subunits. For example, the human βγ subunit (common beta chain, CD131) can combine with distinct receptor subunits specific for IL-3 (IL-3Rα, CD123), IL-5 (IL-5Rα, CD125) or GM-CSF (GM-CSFRα, CD116). Some cytokine receptor subunits are
constitutively expressed by resting cell types and undergo modest upregulation upon cellular activation, while others can be dramatically upregulated (eg, IL-2Rα) by stimulated cells. Cytokine receptors transduce external biological signals into intracellular events by various signal transducing proteins including Protein Tyrosine Kinases. Most of the cytokine receptors are transmembrane proteins, although in some cases measurable (even high) levels of circulating, soluble forms (extracellular domains) of the receptors are observed (eg, soluble TNFR, IL-2R, IL-4R, and IL-6R). Soluble cytokine receptors may regulate cytokine actions by specifically binding their cognate cytokine and thus inhibiting its interaction with receptors expressed on target cells. Alternatively, soluble receptors may potentiate the effects of their bound cytokine by extending its half-life in the circulation. The failure to control the levels of circulating cytokines may contribute to pathological situations including sepsis, tissue damage, inflammation, and autoimmunity.

Chemokine receptors belong to the Rhodopsin Superfamily (seven transmembrane receptors) and are G-protein-coupled. Chemokine receptors can be divided into several families based on their ligand specificity, including CXC receptors, CC receptors, CX3C receptor, and orphan receptors.

Inflammatory mediator receptors are very diverse as one would expect given the tremendous variety of ligands. This group of molecules includes receptors that bind products of complement activation cascades such as C3a and C5a fragments. Other inflammatory receptors engage products of the arachidonic pathway (eg, prostaglandins and leukotrienes), specific molecules made by infectious organisms (eg, CD14, Toll-like receptors), or protein mediators (ie, acute phase proteins, granzymes, and defensins).

Biological Response Modifier Receptors and Flow Cytometry

Some receptors (eg, cytokine receptors) are expressed at relatively low levels by unstimulated cells (10 – 1000 molecules/cell), but their surface levels can be considerably upregulated following activation (>10,000 molecules/cell). In certain cases, the level of cell surface receptors remains quite low even after cellular activation, (eg, 100 – 1000 molecules/cell). In the past, the measurement of surface BRM receptors expressed by cell populations was made by using a receptor binding assay with radioactively-labeled ligands (radioceptor assay). Although the radioceptor assay is useful, it primarily measures high affinity receptors that are often comprised of multiple subunits, not individual receptor subunits. This assay can be successfully used to estimate the numbers of receptors expressed by cells within homogeneous cell populations, such as cell lines. However, it can provide only an average value of receptor levels expressed per cell when the sample is comprised of a mixture of various cell types.

To better understand the physiology of a particular BRM ligand, it is necessary not only to measure its levels in biological fluids (eg, serum, plasma, cell culture supernatants), but also to characterize the frequencies and types of cells that produce the BRM and determine the nature of the target cells that express its
cognate receptors. Multiparameter flow cytometric analysis is a quick, specific, high-throughput method that makes these latter types of studies feasible. Even mixed cell populations, which are routinely prepared from peripheral blood or lymphoid tissues, are amenable to high resolution analysis by using multiparameter flow cytometry.

A large number of fluorescent antibodies specific for cell surface and intracellular markers can be used to characterize cells within populations by multiparameter flow cytometric analysis. In this way, it is possible to gather information regarding each cell’s state of activation and differentiation, lineage, migration potential, and functional responsiveness (Figures 1–4). For example, it is known that receptors for some cytokines increase upon cellular activation. Evidence of their reduced expression could be indicative of a pathologic condition (eg, HIV infection).

Multicolor flow cytometric analysis also enables analysis of complex cellular interactions in mixed cell populations. For instance, analysis of the expression of cell surface markers or intracellular molecules along with cytokine receptor subunits may provide insights into the potential of individual cells within subsets to produce and/or respond to certain cytokines. This type of analysis allows the researcher to make predictions regarding the types of immune responses that could result from interactions amongst cells within sample populations. These predicted cellular response pathways can then be tested by further experimentation (eg, through the use of differentiation cultures that can generate Th1 versus Th2 types of responses).

A great advantage for cells that can be identified by immunofluorescent staining and flow cytometric analysis in mixed cell populations is that they can also be purified by fluorescent-activated cell sorting or by other means (eg, the BD™ IMag Magnetic Cell Separation System). This can allow isolation of individual cells based on lineage, activation, or cellular differentiation.

Other Assays Used to Study BRM Receptor Biology

Enzyme-Linked Immunosorbent Assay (ELISA): Sandwich ELISAs can be used to quantitate soluble BRM ligands and their receptors that are present in serum, plasma, or in tissue culture supernatants (described in Chapter 9.)

BD™ Cytometric Bead Array (CBA): BD CBA (described in Chapter 2) is designed for the multiplexed quantitation of multiple BRM ligands, soluble adhesion receptors, and complement proteins in soluble samples. Employing a series of suspended beads, up to 30 proteins can be analyzed simultaneously using just 25 to 50 μL of sample, providing more data per volume of sample in comparison with traditional ELISA and Western blot.

Biological assays: A variety of bioassays can be used to evaluate whether a test cell population expresses functional BRM receptors by the ability of a test cell population to respond to a given BRM ligand (described in Chapter 12).
Figure 1. Differential expression of human IL-6Rα (CD126) chain on CD4+ and CD8+ T cells. Human PBMCs were isolated by density gradient centrifugation (Ficoll-Paque™) and were stained with FITC-anti-human CD4 (Cat. No. 555346, Panels A and B), PerCP-anti-human CD8 (custom made by the Custom Technology Team, BD Biosciences, Panels C and D), APC-anti-human CD45RO (Cat. No. 559865, all Panels) and PE-anti-human IL-6Rα (CD126, Cat. No. 551850, Panels A and C), and PE mouse IgG1,κ isotype control (Cat. No. 555749, Panels B and D) antibodies. Staining with the anti-human IL-6Rα (CD126) antibody is compared to staining derived with an isotype control antibody (B, D). Two-color dot plots showing the correlated expression patterns of IL-6Rα (CD126) or Ig isotype control and CD45RO were derived from immunofluorescent-gated events with the forward and side light-scatter characteristics of viable CD4+ or CD8+ lymphocytes.

Figure 2. Detection of human CCR7 expression on CD4+ and CD8+ human lymphocytes by PE-conjugated anti-human CCR7 antibody. Human PBMCs were stained with PE-conjugated anti-human CCR7 (clone 3D12, Cat. No. 552176, Panels A and B) and FITC-conjugated anti-human CD45RA (Cat. No. 555488, Panels A and B). The two-color data shown are derived from the CD4+ (based on staining with APC-conjugated anti-human CD4, Cat. No. 555349, Panel A) and CD8+ (based on staining with APC-conjugated anti-human CD8, Cat. No. 555369, Panel B) lymphocyte-gated populations.
Figure 3. Detection of TLR1 and TLR4 expression on human peripheral blood monocytes. Human peripheral blood mononuclear cells were either treated with BD Pharm Lyse™ lysis buffer (Cat. No. 555899) to lyse red blood cells (Panel A) or were purified by density gradient centrifugation (Ficoll-Paque™) to isolate PBMCs (B). The cells were subsequently stained with either purified anti-human TLR1 (CD281; clone GD2, Cat. No. 552033, Panel A), or purified anti-human TLR4 (CD284; clone HTA125, Cat. No. 551964, Panel B). The anti-human TLR1 and anti-human TLR4 antibodies were then detected by either biotinylated F(ab')2 goat anti-mouse IgG (Caltag, Cat. No. M35015, Panel A) or biotinylated anti-mouse IgG2a (Cat. No. 553388, Panel B), respectively, followed by PE-streptavidin (Cat. No. 554061, both Panels) and FITC-rat anti-human CD14 (Cat. No. 555397, both Panels). Gates in panel A were set to include cells that were CD14+. The two-color data shown in panel B are derived from ungated mononuclear cell populations.

Figure 4. Expression of human C5aR on C5aR transfectants and granulocytes. Human C5aR transfected and wild type mouse L cells were treated with Mouse BD Fc Block™, CD16/CD32 (FcγRIII/II Receptor, Cat. No. 553141 and 553142) to block Ig Fc- receptors and were stained with PE-conjugated anti-human-C5aR antibody (clone C85-4124, Cat. No. 552993, Panel A). Human granulocytes were isolated from human peripheral blood by density gradient centrifugation using Polymorphoprep™ (Nycomed). Isolated granulocytes were subsequently stained with PE-conjugated anti-human-C5aR antibody (Panel B) and FITC-conjugated anti-human CD16 (Cat. No. 555406, Panel B). Gates were set to include cells that were CD16+ and had the forward and side light-scatter characteristics of granulocytes (Panel B). Histograms defined as negative control indicate C5aR transfectants (Panel A) or human granulocytes (Panel B) stained with PE streptavidin only.
Reagents for Immunofluorescent Staining of Cell Surface Molecules

BD Biosciences offers the widest range of conjugated and unconjugated antibodies, including BD Pharmingen™ brand antibodies, for immunological research, immune function studies and immune monitoring.

- **Validated antibodies for a wide range of applications:** Multicolor Flow Cytometry, ELISA, Immunohistochemistry, Fluorescence Microscopy, Bioimaging and Western Blotting.
- **Reproducible results:** BD™ and BD Pharmingen™ reagents are manufactured and tested to meet your high standards of purity, stability, low background and lot-to-lot consistency.
- **More information from a single sample:** conjugated antibodies now include PE-Cy™7, APC-Cy™7, Alexa Fluor®, Pacific Blue™, AmCyan and the New BD™ APC-H7 and BD Horizon™ V450 fluorochromes.

Our list of specificities includes an extensive selection of CD antigens and other markers for defining different immune cell lineages and functional populations. Of particular note is our wide range of antibodies to cytokine, chemokine and inflammatory mediator receptors, which can be found in not only in the sections for “Cell Surface Molecules” for the particular species (Human, Mouse, Rat, Non-Human Primate or Other Non-Human Species), but also in the section “Cytokines, Chemokines and Inflammatory Mediators”.

For an updated list of antibodies and other reagents for immunofluorescent staining of cell surface molecules, please refer to the BD Biosciences online product catalog website at [www.bdbiosciences.com](http://www.bdbiosciences.com).

Go to Research Reagents > (Species) Cell Surface Molecules.

**Protocol: Multicolor Immunofluorescent Staining for Receptors and Other Cell Surface Antigens.**

1. **Harvest cells**

Viable leukocytes can be obtained from peripheral blood or lymphoid tissues. Activated cell populations can also be prepared from in vivo-stimulated tissues or from in vitro-activated cultures. Single cell suspensions are prepared and the cell concentrations are adjusted to $2 \times 10^7$/ml (for staining in microwell plates; BD Falcon™ Cat. No. 353910) or $10^7$/ml (for staining in tubes; BD Falcon 12 × 75 polystyrene Cat. No. 352008). All incubations and reagents are kept at 4°C with sodium azide to minimize receptor modulation (eg, internalization or shedding). The cells should be protected from light throughout staining and storage.

2. **Block Immunoglobulin Fc Receptors**

Reagents that block immunoglobulin Fc receptors (FcR) may be useful for reducing nonspecific immunofluorescent staining.

   a. In the mouse and rat systems, purified antibodies directed against mouse FcγII/III (Mouse BD Fc Block™, CD16/CD32, Cat. No. 553141 and
553142) and rat FcγIII Receptor (Rat BD Fc Block™, CD32, Cat. No. 550270 and 550271) respectively, can be used to block nonspecific staining due to FcR. To block FcR with BD Fc Block reagents, preincubate the cells with 10 μg/ml of BD Fc Block antibody per 2 × 10^7 cells for 15–20 min at 4°C. The cells are then transferred (10^6 cells/test) to either microwell plates or plastic tubes for immunofluorescent staining. The cells are not washed before the first staining step.

b. FcR on human cells can be pre-blocked by incubating cells (10^6 cells) with human IgG (polyclonal human IgG, Sigma, Cat. No. I–4506). Alternatively, one can use 10% normal human serum in PBS for 20 minutes at 4°C to block Fc receptors.

3. Stain for Receptors and Other Cell Surface Antigens

a. Direct immunofluorescent staining

1. Incubate ~10^6 cells in 100 μl of staining buffer (see Buffers for more information) containing a pre-titrated, optimal concentration (usually ≤ 1 μg) of a fluorescent monoclonal antibody specific for a receptor or with an immunoglobulin (Ig) isotype-matched control for 30 – 45 min at 4°C. In cases of multicolor staining, other fluorescent antibodies directed at various cell surface antigens can be added at the same time with the receptor-specific antibody.

2. After the incubation, add 100 – 200 μl of staining buffer and pellet the cells by centrifugation (250 x g for 5 min). Wash the cells 1x with 200 μl of staining buffer, pellet by centrifugation (250 x g for 5 min), and remove supernatant.

For staining in tubes, wash the cells 1x with 2 ml of staining buffer and pellet the cells by centrifugation (250 x g for 5 min), and remove supernatant.

3. For staining in microwell plates, add 200 μl of staining buffer to each well, transfer the contents to staining tubes (BD Falcon, 12 × 75mm tubes, Cat. No. 352008) and bring up the volume to 0.5 ml with staining buffer and keep them at 4°C until flow cytometric analysis. For staining in tubes, resuspend cell samples in 0.5 ml of staining buffer and keep them at 4°C until flow cytometric analysis. If desired, cells may be fixed with BD Cytofix™ Buffer (Cat. No. 554655, 100 μl/test) prior to flow cytometric analysis. After fixation, cells are washed as indicated in step 3.a.2 and stored at 4°C until analysis. However, it should be noted that some antigens are sensitive to fixation, resulting in a reduced level of staining (eg, anti-mouse CD21/CD335, clone 7G6).

b. Indirect immunofluorescent staining – 2 Layer Staining

1. Incubate ~10^6 cells in 100 μl with a pre-titrated, optimal concentration (≤ 1 μg) of a purified or biotinylated monoclonal antibody specific for a receptor or with an Ig isotype-matched control antibody for 30 – 45 min at 4°C.

2. After the incubation, add 100–200 μl of staining buffer and pellet the cells by centrifugation (250 x g for 5 min). Wash the cells 1x with 200 μl of staining buffer, pellet by centrifugation (250 x g for 5 min), and remove supernatant.
For staining in tubes, wash the cells 1x with 2 ml of staining buffer and pellet the cells by centrifugation (250 × g, 5 min), and remove supernatant.

3. Resuspend and incubate cells in 100 μl of staining buffer containing a pre-titrated, optimal concentration (usually ≤ 1 μg per 10^6 cells) of a fluorescent anti-Ig secondary antibody (for troubleshooting see Critical Parameters for Detection of Cell Surface Antigens by Flow Cytometry, 7. Background Staining, page 26) or fluorescently conjugated streptavidin (usually ≤ 0.06 μg per 10^6 cells) for 30 min at 4°C.

Note: In cases of multicolor staining, other fluorescent antibodies may be used to detect various cell-surface antigens. When the fluorescent antibodies used for staining additional cell surface antigens originate from the same species as the primary antibody, they have the potential to bind to the fluorescent secondary anti-Ig antibody. To eliminate this possibility, after incubating cells with the fluorescent anti-Ig antibody, wash the cells and then block the unoccupied binding sites of the fluorescent anti-Ig antibody with Ig contained within normal serum obtained from the same species as the primary antibodies (25 μl of neat serum for 20 min). After blocking, add the other fluorescent antibodies and incubate for 20 – 30 min at 4°C.

4. Wash cells as indicated in step 3.b.2.

5. For staining in microwell plates, add 200 μl of staining buffer to each well, transfer the contents to staining tubes and bring up the volume to 0.5 ml with staining buffer and keep them at 4°C until flow cytometric analysis. For staining in tubes, resuspend cell samples in 0.5 ml of staining buffer in tubes and keep them at 4°C until flow cytometric analysis. If desired, cells may be fixed with BD Cytofix Buffer (Cat. No. 554655, 100 μl/test) prior to flow cytometric analysis. After fixation, cells are washed as indicated in step 3.b.2 and stored at 4°C until analysis.

c. Indirect immunofluorescent staining – 3 Layer Staining. For certain BRM receptors that are expressed at very low levels, it may be necessary to use “3 layer” indirect immunofluorescent staining method to “amplify” the fluorescent signal.

1. Incubate ~10^6 cells in or 100 μl of staining buffer containing a pre-titrated, optimal concentration (usually ≤ 1 μg) of a purified monoclonal antibody specific for a receptor or with an Ig isotype-matched control antibody for 30 – 45 min at 4°C.

2. After the incubation, add 100 – 200 μl of staining buffer and pellet the cells by centrifugation (250 × g for 5 min). Wash the cells 1x with 200 μl of staining buffer, pellet by centrifugation (250 × g for 5 min), and remove supernatant.

For staining in tubes, wash the cells 1x with 2 ml of staining buffer and pellet the cells by centrifugation (250 × g for 5 min), and remove supernatant.
3. Resuspend and incubate cells in 100 μl of staining buffer containing a pre-titrated, optimal concentration (usually ≤ 1 μg) of a biotinylated anti-Ig secondary antibody (for troubleshooting see Critical Parameters for Detection of Cell Surface Antigens by Flow Cytometry, 7. Background Staining, Page 26 for 30 min at 4°C.

4. Wash cells as indicated in step 3.c.2.

5. Resuspend and incubate cells for 30 min at 4°C cells in 100 μl of staining buffer containing a pre-titrated, optimal concentration (usually ≤ 0.25 μg) of a fluorescent streptavidin (eg, phycoerythrin- or allophycocyanin-streptavidin for maximum fluorescently conjugated signal intensities and minimal cellular autofluorescence).

Note: In cases of multicolor staining, other fluorescent antibodies may be used to detect various cell surface antigens. When the fluorescent antibodies used for staining additional cell surface antigens originate from the same species as the primary antibody, they have the potential to bind to the biotinylated secondary anti-Ig antibody. To eliminate this possibility, after incubating cells with the biotinylated anti-Ig antibody, wash them and then block the unoccupied binding sites of the biotinylated anti-Ig antibody with Ig contained within normal serum obtained from the same species as the primary antibody (25 μl of neat serum for 20 min). After blocking, add the other fluorescent antibodies and incubate for 20 – 30 min at 4°C.

6. For staining in microwell plates, add 200 μl of staining buffer to each well, transfer the contents to staining tubes and bring up the volume to 0.5 ml with staining buffer and keep them at 4°C until flow cytometric analysis. For staining in tubes, resuspend cell samples in 0.5 ml of staining buffer in tubes and keep them at 4°C until flow cytometric analysis. If desired, cells may be fixed with BD Cytofix Buffer (Cat. No. 554655, 100 μl/test) prior to flow cytometric analysis. After fixation, cells are washed as indicated in step 3.c.2 and stored at 4°C until analysis.


1. Dilute whole blood 1:10 with (1x) BD Pharm Lyse™ lysis buffer (Cat. No. 555899), mix well, and incubate 10 min at room temperature (RT) in the dark.

2. Spin for 5 min at 500 × g.

3. Aspirate supernatant. Wash 2× with 2 ml of staining buffer. Spin for 5 min at 500 × g. Aspirate supernatant.

4. Continue with FcR blocking and staining (see Stain for Receptors and Other Cell Surface Antigens, page 17).

Note: The detection of certain cytokine receptors (eg, IL-6R, IL-4R) may be affected by the lysis step if this is performed prior to...
staining. In those cases, it is recommended to lyse after staining cytokine receptors as indicated below:

1. Add 100 μl of anti-coagulated whole blood to plastic tubes.
2. Stain with receptor-specific antibodies (see *Stain for Receptors and Other Cell Surface Antigens, page 17*).
3. Wash cells 2× with staining buffer (2 ml/tube), pellet by centrifugation (250 × g for 5 min), and remove supernatant.
4. Resuspend cells with 200 μl BD Pharm Lyse lysis buffer, vortex, incubate at RT for 10 min.
5. Wash 2× as indicated in step 3 and proceed step a.2 (see *Stain for Receptors and Other Cell Surface Antigens, pages 17 and 18*).

### Staining Controls

#### 1. Positive Staining Controls

Certain cell surface antigens such as cytokine receptors are upregulated upon cell stimulation. The Technical Data Sheets (TDS) for the BD Pharmingen cytokine-receptor-specific antibodies may describe *in vitro* culture systems that induce detectable frequencies of cytokine-receptor-expressing cells at specific timepoints. Cells stimulated by these methods can be used as positive controls for experimental systems. Alternatively, cell lines that are widely available may also be recommended in the TDS. For those receptor subunits that are constitutively expressed, unstimulated cells can be used as controls.

#### 2. Negative Staining Controls

The following controls can be used to discriminate specific from nonspecific staining:

a. **Staining of a negative cell population:** Staining of a cell line or a specific cell subset within a mixed cell population that is known not to express a specific receptor chain can serve as a negative staining control.

b. **Immunoglobulin isotype control:** Stain with an immunoglobulin (Ig) isotype control of irrelevant specificity. Stain as described in the aforementioned procedure for receptors and other cell surface antigens. Ig isotype controls should be used at the same concentration as the receptor-specific antibody. Guidelines for isotype control selection can be found on our website: [www.bdbiosciences.com/pharmingen/protocols/Isotype.shtml](http://www.bdbiosciences.com/pharmingen/protocols/Isotype.shtml)

c. **Blocking antibody control:** Preincubate cells with unconjugated antibody. This type of negative control can only be used for fluorescent or biotinylated receptor-specific antibodies.

1. Resuspend cells in 50 μl of staining buffer (50 μl for staining in tubes) containing unconjugated receptor-specific antibody (same clone as conjugated antibody) diluted to be in excess when compared to the conjugated antibody (usually 5 μg/10^6 cells), and incubate 30 min at 4°C.
2. After incubation, add fluorescent or biotinylated receptor-specific antibody at an optimal concentration in 50 μl of staining buffer for a final volume of 100 μl, and incubate 30–45 min at 4°C.

3. Wash cells (see *Stain for Receptors and Other Cell Surface Antigens, page 17-18, 3.b.2 – 3.b.5*)

   *Note:* The purified antibody should significantly (>90%) block staining by the fluorescent or biotinylated antibody that subsequently is added to cells.

3. Other Controls

The following controls can be used to optimize instrument settings:

a. Autofluorescence controls

   Autofluorescence results from fluorescent emissions occurring when intracellular materials are excited at the same wavelength as the fluorescent probes used for staining. *In vitro*-cultured cells, tumors, or cells high in granule content may have relatively higher autofluorescence when compared with other cells. To determine the baseline fluorescence of each cell population studied, controls that include only unstained (ie, not stained for the marker of interest) cells can be used.

b. Compensation controls

   Electronic compensation may be necessary to correct the spectral overlap of fluorescent emissions when multiple fluorescent probes excited by a single wavelength are used. Cell samples stained with individual fluorescent probes (ie, cells stained with a FITC-conjugated antibody alone, cells stained with a PE-conjugated antibody alone) can be compared with cells labeled with both fluorescent probes to determine the level of fluorescence signal overlap and to establish proper compensation. For more detailed information see reference 19. For detailed guidelines on setting compensation, please refer to: www.bdbiosciences.com/pharmingen/protocols/Multi-Color_Flow_Cytometric_Analysis.shtml

Buffers

**Staining Buffer**

- Dulbecco’s PBS (DPBS)
- 2% heat inactivated FCS
- 0.09% (w/v) sodium azide
- Adjust buffer pH to 7.4 – 7.6, filter (0.2 μm pore membrane), and store at 4°C.

BD Biosciences offers two buffers: BD Pharmingen™ Stain Buffer (FBS) (Cat. No. 554656) and BD Pharmingen™ Stain Buffer (BSA) (Cat. No. 554657) that are rigorously tested for their ability to optimize immunofluorescent staining and maintain cell viability.
Critical Parameters for Detection of Cell Surface Antigens by Flow Cytometry.

1. Stimulation and Harvesting of Cells

Certain cell surface antigens such as cytokine receptors (eg, mouse IL-12Rβ2) are expressed in very low numbers in non-stimulated cells but can be upregulated up to ten-fold higher levels following cell activation. Therefore, it is necessary to determine the cell activation conditions that enhance their surface expression (Figure 5). In other cases, the level of surface expression for an antigen may decline after cell activation due to shedding (eg, certain cytokine receptors). Use of inhibitors in the tissue culture medium that block receptor shedding has been used successfully to reverse this effect (eg, see TNFRI and TNFRII), (Figure 6).

**Figure 5. Mouse IL-12Rβ2 expression on in vitro-activated cells.** C57BL/6 mouse splenocytes were treated to lyse erythrocytes and were cultured for 5 days with either plate-bound anti-mouse CD3 antibody (Cat. No. 553057) plus recombinant mouse IL-2 (Cat. No. 554578) and IL-4 (Cat. No. 550067) (Panel A) or with ConA (2 µg/ml), PMA (5 ng/ml), dextran sulfate (10 µg/ml), LPS (5 µg/ml), anti-IL-4 antibody (5 µg/ml, Cat. No. 554432), recombinant mouse IL-2 (10 ng/ml, Cat. No. 550069) and IL-12 (20 ng/ml Cat. No. 554592) (Panel B). Five days later, cells were harvested, washed and blocked with mouse BD Fc Block (10 µg/ml, Cat. No. 553141, both Panels). Cells were subsequently stained with purified anti-mouse IL-12Rβ2 (clone HAM10B9, Cat. No. 552819, both Panels) followed by PE-labeled anti-hamster IgG (cocktail) (Cat. No. 554056, both Panels) and BD Via-Probe™ (Cat. No. 555815, both Panels). Staining with the anti-mouse IL-12Rβ2 antibody (filled histograms) is compared to staining obtained using a Purified Hamster IgGκ Isotype control (Cat. No. 553951, both Panels, open histograms). Histograms were derived from gated events of viable (7-AAD negative) lymphocytes.
Figure 6. TACE (Tumor Necrosis Factor-Alpha Converting Enzyme) inhibitors block activation-induced shedding of TNFRs and membrane TNF. Human PBMCs isolated by density gradient centrifugation (Ficoll-Paque™) were stimulated with plate-bound anti-human CD3 antibody (10 µg/ml, Cat. No. 555336) and soluble anti-CD28 antibody (2 µg/ml, Cat. No. 555725) in the presence of human IL-2 (10 ng/ml, Cat. No. 554603) and IL-4 (40 ng/ml, Cat. No. 554605) for 2 days. The cells were subsequently washed and expanded in IL-2 and IL-4 for three days. Following expansion, the cells were washed and stimulated for 2 hr with PMA (5 ng/ml) and ionomycin (500 ng/ml) with or without 25 µM of metalloprotease inhibitors (TAPI) or were used without further stimulation. Following incubation, the cells were harvested and their surface expression of human TNFRI and TNFRII were detected by immunofluorescent staining and flow cytometric analysis using biotinylated anti-human TNFRI (clone MABTNFR1-B1, Cat. No. 550900, Panel A) and purified anti-human TNFRII (clone hTNFR-M1, Cat. No. 551311, Panel B), respectively. The anti-human TNFRI and anti-human TNFRII antibodies were subsequently detected with PE-streptavidin (Cat. No. 554061, Panel A) and biotinylated F(ab')2 goat anti-rat IgG (Jackson ImmunoResearch, Cat. No. 112-066-062, Panel B) followed by PE-streptavidin, respectively. Expression of membrane TNF was detected using the PE-labeled anti-human TNF antibody (clone MAb11, Cat. No. 559321, Panel C). Histograms were derived from gated events with the forward and side light-scatter characteristics of viable lymphocytes.

2. Quality of Antibody

The choice of high affinity, receptor-specific antibodies for immunofluorescent staining is very critical. Certain antibody isotypes may be problematic because they tend to nonspecifically bind to FcRs. For example, antibodies with the mouse IgG1 isotype tend to nonspecifically bind less than other mouse and rat Ig isotypes to surface FcRs expressed by human PBMCs.

3. Choice of Protocol—Direct versus Indirect Staining

In cases where direct immunofluorescent staining is employed, high sensitivity can be achieved using antibodies conjugated to “bright” fluorochromes.11,13 Therefore, in multicolor flow cytometric analysis for cytokine receptors and other weakly-expressed cell surface antigens, it is recommended that PE, APC, PE-Cy™5, PE-Cy™7 or Alexa Fluor® 647-conjugated antibodies be considered, when available.* Fluorescein isothiocyanate (FITC)- and PerCP-labeled reagents should be used for staining antigens that are coexpressed at relatively higher levels.

The limit of sensitivity for flow cytometry is typically around 200 – 500 molecules/cell (depending on the nature of the cells, reagents, staining protocol and flow cytometer that is used). Sensitivity is defined as
the significant separation between the signal from positive cells when compared with signals given by negative cell controls. For those receptors that are expressed at such low levels, signal amplification can be achieved by increasing the “layers” of immunofluorescent staining.\textsuperscript{11,13} For example, use of biotinylated, polyclonal secondary antibodies followed by PE- or APC-streptavidin (“3 layer staining”) has proven to be the preferred method for increased sensitivity (Figure 7).

Each primary antibody can theoretically be bound by at least two secondary antibodies, each one of which carries several biotin molecules (which in turn can bind PE- or APC-streptavidin).

\textit{Note:} PerCP-labeled reagents are not recommended for immunofluorescent staining of cells that are used for sorting because they tend to photobleach after excitation by the high energy laser excitation used by cell sorters.

* Selection of the optimal fluorochrome for each antigen in multicolor experiments involves consideration of multiple factors. For more tips on choice of fluorochromes and design of multicolor flow cytometry experiments in general, please refer to Reference 20, and to the resources listed under Relevant BD Biosciences Literature and Links on p. 29.

\textbf{Figure 7. Analysis of IL-4R\textsubscript{\alpha} chain expression on human B cells.} Human PBMCs were isolated by density gradient centrifugation (Ficoll-Paque\textsuperscript{TM}) and were treated with human IgG (5 \(\mu\)g/10\(^6\) cells) to block Ig Fc receptors. The cells were subsequently stained with either purified anti-human IL-4R (clone hiL4R-M57, Cat. No. 551850, Panel A) antibody followed by biotinylated anti-mouse IgG, (Cat. No. 553441, Panel A) and PE-streptavidin (Cat. No. 554061, Panel A) or PE anti-human IL-4R (clone hiL4R-M57, Cat. No. 552178, Panel B). Samples were blocked with mouse serum (25 \(\mu\)l/10\(^6\) cells) and stained with FITC anti-human CD19 antibody (clone HIB19, Cat.No. 555412, both Panels). Staining with the anti-human IL-4R antibody (filled histograms) is compared to staining obtained using a Mouse IgG\(_{\kappa}\), \(\kappa\) isotype control antibody (Cat. No. 555746, both Panels, open histograms). Histograms were derived from gated events with the forward and side light-scatter characteristics of viable CD19\(^+\) lymphocytes.
Figure 8. Effect of FcR blocking on the analysis of TNFRII expressed by human PBMCs. Human PBMCs were isolated by density gradient centrifugation (Ficoll-Paque™) and were treated with human IgG (5 µg/10^6 cells, Panels B and D) to block FcR. The cells were subsequently stained with purified anti-human TNFRII (clone hTNFR-M1, Cat. No. 551311, all Panels) followed by biotinylated F(ab')2, goat anti-rat IgG (Jackson ImmunoResearch, Cat. No. 112-066-062, all Panels) and PE-streptavidin (Cat. No. 554061, all Panels). Staining with the anti-human TNFRII antibody (filled histograms) is compared to staining obtained using a Rat IgG₂κ isotype control antibody (Cat. No. 553986, all Panels, open histograms). Histograms were derived from gated events with the forward and side light-scatter characteristics of viable lymphocytes and monocytes.

4. Antigen Modulation and Receptor Internalization

Certain surface antigens, such as cytokine receptors, may be susceptible to internalization or shedding (eg, mouse TNFRI). Therefore, shortly after cell harvesting for immunofluorescent staining, it is necessary to minimize cell handling at room temperature and carry out all incubations at 4°C. To further prevent antigen modulation and internalization, it is recommended that the metabolic inhibitor sodium azide be used in the staining buffer.

5. FcR Blocking

To eliminate or reduce non-specific binding of antibodies caused by FcR, cells should be pretreated with FcR-blocking reagents. For example, in the mouse and rat systems, specific monoclonal antibodies are available that are directed against FcγII/III and FcγII receptors respectively. They have been proven to successfully reduce non-specific immunofluorescent staining caused by FcRs. In the human system, an excess of purified Ig from human or other species (or autologous serum that contains Ig) can be used (Figure 8). Alternatively, fragmented F(ab')2 antibodies may be available that can be used for immunofluorescent staining.

6. Immunoglobulin Isotype Controls

Certain antibody isotypes have a greater tendency than others to bind non-specifically to FcRs. To extract meaningful conclusions from experiments that involve immunofluorescent staining, it is recommended that Ig isotype-matched controls be run in the same experiment at the same
dose as the antigen-specific antibodies. Ideally, if the test antibodies are conjugated, the isotype controls must be conjugated in the same way.

7. Background Staining

In cases of indirect immunofluorescent staining where a two- or three-layer staining protocol is employed, the secondary anti-Ig reagent might cross-react with cell-surface immunoglobulin of the species being studied. To eliminate such background staining, the use of monoclonal isotype-specific anti-Ig secondary reagents (rather than polyclonal antibody preparations) or F(\(\text{ab}\)')\(_2\) secondary antibodies are recommended (Figure 9). Frequently, it is necessary to screen a number of secondary anti-Ig reagents for sensitivity versus background staining before choosing the most suitable secondary reagent.

![Figure 9. Analysis of IFN-\(\gamma\)R\(\alpha\) chain expression on human PBMCs.](Figure 9)

**Figure 9. Analysis of IFN-\(\gamma\)R\(\alpha\) chain expression on human PBMCs.** Human PBMCs were isolated by density gradient centrifugation (Ficoll-Paque\textsuperscript{TM}) and were stained with purified anti-human IFN-\(\gamma\)R\(\alpha\) (clone GIR-208, Cat. No. 558932, all Panels) followed with either biotinylated anti-mouse IgG, (Cat. No. 553441, Panels A and C) or biotinylated goat anti-mouse IgG (Cat. No. 553999, Panels B and D) and PE-streptavidin (Cat. No. 554061, all Panels). Staining with the anti-human IFN-\(\gamma\)R\(\alpha\) antibody (filled histograms) is compared to staining obtained using a Mouse IgG\(_k\), \(\kappa\) isotype control antibody (Cat. No. 555746, all Panels, open histograms). Histograms were derived from gated events with the forward and side light-scatter characteristics of viable lymphocytes (Panels A and B) and monocytes (Panels C and D).

8. Cell Viability

Cell viability is particularly an issue when dealing with cultured cells. Dead cells tend to aggregate and nonspecifically adsorb fluorescent antibodies. Large numbers of dead cells in cell suspensions can be removed by centrifugation on density separation media (eg, Ficoll-Paque\textsuperscript{TM}, Pharmacia). Smaller numbers of dead cells can be excluded from the flow cytometric analysis by using either propidium iodide (Propidium Iodide Solution, Cat. No. 556463) or 7–AAD (BD Via-Probe\textsuperscript{TM}, Cat. No. 555815).\(^{18}\)
9. Data Analysis

Single parameter data files can be displayed as histograms (frequency distributions) with fluorescence intensity on the x-axis and relative cell number on the y-axis. Using appropriate software, single-parameter data can also be displayed as overlapping histograms. The percentages of positive cells can be calculated by the placement of a marker (e.g., whose placement is determined due to unstained, Ig-isotype-stained, or stained negative cell controls) or by channel-by-channel subtraction methods when histograms are overlaid. Alternatively, bivariate (two-parameter) plots of light scatter signals and fluorescence intensities can be generated for single-color (as well as multicolor) immunofluorescent staining and flow cytometric experiments. Bivariate plots can be displayed in either a dot plot or a contour plot format with parameter intensities on the x- and y-axes. In this case, positive and negative controls should be compared to identify specific areas of staining so that quadrant markers or other gates can be applied to enumerate the frequencies of cells that coexpress the two parameters in a particular manner. For more details on data analysis please refer to unit 5.2 of *Current Protocols in Immunology*.19
References


Relevant BD Biosciences Literature and Links


Mini-website on Fluorochromes and Instrumentation for Multicolor Flow Cytometry: www.bdbiosciences.com/colors

Interactive spectrum viewer - online at www.bdbiosciences.com/spectra

Multicolor Flow Cytometry Reference Chart: Request a printed copy by contacting your local BD Biosciences office or representative

Additional Technical Resources

Our Scientific Support Team is trained to help you with technical questions related to flow cytometry and immunostaining. Just contact them at the numbers listed at the end of this manual, or e-mail: help.biosciences@europe.bd.com.
Chapter 2

BD™ Cytometric Bead Array (CBA) for Multiplexed Quantitation of Biological Response Modifiers

Introduction

Flow cytometry is a powerful analytical tool that enables the characterization of cells and subcellular organelles as well as particles (e.g., polystyrene beads) on the basis of size and granularity (light scatter characteristics) and a number of different parameters defined by fluorescent probes (including fluorescent antibodies and dyes). Today, flow cytometry is widely applied to the development of multiplex sandwich immunoassays. These particle-based, flow cytometric immunoassays are capable of simultaneously identifying the types and measuring the levels of multiple soluble analytes within small samples of biological fluids. The broad dynamic range of fluorescent detection offered by flow cytometry and the efficient capturing of analytes by suspended particles enable these assays to use fewer sample dilutions and to obtain multiple sample measurements in a short time period. For these reasons, this technology provides an extremely important tool for analyzing the networks of biological response modifiers (BRMs) that are coexpressed by cells that mediate immune and inflammatory responses. BRMs such as cytokines, chemokines, inflammatory mediators (e.g., bioactive complement fragments), and their receptors, as well as immunoglobulins, are popular target molecules for study. In addition, these assays can be applied to the multiplex quantitation of cell signaling molecules that act in complex pathways to orchestrate cellular responses.

BD™ Cytometric Bead Array (CBA) employs a series of suspended beads to simultaneously detect and quantify multiple soluble analytes. Each bead has a unique fluorescence intensity so that they can be mixed and run simultaneously in a single tube to significantly reduce sample requirements and time to results in comparison with traditional ELISA and Western blot techniques. BD CBA is a method of choice for studies requiring high throughput, quantitative measurement of multiple analytes, where ELISA appears less suited.

BD CBA solutions are designed for multiplexed analysis, providing more data using a single sample. Thus multiplexing is especially useful when only a small amount of sample is available, maximizing the number of proteins that can be analyzed. With BD CBA, up to 30 proteins can be analyzed with just 25 to 50 μL of sample. By comparison, other methods such as ELISA and Western blot require a similar amount of sample, however only one protein can be analyzed from the same volume.

The BD CBA portfolio includes assays for measurement of a variety of soluble and intracellular proteins, including cytokines, chemokines, growth factors and phosphorylated cell signaling proteins. BD CBA solutions exist in two formats to meet diverse needs:
• **BD™ CBA Flex Sets** provide an open and configurable method of detection, allowing the user to build their own multiplex.

• **BD™ CBA Kits** are preconfigured for achieving consistent results for routine panels.

BD CBA solutions are available for most BD FACSTM flow cytometers, combining leadership in instrumentation with innovation in application development to deliver a flexible and robust assay system to fulfill diverse research requirements.

**Principle of the Assay**

BD CBA employs a series of different single size particles that are stably labeled with one (Kits) or two (Flex Sets) fluorescent dyes whose emission wavelength is read at ~660 nm and over 680 nm. Each different group of beads is labeled with a discrete level of fluorescent dye so that it can be distinguished by its median fluorescence intensity (MFI) upon flow cytometric analysis.

For BD CBA Flex Sets, each bead population is given an alphanumeric position designation indicating its position relative to other beads (*Figure 1*). Beads with different positions can be combined to create a multiplex assay.

*Figure 1a. BD CBA 30-plex assay resolved on the BD FACSArray™ bioanalyzer.*

*Figure 1b. BD CBA Th1/Th2 Kit assay resolved on the BD FACSCalibur™ flow cytometer.*

In addition, beads within each group are covalently coupled with antibodies that can specifically capture a particular type of molecule present within biological fluids including sera, plasma, tears, tissue culture supernatants, or cell lysates. By analogy with the ELISA method (described in *Chapter 9*), the antibody-coupled
Capture Beads serve as the solid capture phase for the cytometric bead array. The immobilized, high-affinity antibodies function to specifically capture and localize analytes of interest that may be present in biological fluids tested (Figure 2).

The captured analyte is then specifically detected by the addition of a fluorescent antibody (Figure 2). Phycoerythrin-coupled (PE, emission at ~585nm) detection antibodies are most frequently used since the emission wavelength of PE is easily distinguishable from the emission wavelength(s) of the BD CBA capture beads. By including serial dilutions of a standard analyte solution (e.g., a mixture of cytokine protein standards with known concentrations), the BD CBA supports the development of standard curves for each analyte. With multicolor flow cytometric analysis, the levels of analytes captured by the different bead groups are distinguished by looking at the MFI of the PE signal, which is in direct proportion to the amount of analyte present.

The data is analyzed using FCAP Array™ software to calculate the concentrations of multiple analytes by plotting standard curves and calculating sample concentrations based on these curves. Due to the complexity of the BRM and cell signaling networks that underlie immune function, the capacity of the BD CBA to simultaneously measure multiple analytes in a single small-volume sample is highly advantageous.

BD CBA technology has been optimized for digital flow cytometers but is compatible with virtually all dual-laser flow cytometers capable of detecting and distinguishing fluorescence emissions at 576nm, 670nm, and >680nm (Table 1).

**Table 1.** BD CBA instrument compatibility and associated parameters.

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Reporter Parameter</th>
<th>Clustering Parameters for BD CBA Flex Sets</th>
<th>Clustering Parameters for BD CBA Kits</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD FACSArray™ bioanalyzer</td>
<td>Yellow</td>
<td>Red and NIR</td>
<td>Red</td>
</tr>
<tr>
<td>BD FACSCanto™ II flow cytometer</td>
<td>PE</td>
<td>APC and APC-Cy™7</td>
<td>APC</td>
</tr>
<tr>
<td>BD™ LSR II flow cytometer</td>
<td>PE</td>
<td>APC and APC-Cy7</td>
<td>APC</td>
</tr>
<tr>
<td>BD FACSAria™ II cell sorter</td>
<td>PE</td>
<td>APC and APC-Cy7</td>
<td>APC</td>
</tr>
<tr>
<td>BD FACSCalibur™ flow cytometer</td>
<td>FL2</td>
<td>FL4 and FL3</td>
<td>FL3 or FL4</td>
</tr>
</tbody>
</table>

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BD™ CBA Flex Sets

Introduction

The BD™ CBA Flex Set system provides an open and configurable menu of bead-based reagents designed to make it easy to create multiplex assays. Available assays include soluble protein assays for detection of human, mouse, or rat cytokines, chemokines, and growth factors; human immunoglobulins; and cell signaling assays for detection of phosphorylated cell signaling proteins. Using the BD CBA Flex Set system, up to 30 analytes can be measured simultaneously on a flow cytometer equipped with 488 nm and 633 nm lasers.

BD CBA Soluble Protein Flex Set Assays for Detection of Cytokines, Chemokines, Growth Factors and Human Immunoglobulins

BD CBA Soluble Protein Flex Set assays are available for the detection of cytokines, chemokines, growth factors and human immunoglobulins from serum, plasma, or tissue culture supernatant samples. These include T cell differentiation factors, modulators of inflammation, and other key markers for monitoring the immune response. The assays have been formulated to be mixed into any size plex and they are all sold individually, to provide a highly flexible system. All products are available off-the-shelf so custom orders are not required. Each product area (ie, Human Soluble Protein, Mouse/Rat Soluble Protein, and Human Immunoglobulin) has a unique Master Buffer Kit and all assays within each product area have been validated for performance in a single-plex and in multiplexed scenarios to ensure consistent data.

The antibody pair that comprises each assay is evaluated for dynamic range, sensitivity, and parallel titration to native biological samples. The detection antibodies are directly labeled with phycoerythrin (PE). By avoiding the streptavidin-biotin-PE detection method employed by other assays, 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 and flexible method for quantitative detection of multiple analytes in a single serum, plasma, tissue culture supernatant, or cell lysate sample, saving time and conserving precious samples.

BD CBA Cell Signaling Flex Set Assays for Detection of Phosphorylated Signaling Proteins

With the BD CBA Cell Signaling Flex Set system, the benefits of BD CBA assays are extended to researchers investigating cell signaling pathways. The assays cover key signaling molecules involved in B cell and T cell receptor signaling as well as other pathways in the immune response such as signaling via growth factor receptors and MAP kinase signaling (Figure 3).

The assays include a recombinant peptide standard that provides an internal control as well as a means to generate a standard curve for subsequent quantitative analysis. The intuitive analysis software generates a numerical readout in relative units/mL for each protein assayed, delivering the answers needed without additional steps (Figure 3). Low inter- and intra-assay CVs allow researchers to have greater confidence in results.

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Figure 3. Analysis of phospho-ERK1/2 protein levels in HEK 293 and NIH 3T3 cells in response to EGF, TNF, and PDGF stimulation. Panel (A) shows the results of Western Blot analysis. Panel (B) shows the results of BD CBA Flex Set analysis. Data courtesy of Dr. Tony Pawson and Dr. Jay Park, Mount Sinai Hospital, Toronto, Canada.

BD™ CBA Flex Set Assay: Step by Step

BD CBA Flex Sets make it easy to build a multiplex by following three simple steps. First choose the analytes to be measured and the corresponding buffer kit. Then follow the simple formulation instructions in the Master Buffer Kit manual. Assay components are all formulated at 1μL/test for easy calculations and the unique lyophilized standard pellets facilitate the combination of proteins to make the standards mix. The finished assay can be acquired on a variety of dual-laser flow cytometers and analyzed using FCAP Array software.

1. Choose from our menu of Human, Mouse, Rat, and Cell Signaling BD CBA Flex Set Assays
   Each BD CBA Flex Set comes with capture beads, detection reagent, and standards. Sufficient reagents are provided to run 100 tests including two standard curves. All assays are available off-the-shelf and ready for mixing.

2. Choose a 100 or 500 test size BD CBA Flex Set Master Buffer Kit
   Each BD CBA Flex Set Master Buffer Kit contains all the assay reagents and instrument setup beads necessary for any size multiplex configured from compatible BD CBA Flex Sets. This means that running a single-plex assay, a 10-plex assay, or larger, the buffer reagents are optimized to perform with the customized mixture selected and yield the correct number of assay tests.

3. Perform the assay following the instructions in the Master Buffer Kit manual.

4. Acquire samples on a dual-laser flow cytometer
   The BD CBA Flex Set reagents have been validated on a number of BD dual-laser flow cytometry platforms (Table 1). The plate-based BD FACSArray™ bioanalyzer can be used to optimize assay throughput and workflow. Simply prepare samples and standards in a 96-well filter-bottom plate, launch the previously designed FCAP Array template, load the plate, and experience truly hands-free sample acquisition.
5. Analyze data files using FCAP Array multiplex analysis software

Use the intuitive, wizard-driven FCAP Array software to plot standard curves and calculate sample concentrations.

Important notes

Quantitative results or protein levels for the same sample or recombinant protein run in ELISA and BD™ CBA assays may differ. A spike recovery assay can be performed using an ELISA standard followed by BD CBA analysis to assess possible differences in quantitation. So-called “Gold Standards” further allow for comparing values obtained using different assay methods. Please refer to the section “Standardization of BD CBA Standards to the NIBSC/WHO International Standards” for details (see page 65).

When several BD CBA Flex Sets assays are multiplexed, it is possible that the background (MFI of the 0 pg/mL standard point) may increase and the overall assay signals of other standard points may be reduced. This can result in lower dynamic range or loss in sensitivity in some assays. This effect may be greater as more assays are added to the multiplex.

For assays that will be acquired on a BD FACSCalibur flow cytometry instrument, it is recommended that additional dilutions of the standard be prepared (ie, 1:512 and 1:1024) as it is possible that in multiplex experiments containing a large number of assays, the Top Standard, 1:2, and 1:4 standard dilutions will not be analyzable by the FCAP Array software. In those cases, the Top Standard, 1:2, and 1:4 standard dilutions can be run on the experiment but may need to be excluded from the final analysis in the FCAP Array software. Please see the BD FACSCalibur Setup Manual for further limitations.

Reagents Provided

Each BD CBA Flex Set includes specific Capture Beads, Detection Reagents and Standards. Assay buffers, Flow Cytometer Setup Reagents and Instruction Manual are provided in each BD CBA Flex Set Master Buffer kit. Instruction Manuals, Instrument Setup instructions and Acquisition Templates can be downloaded at www.bdbiosciences.com/flexset

Materials Required but not Provided

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

- A dual-laser flow cytometer equipped with a 488 nm or 532 nm laser, and a 633 nm or 635 nm laser and capable of distinguishing 576 nm, 660 nm, and >680 nm fluorescence. Refer to Table 1 for examples of compatible instrument platforms
- 12 × 75 mm sample acquisition tubes for a flow cytometer (eg, BD Falcon™ Cat. No. 352008)
- FCAP Array software (Cat. No. 641488)
• Microcentrifuge
• Microcentrifuge tubes (polypropylene)

**Required for running experiments on a BD FACSCalibur flow cytometer:**

- BD Calibrite™ 3 beads (Cat. No. 340486)
- BD Calibrite™ APC beads (Cat. No. 340487)
- BD FACSComp™ software

**Required for plate loader-equipped flow cytometers:**

- Millipore MultiScreenHTS-BV 1.2 µm clear non-sterile filter plates [Cat. No. MSBVN1210 (10 pack) or MSBVN1250 (50 pack)]
- Millipore MultiScreenHTS Vacuum Manifold (Cat. No. MSVMHTS00)
- MTS 2/4 Digital Stirrer, IKA Works, VWR (Cat. No. 82006-096)
- Standard microtiter plate for BD FACSArray Bioanalyzer Setup (BD Falcon Cat. No. 353910)
- Vacuum source
- Vacuum gauge and regulator (if not using recommended manifold)

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**BD™ CBA Soluble Protein Flex Set Assay Procedure**

**Overview**

Summarized below is the BD CBA Flex Set assay procedure for measuring soluble proteins in human, mouse, or rat (see sections below for details).

1. Perform instrument setup procedure.
2. Dilute samples as appropriate.
3. Reconstitute standards mix and prepare serial dilutions.
4. Prepare (dilute) Capture Beads.
5. Prepare (dilute) PE Detection Reagents.
6. Transfer Capture Beads to tubes or wells.
7. Add standard and sample dilutions to the appropriate tubes or wells. Incubate 1 Hour at RT (protect from light).
8. Add mixed PE Detection Reagent to tubes or wells. Incubate at RT: 2 Hours for Human and Rat, 1 Hour for Mouse (protect from light).
9. Wash.
10. Analyze.

**Figure 4.** Overview of the BD CBA Flex Set Soluble Protein assay procedure for human, mouse and rat.
Instrument Setup

In order to ensure that the flow cytometer is performing optimally, perform the instrument setup procedure prior to preparing the BD™ CBA Flex Set Assay. Refer to the appropriate flow cytometry instrument setup in the manual included with the Master Buffer Kit for instructions on how to set up your instrument.

Preparation of Test Samples

The standard curve for each BD™ CBA Soluble Protein Flex Set covers a defined set of concentrations. 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.

1. Dilute test sample by the desired dilution factor (ie, 1:10 or 1:100) using the appropriate volume of Assay Diluent. Serum or plasma samples must be diluted at least 1:4 before transferring the samples to the assay tubes or wells.
2. Mix sample dilutions thoroughly before transferring samples to the appropriate assay tubes containing Capture Beads.
3. In order to facilitate analysis in FCAP Array software, load serially diluted samples in sequential wells from most concentrated to least concentrated (eg, Sample 1 - 1:4, 1:8, 1:16; Sample 2 - 1:4, 1:8, 1:16; etc).

Preparation of BD CBA Soluble Protein Flex Set Standards

For each single bead or multiplex assay a standard curve will need to be prepared. The protocol below indicates how standards should be mixed and diluted for use in a BD CBA Soluble Flex Set assay.

1. Remove one lyophilized standard vial from each BD CBA Soluble Flex Set that will be tested.
2. Open each vial of lyophilized standard.
3. Pool all lyophilized standard spheres into a single polypropylene tube (Recommended 15 mL Conical Tube, BD Falcon Cat. No. 352097). Label the tube “Top Standard”.
4. Reconstitute the standards with 4.0 mL of Assay Diluent. Allow the reconstituted standard to equilibrate for at least 15 minutes before making dilutions. Mix reconstituted protein by pipette only. Do not vortex or mix vigorously.
5. Label 12 x 75 mm tubes (BD Falcon Cat. No. 352008) and arrange them in the following order: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256.
6. Pipette 500 μL of Assay Diluent to each of the remaining tubes.
7. 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 5). Mix by pipette only, do not vortex. Prepare one tube containing Assay Diluent to serve as the 0 pg/mL negative control.

![Diagram](https://via.placeholder.com/150)

**Figure 5.** Preparation of the BD CBA Soluble Protein Flex Set standard dilutions.

**Table 2.** Typical BD CBA Human Soluble Protein Flex Set standard concentrations after dilution.

<table>
<thead>
<tr>
<th>BD CBA Human Soluble Protein Flex Set Standard</th>
<th>Top Standard</th>
<th>1:2 Dilution Tube</th>
<th>1:4 Dilution Tube</th>
<th>1:8 Dilution Tube</th>
<th>1:16 Dilution Tube</th>
<th>1:32 Dilution Tube</th>
<th>1:64 Dilution Tube</th>
<th>1:128 Dilution Tube</th>
<th>1:256 Dilution Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (pg/ml)</td>
<td>5000</td>
<td>2500</td>
<td>1250</td>
<td>625</td>
<td>312.5</td>
<td>156</td>
<td>80</td>
<td>40</td>
<td>20</td>
</tr>
</tbody>
</table>

**Note:** Refer to the Technical Data Sheet for each individual assay to verify the concentration of the Top Standard.

**Table 3.** Typical BD CBA Mouse/Rat Soluble Protein Flex Set standard concentrations after dilution.

<table>
<thead>
<tr>
<th>BD CBA Mouse Soluble Protein Flex Set</th>
<th>Top Standard</th>
<th>1:2 Dilution Tube</th>
<th>1:4 Dilution Tube</th>
<th>1:8 Dilution Tube</th>
<th>1:16 Dilution Tube</th>
<th>1:32 Dilution Tube</th>
<th>1:64 Dilution Tube</th>
<th>1:128 Dilution Tube</th>
<th>1:256 Dilution Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (pg/ml)</td>
<td>2500</td>
<td>1250</td>
<td>625</td>
<td>312.5</td>
<td>156</td>
<td>80</td>
<td>40</td>
<td>20</td>
<td>10</td>
</tr>
</tbody>
</table>

**Note:** Refer to the Technical Data Sheet for each individual assay to verify the concentration of the Top Standard.

8. It is recommended that the first ten wells or tubes in the experiment be the standards. Standards should be run in order from least concentrated (0 pg/mL) to most concentrated (Top Standard, see Table 4).
Table 4. Essential controls.

<table>
<thead>
<tr>
<th>Tube No. (Negative Control)</th>
<th>Reagents (All reagent volumes are 50 µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 pg/ml Standard)</td>
<td>Capture Beads, Standard 1:256 Dilution, PE Detection Reagent</td>
</tr>
<tr>
<td>4 (40 pg/ml Standard)</td>
<td>Capture Beads, Standard 1:128 Dilution, PE Detection Reagent</td>
</tr>
<tr>
<td>8 (80 pg/ml Standard)</td>
<td>Capture Beads, Standard 1:64 Dilution, PE Detection Reagent</td>
</tr>
<tr>
<td>156 pg/ml Standard)</td>
<td>Capture Beads, Standard 1:32 Dilution, PE Detection Reagent</td>
</tr>
<tr>
<td>317.5 pg/ml Standard)</td>
<td>Capture Beads, Standard 1:16 Dilution, PE Detection Reagent</td>
</tr>
<tr>
<td>625 pg/ml Standard)</td>
<td>Capture Beads, Standard 1:8 Dilution, PE Detection Reagent</td>
</tr>
<tr>
<td>1250 pg/ml Standard)</td>
<td>Capture Beads, Standard 1:4 Dilution, PE Detection Reagent</td>
</tr>
<tr>
<td>2500 pg/ml Standard)</td>
<td>Capture Beads, Standard 1:2 Dilution, PE Detection Reagent</td>
</tr>
</tbody>
</table>

Preparation of BD™ CBA Soluble Protein Flex Set Capture Beads

The Capture Beads provided in each BD CBA Soluble Flex Set are at a 50× concentration and must be diluted to their optimal concentration before adding to a given assay tube or assay well.

1. Determine the number of BD CBA Soluble Protein Flex Sets to be used in the experiment (size of the multiplex).
2. Determine the number of tests in the experiment. It is recommended that the user prepare additional tests than they will use in the experiment to ensure that there is enough material prepared for the experiment.
3. Vortex each Capture Bead stock vial for at least 15 seconds to resuspend the beads thoroughly.
4. Determine the total volume of diluted beads needed for the experiment. Each tube/well requires 50 µL of the diluted beads. The total volume of diluted beads can be calculated by multiplying the number of tests (determined in step 2) by 50 µL.
   - eg, 35 tests × 50 µL = 1750 µL total volume of beads
5. Determine the volume needed for each capture bead. Beads are supplied so that 1.0 µL = 1 test. Therefore, the required volume (µL) of beads is equal to the number of tests.
   - eg, 35 tests require 35 µL of each capture bead included in the assay
6. Determine the volume of Capture Bead Diluent needed to dilute the beads.
The volume of Capture Bead Diluent can be calculated by subtracting the volume for each bead tested from the total volume of diluted beads needed to perform the assay.

- eg, 1750 µL total volume of beads - 35 µL for each bead = volume of Capture Bead Diluent

- eg, if testing one analyte: 1750 µL - (35 µL × 1) = 1715 µL Diluent

- eg, if testing 5 analytes: 1750 µL - (35 µL × 5) = 1575 µL Diluent

Refer to Table 5 for more examples of the calculation.

7. Pipette the Capture Beads and Capture Bead Diluent into a tube labeled Mixed Capture Beads.

For Human serum and plasma samples

Due to some unspecific binding observed with Human serum and plasma samples, a specific Capture Beads procedure has been implemented in order to avoid any interference with the assay. This procedure is not recommended for Mouse and Rat serum and plasma samples. The Capture Bead Diluent for Serum/Plasma is included in the Human Soluble Master Buffer kit (Cat. Nos. 558264 and 558265) but not in the Mouse/Rat Soluble Master Buffer kit (Cat. Nos. 558266 and 558267).

1. Determine the number of BD CBA Human Soluble Protein Flex Sets to be used in the experiment (size of the multiplex).

2. Determine the number of tests in the experiment. It is recommended that the user prepare a few additional tests than they will use in the experiment to ensure that there is enough material prepared for the experiment. Beads are supplied so that 1.0 µL = 1 test. Therefore, the required volume (µL) of beads is equal to the number of tests.

- eg, 35 tests requires 35 µL of each capture bead included in the assay

3. Vortex each Capture Bead stock vial for at least 15 seconds to resuspend the beads thoroughly.

4. Pipette the appropriate volume (determined in step 2) of each capture bead into a tube labeled Mixed Capture Beads.

5. Add 0.5 mL Wash Buffer, centrifuge at 200 × g for 5 minutes.

6. Carefully discard supernatant by aspiration. Avoid aspiration of the bead pellet.

7. Resuspend beads in Capture Bead Diluent for Serum/Plasma to a final volume of 50 µL/test, vortex, and incubate for 15 minutes at room temperature prior to use.

- eg, 35 tests × 50 µL = 1750 µL Capture Bead Diluent for Serum/Plasma
Preparation of BD™ CBA Soluble Protein Flex Set PE Detection Reagents

The PE Detection Reagent provided with each BD CBA Soluble Protein Flex Set is a 50× bulk (1 µL/test). The PE Detection Reagents for all BD CBA Protein Flex Sets used in the assay should be combined, and subsequently diluted to their optimal volume per test (50 µL/test) before adding the PE Detection Reagent Mix to a given tube or assay well.

Note: Protect the PE Detection Reagents from exposure to direct light because they can become photobleached and will lose fluorescent intensity.

1. Determine the number of BD CBA Soluble Protein Flex Sets to be used in the experiment (size of the multiplex).

2. Determine the number of tests to be run in the experiment. It is recommended that the user prepare a few additional tests than they will use in the experiment to ensure that there is enough material prepared for the experiment.

3. Determine the total volume of diluted PE Detection Reagent needed for the experiment. Each tube/well requires 50 µL of the diluted PE Detection Reagent. The total volume of diluted PE can be calculated by multiplying the number of tests (determined above) by 50 µL.
   • eg, 35 tests × 50 µL = 1750 µL total volume of PE

4. Determine the volume needed for each PE Detection Reagent. The PE Detection Reagent is supplied so that 1.0 µL = 1 test. Therefore, the required volume (µL) of PE Detection Reagent is equal to the number of tests.
   • eg, 35 tests requires 35 µL of each Detection Reagent included in the assay

5. Determine the volume of Detection Reagent Diluent needed to dilute the PE Detection Reagents. The volume of Detection Reagent Diluent can be calculated by subtracting the volume for each PE Detection Reagent tested from the total volume of diluted PE needed.
   • eg, 1750 µL total volume PE - 35 µL for each Detection Reagent = volume of Detection Reagent Diluent
   • eg, if testing one analyte: 1750 µL – (35 µL × 1) = 1715 µL Diluent
   • eg, if testing 5 analytes: 1750 µL – (35 µL × 5) = 1575 µL Diluent

Refer to Table 5 for more examples.

6. Pipet the Detection Reagents and Detection Reagent Diluent into a tube labeled Mixed PE Detection Reagents. Store at 4°C, protected from light until ready to use.
Table 5. Capture Bead and PE Detection Reagent Diluent calculations.

<table>
<thead>
<tr>
<th>No of Flex Sets to be used</th>
<th>Volume of each Capture Bead or PE Detection Reagent/test*</th>
<th>Total Capture Bead volume/test*</th>
<th>Volume of Capture Bead or Detection Reagent Diluent/test*</th>
<th>Total volume of mixed Capture Beads or PE Detection Reagents/test*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 µl</td>
<td>1 µl</td>
<td>49 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>2</td>
<td>1 µl</td>
<td>2 µl</td>
<td>48 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>3</td>
<td>1 µl</td>
<td>3 µl</td>
<td>47 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>4</td>
<td>1 µl</td>
<td>4 µl</td>
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<td>5</td>
<td>1 µl</td>
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<td>50 µl</td>
</tr>
<tr>
<td>6</td>
<td>1 µl</td>
<td>6 µl</td>
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<td>7</td>
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<td>7 µl</td>
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<td>8 µl</td>
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<td>50 µl</td>
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<td>1 µl</td>
<td>9 µl</td>
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<td>14</td>
<td>1 µl</td>
<td>14 µl</td>
<td>36 µl</td>
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<td>17</td>
<td>1 µl</td>
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<tr>
<td>30</td>
<td>1 µl</td>
<td>30 µl</td>
<td>20 µl</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

BD™ CBA Soluble Protein Flex Set Assay Procedure

Following the preparation and dilution of the individual assay components transfer the Standards or samples, mixed Capture Beads, and mixed PE Detection Reagents to the appropriate assay wells or tubes for incubation and analysis.

Note: Protect Capture Beads and PE Detection Reagents from direct exposure to light.

Overview

<table>
<thead>
<tr>
<th></th>
<th>Standards</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capture Beads Mixture</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>Diluted Standards</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>Samples</td>
<td>n/a</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

Vortex the tubes for 5 seconds or shake the plates for 5min and incubate for 1h at RT.

<table>
<thead>
<tr>
<th></th>
<th>50 µl</th>
<th>50 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE Detection Reagent Mixture</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mix assay tubes gently or shake the plates for 5min and incubate for 1h (Mouse samples) or 2h (Human and Rat samples) at RT in the dark.

Apply the plate to the vacuum manifold and aspirate or add 1.0 mL of Wash Buffer to each assay tube, centrifuge at 200 x g for 5 minutes and discard the supernatant.

<table>
<thead>
<tr>
<th></th>
<th>150 µl for plates or 300 µl for tubes</th>
<th>150 µl for plates or 300 µl for tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wash Buffer</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Detailed Protocol for Plates

1. Prepare all reagents as described in previous sections before starting the experiment.
2. Pre-wet the plate by adding 100 µL of Wash Buffer to each well. To remove the excess volume, apply to vacuum manifold. Do not exceed 10” Hg of vacuum pressure. Vacuum aspirate until wells are drained (2-10 seconds).
3. Vortex the Mixed Capture Beads for at least 5 seconds. Add 50 µL of the Mixed Capture Beads to each assay well.
4. Add 50 µL of Standard or sample to the assay wells.
   Note: See Table 3 for a list of essential control wells to be run in each experiment.
5. Mix the plate for 5 minutes using a digital shaker at 500 RPM (do not exceed 600 RPM) and incubate plate for 1 hour at RT.
6. Add 50 µL of the Mixed PE Detection Reagent to each assay well.
7. Mix the plate for 5 minutes using a digital shaker at 500 RPM and incubate plate at RT for 1h (Mouse samples) or 2h (Human and Rat samples).
8. Apply the plate to the vacuum manifold and vacuum aspirate (do not exceed 10” Hg of vacuum pressure) until wells are drained (2-10 seconds).
9. Add 150 µL of Wash Buffer to each assay well. Shake microwell plate on a digital shaker at 500 RPM for 5 minutes to resuspend beads.
10. Begin analyzing samples on a flow cytometer. Proceed to the appropriate flow cytometry instrument instruction manual (included in the Master Buffer Kit) for acquiring the BD CBA Flex Sets.
   Note: It is best to analyze samples on the day of the experiment. Prolonged storage of samples, once the assay is complete, can lead to increased background and reduced sensitivity.

Detailed Protocol for Tubes

1. Prepare all reagents as described in previous sections before starting the experiment.
2. Vortex the Mixed Capture Beads for at least 5 seconds. Add 50 µL of the Mixed Capture Beads to each assay tube.
3. Add 50 µL of Standard or sample to each assay tube.
   Note: See Table 3 for a list of essential control tubes to be run in each experiment.
4. Mix assay tubes gently and incubate for 1 hour at RT.
5. Add 50 µL of the Mixed PE Detection Reagent to each assay tube.
6. Mix assay tubes gently and incubate at RT for 1h (Mouse samples) or 2h (Human and Rat samples).
7. Add 1.0 mL of Wash Buffer to each assay tube and centrifuge at 200 × g for 5 minutes.
8. Carefully aspirate and discard the supernatant from each assay tube.
9. Add 300 µL of Wash Buffer to each assay tube. Vortex assay tubes briefly to resuspend beads.
10. Begin analyzing samples on a flow cytometer. It is recommended that each tube be vortexed briefly before analyzing on the flow cytometer. Proceed to the appropriate flow cytometry instrument instruction manual (included in the Master Buffer Kit) for acquiring the BD CBA Flex Sets.

Note: It is best to analyze samples on the day of the experiment. Prolonged storage of samples, once the assay is complete, can lead to increased background and reduced sensitivity.

BD™ CBA Cell Signaling Protein Flex Set Assay Procedure

The procedure for performing a cell signaling BD CBA Flex Set assay is similar to the one described for soluble proteins (see page 37) with the exception of the steps detailed in the sections below (preparation of samples and standards, and incubation times).

Overview

1. Perform instrument setup procedure.
2. Prepare and denature cell lysate samples. Samples may be frozen and stored at this point for later analysis (see Preparation of Test Samples, page 46).
3. Reconstitute standard and prepare serial dilutions using the Assay Diluent.
4. Vortex and dilute BD CBA Cell Signaling Flex Set Capture Beads.
5. Dilute BD CBA Cell Signaling Flex Set PE Detection Reagents.
6. Transfer 50 µl of mixed Capture Beads to each assay well/tube. 3 Hour incubation at RT (protect from light)
7. Add Standard Dilutions and test samples to the appropriate sample tubes or wells (50 µl/test).
8. Add mixed PE Detection Reagent to each assay well/tube (50µl/test). 1 Hour incubation at RT (protect from light)
9. Wash samples with 1.0 ml of Wash Buffer (tubes only) and centrifuge. Assays run in wells will aspirate only at this step.
10. Add Wash Buffer to each assay tube (300 ul/tube) or well (150 ul/well) and analyze samples.

Figure 6. BD CBA Cell Signaling Flex Set assay procedure.
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 (Cat. Nos. 560005 and 560006) 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 to 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. In cases where the samples are known or assumed to contain low levels of a given protein, the sample should be lysed in a lower volume of lysis buffer thereby concentrating the protein in the sample. 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/10⁶ 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× after being mixed with cells.

The process is basically the same as preparing a sample for gel electrophoresis and Western blotting except that Denaturation Buffer is used instead of SDS-PAGE sample buffer.

In order to facilitate analysis in FCAP Array software, load serially diluted samples in sequential wells from most concentrated to least concentrated (eg, Sample 1 – 1:2, 1:4, 1:8; Sample 2 – 1:2, 1:4, 1:8; etc.).

Cells in Suspension

1. Count cells in sample. This gives an approximate idea of protein concentration, which should be greater than 1 mg/mL (protein concentration is dependent on cell type, eg, 100-200 μg/10⁶ cells for Jurkat; 25-50 μg/10⁶ cells for peripheral blood lymphocytes [PBL]). Proceed to point 2 below.

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. Proceed to point 2.
2. Treat cells to induce or inhibit protein phosphorylation as required for the experiment.
3. Use one of the following methods to prepare samples for denaturation:
   - Halt activation of the cells by adding the appropriate amount of 5× Denaturation Buffer so that the final concentration of Denaturation
Buffer is 1×.

- Add ice-cold PBS to the activated cells and pellet by centrifugation. Add an appropriate amount of 1× Denaturation Buffer (prepared by diluting the 5× Denaturation Buffer with water) to resuspend the cell pellet.

- Add ice-cold lysis buffer containing a detergent (eg, Triton® X-100, NP40, etc) to the cells. Incubate for 15 – 30 minutes at 4°C and pellet insoluble material by centrifugation. Transfer the supernatant to a clean tube and add the appropriate amount of 5× Denaturation Buffer so that the final concentration of Denaturation Buffer is 1×.

**Note:** Regardless of the method used, recoveries may be enhanced by adding protease inhibitors and phosphatase inhibitors.

4. Denature sample by immediately placing in a boiling water bath for 5 minutes. The sample may be very viscous and difficult to pipet due to the presence of DNA. This can be remedied by one of the following methods:

- Shear the DNA using a probe sonicator. The sample should be sonicated until it is easy to pipet and the liquid falls as discrete drops.

- Pass the sample through a 26 gauge needle several times.

- Add a very high quality (protease free) DNase I to the denatured sample. DNase I is the best solution if many different lysates will be tested. However, a high quality DNase I must be used, or residual proteases in the DNase I will destroy the samples.

5. Determine protein concentration.

6. Cell lysates may be stored in aliquots 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. Samples should be centrifuged at 14,000 rpm for 3 minutes before use, to pellet debris.

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 1:4 to reduce the percentage of SDS and should not contain more than 20 μg of total protein.

8. Mix sample dilutions thoroughly before transferring samples to the appropriate assay tubes containing Capture Beads.

**Preparation of BD CBA Cell Signaling Flex Set Standards**

The standard provided with each BD CBA Cell Signaling Flex Set is provided in a lyophilized form 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. 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. Transfer a lyophilized sphere of each standard to be used to a 1.5 mL microfuge tube. Reconstitute each standard by adding 100 μL of Assay
Diluent, warming the tube to 37°C, and vortexing (Figure 7). Once reconstituted, standards should be stored at 4°C and are stable for 3 months. If you are using a reconstituted standard, simply warm to 37°C and vortex to mix well.

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 Cell Signaling BD CBA Flex Set Standard to be run in the experiment to the Top Standard tube.

4. Add Assay Diluent (yellow buffer) 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 BD CBA Cell Signaling Flex Set 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. The Assay Diluent serves as the negative control.

![Figure 7. Preparation of BD CBA Cell Signaling Flex Set Standard Dilutions.](image)

7. It is recommended that the first ten wells or tubes in the experiment be the standards. Standards should be run in order from least concentrated (0 U/mL) to most concentrated (Top Standard).

The typical concentration (Units/mL) of each BD CBA Cell Signaling Flex Set Standard in each dilution tube is shown in Table 6.
Table 6. BD CBA Cell Signaling Flex Set Standard concentrations after dilution.

<table>
<thead>
<tr>
<th>BD CBA Cell Signaling Flex Set Standard</th>
<th>Top Standard</th>
<th>1:2 Dilution Tube</th>
<th>1:4 Dilution Tube</th>
<th>1:8 Dilution Tube</th>
<th>1:16 Dilution Tube</th>
<th>1:32 Dilution Tube</th>
<th>1:64 Dilution Tube</th>
<th>1:128 Dilution Tube</th>
<th>1:256 Dilution Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (Units/mL)</td>
<td>1000</td>
<td>500</td>
<td>250</td>
<td>125</td>
<td>62.5</td>
<td>31.25</td>
<td>15.6</td>
<td>7.8</td>
<td>3.9</td>
</tr>
</tbody>
</table>

BD™ CBA Human Immunoglobulin Flex Set Assay Procedure

The procedure for performing a BD CBA Human Immunoglobulin Flex Set assay is similar to the one described above for soluble proteins (see page 37), with the exception of the steps detailed below (preparation of samples and standards; incubation times; limitations; one additional washing step).

The BD CBA Human Immunoglobulin Flex Set system includes the BD CBA Human Immunoglobulin Master Buffer Kit (Cat. No. 558683) and the following BD CBA Human Immunoglobulin Flex Set Assays: Total IgG (Cat. No. 558679), IgG₁ (Cat. No. 558675), IgG₂ (Cat. No. 558676), IgG₃ (Cat. No. 558677), IgG₄ (Cat. No. 558678), IgM (Cat. No. 558680) and IgA (Cat. No. 558681), all available separately.

Limitations

The BD CBA Human IgG₁ and Human Total IgG assays cannot be multiplexed with any other assay and must be run as single plexes.

The BD CBA Human IgE assay is not compatible with the BD CBA Human Immunoglobulin assay system. IgE can be measured using the BD CBA Human Soluble Protein assay system.

Multiplexing of BD CBA Human Immunoglobulin Flex Set assays with assays from the other BD CBA Flex Set Systems is not supported.

The buffers contained in the BD CBA Human Immunoglobulin Master buffer Kit have been formulated specifically for use with the BD CBA Human Immunoglobulin Flex Sets. This buffer kit should not be used with any non-Human Immunoglobulin BD CBA Flex Sets. In addition, do not attempt to substitute any buffers from another non-immunoglobulin Master Buffer Kit as this could lead to poor assay performance.
Overview

1. Perform instrument setup procedure.
2. Dilute samples as appropriate using Assay Diluent.
4. Prepare the diluted BD CBA Human Immunoglobulin Flex Set Capture Beads.
5. Dilute BD CBA Human Immunoglobulin Flex Set Detection Reagents.

6. Wet filter plate with 100 µL of Wash Buffer and Aspirate (Plate Protocol Only).
7. Transfer 50 µL of Capture Beads to each assay tube or well.
8. Add Standard Dilutions and test samples to the appropriate sample tubes or wells (50 µL/test).

1 Hour incubation at RT (protect from light)

9. Tubes: Wash Samples with 1.0 mL of Wash Buffer, centrifuge and aspirate supernatant.
   Plates: Aspirate plate, add 200 µL Wash Buffer to the wells and aspirate plate again.
10. Add mixed PE Detection Reagent to each assay well/tube (50 µL/test).

2 Hour incubation at RT (protect from light)

11. Tubes: Wash Samples with 1.0 mL of Wash Buffer, centrifuge and aspirate supernatant.
   Plates: Aspirate plate, add 200 µL Wash Buffer to the wells and aspirate plate again.
12. Add Wash Buffer to each assay tube (300 µL/tube) or well (150 µL/well) and analyze samples.

Figure 8. Overview of the BD CBA Human Immunoglobulin Flex Set assay procedure.

Preparation of Test Samples

The standard curve for each BD CBA Human Immunoglobulin Flex Set covers a defined set of concentrations. 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 should be serially diluted. When analyzing, determine which dilutions fall within the linear portion of the curve, correct for dilution factors and average the results. Typical serum ranges for each of the human immunoglobulins have been previously reported in the literature.16 Suggested starting sample dilutions for each of the assays are provided in Table 7.
Table 7. Suggested starting sample dilutions for BD CBA Human Immunoglobulin Flex Set assays.

<table>
<thead>
<tr>
<th>Total IgG</th>
<th>IgG1</th>
<th>IgG2</th>
<th>IgG3</th>
<th>IgM</th>
<th>IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:32,000</td>
<td>1:16,000</td>
<td>1:2,000</td>
<td>1:2,000</td>
<td>1:2,000</td>
<td>1:2,000</td>
</tr>
</tbody>
</table>

Note: These are suggested starting dilutions. With normal human serum, these dilutions will most likely fall near the top of the linear range of the assay. It is recommended that these initial dilutions be followed by additional serial dilutions to ensure that at least some sample dilutions fall within the linear portion of the curve.

Dilutions should be prepared as described below:

1. Dilute test sample by the desired dilution factor using the appropriate volume of Assay Diluent (see Table 7). Serial dilutions can be performed from the initial dilution using Assay Diluent.
2. Mix sample dilutions thoroughly before transferring samples to the appropriate assay tubes containing Capture Beads.
3. In order to facilitate analysis in FCAP Array software, load serially diluted samples in sequential wells from most concentrated to least concentrated (e.g., Sample 1 - 1:4,000, 1:8,000, 1:16,000; Sample 2 - 1:4,000, 1:8,000, 1:16,000; etc).

Preparation of BD CBA Human Immunoglobulin Flex Set Standards

All of the assays in the BD CBA Human Immunoglobulin Flex Set System use the same lyophilized standard. This means that for any assay, single plex or multiplex, only one standard vial needs to be reconstituted. The dilutions performed to obtain the Top Standard for some of the assays are different. For this reason, some of the assays cannot be run in a multiplex. See Limitations (page 49) for more details.

For each assay, single plex or multiplex, a single standard curve needs to be prepared. The protocol below indicates how standards should be mixed and diluted for use in a BD CBA Human Immunoglobulin Flex Set assay.

1. Label 12 x 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.
2. Remove one lyophilized standard vial from one of the BD CBA Human Immunoglobulin Flex Sets that will be tested.
3. Open the vial of lyophilized standard and transfer the lyophilized standard sphere into a polypropylene tube (recommended 15 mL conical tube: BD Falcon Cat. No. 352097).
4. Reconstitute the standard sphere with 1.0 mL of Assay Diluent. Allow the reconstituted standard to equilibrate for at least 15 minutes before proceeding. Mix reconstituted protein by pipette only. Do not vortex or mix vigorously.
5. Create the assay-specific Top Standard by following the appropriate set of instructions below.
• For the Human IgG<sub>2</sub>, IgG<sub>3</sub>, IgG<sub>4</sub>, IgM and IgA Flex Set Assays (any single assay or multiplex combination):
  a. Transfer the contents of the reconstituted standard vial directly to the Top Standard tube. The reconstituted material is already at the correct dilution to be used as the Top Standard.

• For the Total Human IgG Flex Set Assay:
  a. Add 1.55 mL of Assay Diluent to the Top Standard tube.
  b. Transfer 50 μL of the reconstituted lyophilized standard to the Top Standard tube.
  c. Mix Top Standard tube by pipette only. Do not vortex or mix vigorously.

• For the Human IgG<sub>1</sub> Flex Set Assay:
  a. Add 1.5 mL of Assay Diluent to the Top Standard tube.
  b. Transfer 100 μL of the reconstituted lyophilized standard to the Top Standard tube.
  c. Mix Top Standard tube by pipette only. Do not vortex or mix vigorously.

6. Pipet 500 μL of Assay Diluent into each of the remaining standard curve tubes.
7. 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 9). Mix by pipette only, do not vortex. Prepare one tube containing Assay Diluent to serve as the 0 ng/mL negative control.

8. It is recommended that the first ten wells or tubes in the experiment be the standards. Standards should be run in order from least concentrated (0 ng/mL) to most concentrated (Top Standard).

![Figure 9. Preparation of BD Human Immunoglobulins Flex Set standard dilutions.](image-url)
The approximate concentration (ng/mL) of each BD CBA Human Immunoglobulin Flex Set Standard in each dilution tube is shown in Table 8.

Table 8. Approximate concentrations of BD CBA Human Immunoglobulin Flex Set standards for the different dilution points of the standard curve.

Cytometer setup, data acquisition and analysis

Note: The BD CBA Instrument Setup Templates for different flow cytometers mentioned in this section can all be downloaded at: www.bdbiosciences.com/flexset

For flow cytometers running BD FACSDiva™ software

For optimal performance of a BD CBA assay, it is necessary to properly set up the flow cytometer. The cytometer setup information in this section is to be used for the BD FACSCanto™, BD FACSCanto II, BD™ LSR II, BD FACSARia™, and BD FACSARia II flow cytometers.

Preparation of Instrument Setup Beads

Prepare 5 tubes labeled: A9, PE-F1, F1, F9, and A1. Vortex the stock vials of beads, add 200 µl of Wash Buffer to each tube followed by 25 µl of the corresponding setup beads.

Instrument Setup

1. Edit the parameters list to display only the following: FSC-A, FSC-W, SSC-A, SSC-W, PE-A, APC-A, and APC-Cy7-A.
2. On a global worksheet, create the following plots: FSC-A/SSC-A dot plot, APC/APC-Cy7 dot plot, and PE histogram.
3. Set FSC-A and SSC-A to Log and create a statistics view showing the FSC-A and SSC-A means. Set the events to display to 500. Using the A9 setup beads, adjust FSC and SSC so that the singlet beads have a mean of 30,000 for each parameter. Stop acquisition to avoid running out of sample.
4. Adjust the FSC-A and SSC-A thresholds using the mean channel as a guideline. Be sure that the thresholds do not cut into the bead population.
5. In the FSC-A vs. SSC-A dot plot, create a region that includes the singlet population of beads. In the Population Hierarchy, rename that region singlet.
6. Edit the statistics view to display the PE, APC, and APC-Cy7 mean of the singlet beads.
7. Through the singlet gate, run the A9 setup beads and adjust the APC and APC-Cy7 voltages until the mean of each parameter is 160,000 ±2,000.

8. Through the singlet gate, run the PE-F1 tube and adjust the PE voltage so that the mean is 65 ±5.

9. Create compensation controls and delete the PE compensation tube. Run beads as follows for compensation controls:
   - Unstained: F1
   - APC Stained: F9
   - APC-Cy7 Stained: A1

10. Calculate compensation.

11. Optional: Verify instrument settings prior to analyzing the assay by recording a sample using the remaining mixed capture beads from the Flex Set assay, export as FCS2.0, and go to Tools > Clustering Test in FCAP Array software to see if it can identify the correct number of bead clusters.

**Verification of instrument setup**

The BD CBA 30 Plex Bead Mixture (Cat. No. 558522) can be included as an additional sample when performing the instrument setup procedure. This cocktail containing 30 bead populations from the BD CBA Flex Set is useful as a control sample to demonstrate that the instrument has been properly set up for analysis and to guarantee that the instrument and FCAP Array Software can analyze a 30 plex assay correctly.

**Recommended Assay Procedure:**

1. Vortex the BD CBA 30 Plex Bead Mixture to bring beads into suspension.

2. Transfer 25 μl of the BD CBA 30 Plex Bead Mixture to the appropriate sample well or tube.

3. Add 175 μl of Wash Buffer (from the BD CBA Master Buffer Kit used) to the bead sample. More Wash Buffer (275 μl) may be needed for samples in tubes.

4. Proceed with analysis on a BD flow cytometer that has been set up according to the instrument setup procedure and export the FCS2.0 file for the 30 plex bead mixture.

5. Analyze the 30 plex bead mixture data file using the FCAP Array software clustering tool (see Technical Data Sheet for details). If the FCAP Array software fails to identify 30 bead clusters, repeat steps 4 and 5 after repeating the instrument setup procedure. If the FCAP Array software is unable to identify 30 bead clusters from the repeat sample, instrument service may be required.

*Note:* Do not store the diluted beads. Prepare a new bead sample for each test.

The same procedure is recommended for verification of instrument setup when using the BD FACSArray bioanalyzer.
Data acquisition

Set events to record to 300 events per analyte (eg, 300 x 6 = 1800 events for a 6plex) and set the singlet gate as the storage gate and stopping gate to ensure that only singlet bead events are recorded. Change events to display to 5,000. Record samples and export as FCS2.0 files for analysis in FCAP Array.

The Experiment can be saved as a template for future experiments, however it is recommended to verify instrument settings (ie, voltages and compensation) prior to each experiment.

Analysis of Sample Data

The analysis of BD CBA data is optimized when using FCAP Array software. Install the software according to the instructions in the FCAP Array User’s Guide (available for download at www.bdbiosciences.com/fcaparray).

1. Transfer FCS data files for the experiment to the computer with the FCAP Array software.

2. Place all data files for a given experiment in a single folder.

Follow the instructions in the FCAP Array Software User’s Guide for creating an experiment and for data analysis.

For the BD FACSArray™ bioanalyzer running BD FACSArray software

For experiments requiring higher throughput, BD CBA reagents can optimally be combined with the BD FACSArray bioanalyzer. Equipped with a 96-well plate loader, the BD FACSArray offers automated data acquisition. BD FACSArray software is used for instrument setup and sample acquisition. BD FACSArray software is compatible with FCAP Array software to allow for quantitative analysis of BD CBA data. FCAP Array software enables researchers to design and set up their BD CBA experiments and import them to the BD FACSArray bioanalyzer in a streamlined workflow. Refer to the BD FACSArray Bioanalyzer Instrument Setup Manual for instrument setup and data acquisition. Follow the instructions in the FCAP Array Software User’s Guide (available for download at www.bdbiosciences.com/fcaparray) for creating an experiment and for data analysis.

For the BD FACSCalibur™ flow cytometer

For optimal performance of a BD CBA assay, it is necessary to properly set up the flow cytometer. The cytometer setup information in this section is to be used for the BD FACSCalibur™ flow cytometer. The BD FACSComp™ Software is useful for setting up the flow cytometer. BD CellQuest™ (or BD CellQuest Pro) Software is required for analyzing samples and formatting data for subsequent analysis using FCAP Array software.

Instrument Setup with BD FACSComp software and BD Calibrite™ Beads

1. Perform instrument startup.

2. Perform flow check.

3. Prepare tubes of BD Calibrite™ beads for a four-color setup.
4. Launch BD FACSComp software.
5. Run BD FACSComp software in Lyse/No Wash mode.
   
   Note: Time-delay calibration must be performed. For detailed information on using BD FACSComp with BD Calibrite beads to set up the flow cytometer, refer to the BD FACSComp Software User’s Guide and the BD Calibrite Beads package insert.

6. Proceed to the next section.

**Preparation of Instrument Setup Beads**

1. Label five 12 x 75 mm tubes A, B, C, D, and E.
2. Add 25 µL of PE Instrument Setup Bead F1 to tube D.
3. Add 50 µL of PE Positive Control Detector to tube D and vortex tube briefly to mix.
4. Incubate tube D for 15 minutes at room temperature, protect from light.
5. Add 25 µL of Instrument Setup Bead A9 to tube A.
6. Add 25 µL of Instrument Setup Bead A1 to tube B.
7. Add 25 µL of Instrument Setup Bead F9 to tube C.
8. Add 25 µL of Instrument Setup Bead F1 to tubes A, B, C, and D.
9. Add 50 µL of the mixed Capture Beads from the BD CBA Flex Set experiment to tube E.
   
   Note: The mixed Capture Beads added in step 9 should be beads only and not contain PE detection reagent, standards, or sample.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Setup Beads</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A9 + F1</td>
</tr>
<tr>
<td>B</td>
<td>A1 + F1</td>
</tr>
<tr>
<td>C</td>
<td>F9 + F1</td>
</tr>
<tr>
<td>D</td>
<td>PE-F1 + F1</td>
</tr>
<tr>
<td>E</td>
<td>Mixed Capture Beads</td>
</tr>
</tbody>
</table>

10. Add 300 µL of Wash Buffer to tube D and vortex tube briefly to mix.
11. Add 350 µL of Wash Buffer to tubes A, B, C, and E. Vortex each tube briefly to mix.
12. Proceed to the next section.

**Instrument Setup with Instrument Setup Beads**

1. Launch BD CellQuest (or BD CellQuest Pro) software and open the BD CBA Flex Set Setup Template.
   
   Note: The BD CBA Flex Set Setup Template file can be downloaded at www.bdbiosciences.com/flexset
2. Set the instrument to Acquisition mode.
3. Set SSC and FSC to Log mode.
4. Decrease the SSC PMT voltage by 100 from what BD FACSCcomp set.
5. Set the Threshold to SSC at 650.
6. In Setup mode, run tube A. Follow the setup instructions on the following pages.

*Note:* Pause and restart acquisition frequently during the instrument setup procedure to reset detected values after settings adjustments.

Adjust gate R1 so that the singlet bead population is located in gate R1 (*Figure 10a*).

![Figure 10a](image1)

Adjust the R2 gate so it gates the brightest bead population (*Figure 10b*). Adjust the FL3 PMT so that the FL3 median of the A9 bead population is around 1500 and then adjust the FL4 PMT so that the FL4 median of the A9 bead population is around 5000.

![Figure 10b](image2)

Adjust gate R3 so the F1 bead population is inside it (*Figure 10c*). Adjust the FL2 PMT so the FL2 median of the F1 bead population is between 2.5 and 4.0. Proceed to the next page of the setup template.

![Figure 10c](image3)
Run tube B to adjust the compensation settings for FL4 – %FL3.

Adjust gate R4 so the A1 bead population (brightest bead in FL3 channel) is inside it (Figure 10d). Ensure that the left edge of gate R4 is touching the y-axis boundary of the dot plot. Adjust gate R5 so the F1 bead population is inside it (Figure 10d). Using the FL4 - %FL3 control, adjust the median of G4 until it is equal to the median of G5 (Figure 10d). Proceed to the next page of the setup template.

![Figure 10d](image)

Run tube C to adjust the compensation settings for FL3 – %FL4.

Adjust gate R6 so the F1 bead population (dimmest bead in FL4 channel) is inside it (Figure 10e). Adjust gate R7 so the F9 bead population is inside it (Figure 10e). Using the FL3 – %FL4 control, adjust the median of G7 until it is equal to the median of G6. Proceed to the next page of the setup template.

![Figure 10e](image)

Run tube D to adjust the compensation settings for FL3 – %FL2.

Adjust gate R8 so the F1 bead population (dimmest bead in FL2 channel) is inside it (Figure 10f). Adjust gate R9 so the PE-F1 bead population is inside it (Figure 10f). Using the FL3 – %FL2 control, adjust the median of G9 until it is equal to the median of G8. Proceed to the next page of the setup template.

![Figure 10f](image)
Run tube E to adjust the compensation settings for FL2 – %FL3.

Adjust gates R10, R11, R12, R13, and R14 so each bead row falls within only one gate (see Figure 10g). Ensure the left edge of each gate is touching the y-axis boundary of the dot plot. Using the FL2 – %FL3 control, adjust until the median of any row (G10, G11, G12, G13, or G14) equals 2 – 4 (Figure 10g).

Note: It is important not to overcompensate the bead populations in this step. No bead population should have a median below 2.

![Figure 10g](image)

Note: Not all populations shown in Figure 10g will be displayed in every setup; the populations displayed are dependent on the Flex Set beads used in the experiment.

Proceed to the next section.

Data Acquisition

1. Return to page 1 on the BD CBA Flex Set Setup Template.
2. Set the instrument to Acquisition mode.
3. In the Acquisition and Storage window, do the following:
   a. Set the Acquisition Gate to Accept G1=R1 events. (This will allow for only the events that fall into R1 to be saved).
      
      Note: Be sure that R1 is set correctly to avoid data loss.
   b. Set Collection Criteria for acquisition to stop at 300 × the number BD CBA Flex Set assays being used (eg, 300 × 6 = 1800 events for a 6 plex). This ensures that the sample file contains approximately 300 events of each bead population. Do not acquire more than 300 beads per population.
   c. Set Resolution to 1024.
   d. Click OK.
4. In setup mode, run tube number 1 and using the FSC vs. SSC dot plot, place the R1 region gate around the singlet bead population (see Figure 10a).
5. Samples are now ready to be acquired.
6. Begin sample acquisition with the flow rate set to LOW. Using the lowest flow rate can improve resolution of the individual bead populations in the bead plex.
To facilitate analysis of data files using FCAP Array software and to avoid confusion, add a numeric suffix to each file that corresponds to the assay tube number (ie, Tube No. 1 containing 0 pg/ml could be saved as KT032598.001). The file name must be alphanumeric (ie, contain at least one letter).

Analysis of Sample Data

The analysis of BD CBA data is optimized when using FCAP Array software. Install the software according to the instructions in the FCAP Array Software User’s Guide.

1. Transfer FCS data files for the experiment to the computer with the FCAP Array software.
2. Place all data files for a given experiment in a single folder.

Follow the instructions for creating an experiment and data analysis in the FCAP Array Software User’s Guide.

Typical Sample Data

Human peripheral blood mononuclear cells (PBMCs) stimulated under two different conditions and measured in a BD CBA Flex Set assay (30 plex)

Human PBMCs were stimulated with immobilized anti-human CD3 antibody (UCHT1, 10 μg/ml, Cat. No. 555329), soluble anti-human CD28 antibody (CD28.2, 2 μg/ml, Cat. No. 555725), recombinant human IL-2 (Cat. No. 554603, 10 ng/ml) and recombinant human IL-4 (Cat. No. 554605, 20 ng/ml) for 2 days. Cells were washed and subsequently cultured in medium containing rhIL-2 and rhIL-4 for 3 days to promote cellular proliferation and differentiation. Finally, cells were harvested and restimulated for 24 hr with PMA (Sigma, Cat. No. P-8139; 5 ng/ml) and Ionomycin (Sigma, Cat. No. I-0634; 500 ng/ml) or with anti-human CD3 and anti-human CD28. Finally, cells were harvested and the supernatants were collected.

Using 50μl of supernatant, the following proteins were measured using the corresponding BD CBA Flex Sets: IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IFNγ, TNF, LT-α, G-CSF, GM-CSF, RANTES, VEGF, IP-10, MIP-1α, MIP-1β, MCP-1, MIG, Angiogenin, bFGF, FasL, Eotaxin, OSM and IgE.
Figure 11a. Standard curves generated using the Human Soluble Protein Master Buffer Kit and the 30 BD CBA Flex sets. Acquisition was performed on a BD FACSArray. The data were analyzed using FCAP Array Software.

Figure 11b. Comparison between PMA/Ionomycin and Anti-CD3/CD28 stimulated PBMCs.

BD™ CBA Kits

Introduction

BD CBA kits enable multiplex analysis of complex biological samples on a flow cytometer. In contrast to the BD CBA Flex Sets, the kits are preconfigured by functional areas of biology (eg, Th1/Th2 or inflammatory cytokines) to measure up to seven analytes simultaneously using capture beads that contain unique amounts of a single red dye. The unique spectral properties of this dye enable analysis of the kits on flow cytometers that have a single 488 nm laser or on dual laser (488 nm and 633 nm) flow cytometers. Each kit comes complete with all of the buffers and reagents necessary to analyze 80 tests.
Figure 12. Representative data generated using the BD CBA Human Inflammatory Cytokines Kit, showing relative bead fluorescence intensities.

Each assay has been stringently developed for ease-of-use, rapid data analysis, sensitivity, reproducibility, and quality. Each antibody pair used in the kits is evaluated for dynamic range, sensitivity, and parallel titration curves to native biological samples. In addition, the assay diluent and wash buffers in each kit have been formulated to reduce detrimental effects of serum and plasma proteins on assay performance.

Reagents Provided
Each BD CBA Kit includes specific Capture Beads, Detection Reagents, Standards, assay buffers, and Flow Cytometer Setup Reagents. All of the reagents required for performing a BD CBA experiment are provided in each BD CBA Kit.

BD CBA Kit Assay Procedures
Each of the BD CBA Kits are specific for proteins in a variety of matrices and often have differences in their specific protocols. For information on the protocol used by a given BD CBA Kit, please refer to the specific BD CBA Kit Manual that can be downloaded from the BD Biosciences website at: www.bdbiosciences.com/pharmingen/cba

Overview

1. Reconstitute Cytokine Standards in Assay Diluent (15 min)
2. Dilute Standards by serial dilutions using the Assay Diluent
3. Mix 10 µl/test of each Cytokine Capture Bead suspension (vortex before aliquoting)
4. Transfer 30 µl of mixed beads to each assay tube
5. Add PE Detection Reagent (50 µl/test)
6. Add Standard Dilutions and test samples to the appropriate sample tubes (50 µl/tube)
7. Wash samples with 1 ml Wash Buffer and centrifuge
8. Add 300 µl of Wash Buffer to each assay tube and analyze samples

Cytometer Setup Bead Procedure
1. Add Cytometer Setup Beads (vortex before adding) to setup tubes A, B and C (50 µl/tube)
2. Add 30 µl of FITC Positive Control to tube B and 50 µl of PE Positive Control to tube C
3. Add 450 µl of Wash Buffer to tubes B and C
4. Add 450 µl of Wash Buffer to tube A
5. Use tubes A, B and C for cytometer setup

Unless otherwise specified, all products are for Research Use Only. Not for use in diagnostic or therapeutic procedures. Not for resale.
Cytometer Setup, Data acquisition and Analysis

For optimal performance of a BD™ CBA assay, it is necessary to properly set up the flow cytometer. For this purpose, each BD CBA Kit uses a simple procedure and templates to enable the operator to optimize their instrument setup.

For the BD FACScan™ and BD FACSCalibur™ flow cytometers, BD FACSComp™ Software is useful for setting up the flow cytometer. BD CellQuest™ (or BD CellQuest Pro) Software is required for analyzing samples and formatting data for subsequent analysis using FCAP Array Software. For detailed information on using BD FACSComp with BD Calibrite™ Beads to set up the flow cytometer, refer to the BD FACSComp Software User’s Guide and the BD Calibrite beads Package Insert. Version 4.2 of the software contains a BD CBA preference setting to automatically save a BD CBA calibration file at the successful completion of any Lyse/No Wash assay. The BD CBA calibration file provides the optimization for FSC, SSC, and threshold settings. Optimization of the fluorescence parameter settings is still required (ie, PMT and compensation settings).

For the 96-well plate-loader-equipped BD FACSArray™ bioanalyzer, BD FACSArray software is used for instrument setup and sample acquisition. BD FACSArray software is compatible with FCAP Array software to allow for quantitative analysis of BD CBA data. FCAP Array software enables researchers to design and set up their BD CBA experiments and import them to the BD FACSArray bioanalyzer in a streamlined workflow. Refer to the BD FACSArray Bioanalyzer Instrument Setup Manual for instrument setup and data acquisition. Follow the instructions in the FCAP Array Software User’s Guide (available for download at www.bdbiosciences.com/fcaparray) for creating an experiment and for data analysis.

The BD CBA Instrument Setup Templates for different flow cytometers can all be downloaded at: www.bdbiosciences.com/pharmingen/cba

Analysis of Sample Data

The analysis of BD CBA data is optimized when using FCAP Array Software.

Install the software according to the instructions in the Software User’s Guide. Refer to the web at www.bdbiosciences.com/cba (select the Downloads tab) for more information on data analysis.
Typical Sample Data

Figure 13. Example of BD CellQuest dot plots of various standard dilutions analyzed with the BD CBA Human Th1/Th2 Cytokine Kit.

Figure 14. Example of standard curves generated using the BD CBA Human Th1/Th2 Cytokine Kit.
Performance Evaluation and Standardization

All BD CBA products are evaluated for performance characteristics including reproducibility, linearity, spike recovery and theoretical limit of detection. These values are reported in the product documentation that is included with each BD CBA product.

Reproducibility

The inter-assay and intra-assay reproducibility are determined by evaluating ten replicates of three different sample levels (intra-assay) and two replicates of three different sample levels from four separate experiments (inter-assay).

Linearity

Cell culture supernatant, serum, or EDTA-plasma are spiked with protein and serially diluted. The diluted samples are assayed and the results are compared with the spiked sample.

Spike Recovery

Cell Culture supernatant, serum or EDTA-plasma are spiked with protein and serially diluted. The diluted samples are assayed and the results are compared with a standard curve to determine recovery.

Theoretical Limit of Detection

The individual standard curve range for a given cytokine defines the minimum and maximum quantifiable levels using the BD CBA assay. The theoretical limit of detection is defined as the corresponding concentration at two standard deviations above the median fluorescence of 20 replicates of the negative control (0 pg or units/ml) using a 4- or 5- parameter curve.

Standardization of BD CBA Standards to the NIBSC/WHO International Standards

Laboratories throughout the world use different assays to measure soluble proteins in biological samples. To support the comparison of values obtained with the BD CBA method, we have evaluated the performance of many of our BD CBA standards with gold standards from the National Institute for Biological Standards and Control (NIBSC). The NIBSC protein standards are recognized by the World Health Organization (WHO) as international biological standards. They meet established requirements for accuracy, consistency, and stability. The NIBSC/WHO standards are assigned potency values in International Units (IU) of biological activity and nominal mass (ie, not absolute mass values), therefore they cannot be used to establish absolute concentrations for a cytokine preparation. However, these standards do provide a means to facilitate comparisons of cytokine concentration values determined by experiments conducted within different laboratories or methods.
The source of a recombinant protein (ie, insect cell, *E. coli*, etc.) and the affinity of antibodies used can affect the measurement and performance of a protein in an immunoassay. The conversion factors provided in Tables 9 and 10 make it possible to compare protein concentrations present in samples measured by different immunoassays that have been standardized to the same NIBSC/WHO standards.

### Table 9. NIBSC conversion factor summary for BD CBA Flex Set Standards.

<table>
<thead>
<tr>
<th>Code No.</th>
<th>Mass Units / Vial</th>
<th>IU Value</th>
<th>Calculated Concentration (pg/ml)</th>
<th>Nominal Mass Concentration (ng)</th>
<th>IU Value</th>
<th>Conversion Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD CBA FLEX SET: NIBSC/WHO MASS CONVERSION FACTOR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 10. NIBSC conversion factor summary for BD CBA Kit Standards.

<table>
<thead>
<tr>
<th>Code No.</th>
<th>Mass Units / Vial</th>
<th>IU Value</th>
<th>Calculated Concentration (pg/ml)</th>
<th>Nominal Mass Concentration (ng)</th>
<th>IU Value</th>
<th>Conversion Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD CBA KIT: NIBSC/WHO MASS CONVERSION FACTOR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The conversion factor may change based on the batch of either standard. Therefore, the conversion factor is intended to be a guideline indicating whether a BD CBA assay over- or under-estimates analyte concentrations relative to the NIBSC/WHO standards. Researchers are advised to incorporate both sets of standards in their assays if they wish to derive data from the NIBSC/WHO standards.

**Figure 15.** Titration curve comparing the BD CBA Human IFN-γ recombinant standard to the NIBSC/WHO International Standard.

### BD™ CBA Functional Beads to Conjugate for Unique Requirements

**Introduction**

The BD CBA Flex Set Functional Beads are unconjugated beads that allow researchers to conjugate their own antibody or protein of interest using sulfo-SMCC chemistry. The conjugation procedure takes less than four hours using common laboratory supplies along with the buffers in the BD CBA Functional Bead Conjugation Buffer Set. The beads, once linked to a protein, can be used as a capture matrix for analytes of interest.

This is an ideal platform for converting existing ELISAs into bead-based immunoassays that can be mixed with our portfolio of BD CBA Flex Set assays. The availability of multiple bead positions enables generation of novel multiplex panels while the ability to prepare up to 1,000 tests in a single reaction ensures consistency across a large number of tests.

**Principle**

The Functional Bead Conjugation Buffer Set (Cat. No. 558556) is used to covalently link water-soluble proteins to the surface of color-coded functional beads. The bond between the bead and the protein is formed using sulfo-SMCC chemistry (see Figure 16). Before attempting to conjugate a protein to the beads, it is important to ensure the protein has been purified and is free of BSA, glycine, Tris, or any other proteins or protein stabilizing additives. Presence of stabilizing or contaminating proteins may affect the performance of the beads after conjugation. The conjugation method will activate and bind any free amino group in the sample to the beads. For a successful conjugation the protein needs to be suspended in PBS, pH 7.2 ± 0.2.
Figure 16. Overview of the Functional Beads conjugation procedure.

The Functional Bead Conjugation Buffer Set contains enough material for 15 coupling reactions. Each coupling reaction yields approximately $3 - 6 \times 10^6$ beads which is enough material to run 500 or 1000 tests at a concentration of 6,000 beads per well. For a 500 test conjugation reaction 90 $\mu$g of protein is needed, and for a 1000 test conjugation reaction 180 $\mu$g of protein is needed.

The procedure to conjugate a protein to a functional bead consists of 4 major steps:

- Step I – Bead Preparation
- Step II – Protein Modification
- Step III – Buffer Exchange to Remove Unreacted Components
- Step IV – Protein Conjugation

The estimated time to completion for the entire conjugation procedure is approximately 3.5 hrs.

Once the beads are conjugated with a protein, it is recommended that the success of the reaction be confirmed. If the functional bead was conjugated with an antibody, it is recommended to use one of the three PE (Phycoerythrin) Functional Bead Ig Detectors (see page 70) to confirm the presence of antibody bound to the beads. The procedure to confirm the conjugation of an antibody will take approximately 45 minutes.

If the protein cannot be identified using one of the Functional Bead Ig Detectors, then a biotinylated antibody specific to that protein can be used in conjunction with streptavidin-PE. A flow cytometer will be used to analyze any sample.

Important notes

- For Research Use Only.
- Protein must be purified and free of BSA, glycine, Tris, or any other proteins or protein stabilizing additives. The protein must be suspended in PBS, pH 7.2 ± 0.2 before attempting the coupling reaction.
- Not suitable for conjugation of ascites or crude antiserum.
• Although suitable for conjugation of any protein with a free amino group, the protocol has only been validated for IgG subclass antibodies.

• Functional Beads are stable at 4°C for 1 year after conjugation.

• Do not use expired reagents.

Reagents Provided

The Functional Bead Conjugation Buffer Set (Cat. No. 558556) contains:

• Coupling Buffer: 1 bottle, 150 mL (1×)

• Storage Buffer*: 1 bottle, 90 mL (1×)

Store reagents provided undiluted at 4°C.

* The Storage Buffer contains serum proteins. Source of all serum proteins is from USDA inspected abattoirs located in the United States.

Equipment and Materials Required but Not Provided

The following items are required for optimal results:

• A dual-laser flow cytometer equipped with a 488 nm or 532 nm laser and a 633 nm or 635 nm laser and capable of distinguishing 576 nm, 660 nm, and >680 nm fluorescence

• Analytical Balance: Readability of 0.1 mg (VWR Cat. No. XS205DU) or equivalent

• Microcentrifuge model 5415C (VWR Cat. No. 20901-051) or equivalent

• Swing Bucket Centrifuge International Equipment Company (IEC/ CENTRA CL2 Benchtop) or equivalent

• Vortex-Genie Mixers, Scientific Industries (VWR Cat. No. 58816-121) or equivalent

• Table-top ultrasonic cleaner (Branson 1510-DTH or equivalent)

• Eppendorf Thermo R mixer (VWR Cat. No. 21516-166) or equivalent

• Bio-Spin Columns (Bio-Rad Cat. No. 732-6231)

• DTT (Dithiothreitol, Pierce Cat. No. 20290)

• Sulfo-SMCC (Sulfo succinimidyl 4-N-maleimidomethyl cyclohexane 1-carboxylate, Pierce Cat. No. 22322)

• NEM (N-Ethylmaleimide, Pierce Cat. No. 23030)

• DMSO (Dimethylsulfoxide, Pierce Cat. No. 20684)

• Eppendorf Safe-lock Tubes, 1.5 mL (VWR Cat. No. 21008-959) or equivalent test tubes

• 12 x 75 mm, 5 mL Polystyrene Round Bottom Test Tubes (BD Falcon Cat. No. 352008)

• PBS (Dulbecco’s Phosphate Buffered Saline 1x, Invitrogen Cat. No. 14040-117)
Supporting Reagents (Recommended)

The following items from BD Biosciences are recommended for optimal results:

- **Functional Bead Ig Detectors** (for validating conjugation):
  - PE Goat anti-Mouse Ig Detector, 25 tests (Cat. No. 558550)
  - PE Goat anti-Rat Ig Detector, 25 tests (Cat. No. 558551)
  - PE Goat anti-Rabbit IgG Detector, 25 tests (Cat. No. 558553)

- **Assay Buffer Kits** (for running assays; choose one depending on protein being assayed):
  - Cell Signaling Master Buffer Kit, 100 or 500 tests (Cat. Nos. 558223 or 558224)
  - Human Soluble Protein Master Buffer Kit, 100 or 500 tests (Cat. Nos. 558264 or 558265)
  - Mouse/Rat Soluble Protein Master Buffer Kit, 100 or 500 tests (Cat. Nos. 558266 or 558267)
  - Streptavidin-PE (Cat. No. 554061) (for validating conjugation with biotinylated protein)

Conjugation Procedure

Before starting the procedure:

1. Bring all provided reagents to room temperature.
2. Remember to protect functional beads from light at all times. Cover tubes with aluminum foil during procedure.
3. Protein sample must be free of BSA, glycine, Tris, or any other proteins or protein stabilizing additives. The protein must be suspended in PBS, pH 7.2 ± 0.2 before attempting the coupling reaction.
   a. The protein sample must be in a buffer that is free of other proteins (e.g., BSA or FBS). Purification may be required.
   b. If the protein is in a buffer containing amino groups (e.g., Tris or glycine buffer), then a buffer exchange is required. For buffer exchange dialyze overnight in PBS or use a buffer exchange column (recommended: Bio-Rad Cat. No. 732-6221).
4. You will need 90 μg of protein for a 500 test conjugation reaction or 180 μg of protein for a 1000 test conjugation reaction. The protein must be at a stock concentration of 1 mg/mL.

   *Note:* This protocol has not been validated for scalability at anything other than 500 or 1000 tests.

**Step I. Bead Preparation**

2. Transfer the functional beads to a microcentrifuge tube. Cover tube with aluminum foil to protect from light.
   a. 500 test reaction: Transfer 75 μL of functional beads.
   b. 1000 test reaction: Transfer 150 μL of functional beads.
3. Sonicate functional beads for 1 min.
4. Prepare 1M DTT in H₂O.

   *Note:* It is recommended to prepare aliquots of DTT to be stored at –20°C. Use a fresh aliquot for each conjugation.
5. Add 1M DTT to microcentrifuge tube containing the functional beads.
   a. 500 test reaction: Add 1.9 μL of 1 M DTT.
   b. 1000 test reaction: Add 3.8 μL of 1 M DTT.
7. Incubate beads on an orbital shaker for 1 hour at room temperature, protect from light. If a shaker is not available, vortex the beads every 15 min, protect from light.

   *Note:* Incubation time can be used to prepare the sulfo-SMCC reagent for protein modification (See Step II.#2 below).
8. Add 1 ml of Coupling Buffer and vortex for 5 sec. (use same volume for 500 and 1000 test reactions).
9. Pellet beads by centrifugation at 900 × g for 3 min. Aspirate and discard the supernatant – do not aspirate beads or disturb bead pellet.
10. Repeat steps 8 and 9 three times.

   a. 500 test reaction: Resuspend with 20 μL of coupling buffer.
   b. 1000 test reaction: Resuspend with 40 μL of coupling buffer.

**Step II. Protein Modification**

1. Prepare a microcentrifuge tube containing protein stock solution at 1 mg/ml in PBS, pH 7.2 ± 0.2. Cover tube with aluminum foil to protect from light.

   a. 500 test reaction: Prepare a tube with 90 μL protein stock solution.
   b. 1000 test reaction: Prepare a tube with 180 μL protein stock solution.
2. Prepare sulfo-SMCC stock solution (2 mg/mL in DI H₂O) immediately before use.

   *Note:* Do not store or reuse sulfo-SMCC stock solution.
3. Add sulfo-SMCC to protein stock solution.
   a. 500 test reaction: Add 2 μL of sulfo-SMCC.
   b. 1000 test reaction: Add 4 μL of sulfo-SMCC.
4. Vortex microcentrifuge tube for 5 sec. Avoid excessive splashing on top of tube.
5. Incubate protein on an orbital shaker for 1 hour at room temperature, protect from light. If a shaker is not available, vortex the beads every 15 min, protect from light.
   Note: Incubation time can be used to equilibrate the spin column with Coupling Buffer (See step III.#1 below).

Step III. Buffer Exchange to Remove Unreacted Components
1. To a Bio-Rad Spin Column (Cat. No. 732 - 6231) add Coupling Buffer until full. Let Coupling Buffer drain from column by gravity, repeat twice (same for 500 and 1000 test reactions).
   Note: This takes approximately 35 min. Refer to the instruction manual from Bio-Rad for detailed instructions.
2. Place the spin column in a 12 × 75 mm test tube and centrifuge the spin column at 1,000 × g for 2 min. Discard the 12 × 75 mm tube after centrifugation.
3. Place the spin column in a new 12 × 75 mm test tube.
4. Transfer the entire volume of protein/sulfo-SMCC solution to the spin column.
5. Centrifuge the spin column at 1,000 × g for 2 min 15 sec. This transfers the modified protein into the 12 × 75 mm test tube in Coupling Buffer. Discard spin column.
6. It is important to keep the protein/sulfo-SMCC reaction time to one hour as specified in step II. After buffer exchange, proceed immediately to step IV.

Step IV. Protein Conjugation
1. Transfer all the modified protein from the 12 × 75 mm tube to the microcentrifuge tube containing the functional beads previously prepared in Step I.
2. Vortex microcentrifuge tube for 5 sec. Avoid excessive splashing on top of tube.
3. Incubate protein and beads on an orbital shaker for 1 hour at room temperature, protect from light. If a shaker is not available, vortex the beads every 15 min, protect from light.
4. Prepare NEM (2 mg/ml in DMSO).
   Note: It is recommended to prepare 20 μl aliquots of NEM solution and store at -20°C. Use a fresh aliquot for each conjugation.
5. Add NEM solution to the microcentrifuge tube containing the functional beads and modified protein.
   a. 500 test reaction: Add 2 µL of NEM.
   b. 1000 test reaction: Add 4 µL of NEM.


7. Incubate microcentrifuge tube for 15 min on an orbital shaker. If a shaker is not available, vortex periodically during the incubation.

8. Add 1 ml of Storage Buffer to the microcentrifuge tube (use same volume for 500 and 1000 test reactions).

9. Pellet beads by centrifugation at 900 × g for 3 min. Aspirate and discard the supernatant – do not aspirate beads or disturb bead pellet.

10. Repeat steps 8 and 9 three more times.

11. Resuspend bead pellet in Storage Buffer. The beads are now at a final concentration of approximately 6 × 10^6 beads/ml and are ready to be validated.
   a. 500 test reaction: Resuspend pellet in 0.5 mL of Storage Buffer.
   b. 1000 test reaction: Resuspend pellet in 1.0 mL of Storage Buffer.

   *Note:* Store beads at 4°C and protect from light. For optimal results let beads sit in storage buffer overnight prior to first use. The background of the beads will decrease slightly overnight. The beads are stable for 1 year after conjugation.

**Conjugation Confirmation**

Once the beads are conjugated it is recommended to confirm the success of the conjugation procedure before using the conjugated beads in an assay. This confirmation can be performed using a flow cytometer and a phycoerythrin (PE) conjugated molecule that binds specifically with the protein conjugated to the bead. Depending on the reagents used, a successful conjugation normally gives a PE signal greater than 500 MFI. Below are two suggested methods for determining a successful conjugation.

1. To test for antibodies that have been conjugated to the functional beads, BD Biosciences offers PE anti-Ig Detectors (Cat. Nos. 558550: Anti-Mouse; 558551: Anti-Rat; 558553: Anti-Rabbit, see page 70). Ensure the correct reagent is used to confirm conjugation by matching the species of the antibody that was conjugated to the functional beads with the PE anti-Ig Detector (eg, mouse, rat, rabbit).

   *Note:* See the Functional Bead Ig Detectors Procedure below for the Conjugation Confirmation protocol using a Functional Bead Ig Detector.

2. To test for non-antibody proteins that have been conjugated to the functional beads:
   a. Use a PE-labeled antibody that is specific for that protein.
b. Use a biotinylated antibody specific for that protein followed by streptavidin-PE.

Functional Bead Ig Detector Procedure

Before starting the procedure:

- Bring all provided reagents to room temperature.
- Remember to protect functional beads from light at all times. Cover tubes with aluminum foil during procedure.

Procedure

1. Label three 12 x 75 mm test tubes, (1) diluted functional beads, (2) negative control, and (3) test sample.

2. Vortex the bulk conjugated functional beads for 5 sec.

3. To the diluted functional beads tube (1) add 245 µL of Wash Buffer from the Master Buffer Kit used.

   Note: Do not use Capture Bead Diluent from Master Buffer Kit to dilute beads when confirming conjugation.

4. Add 5 µL of the bulk functional beads to the diluted functional beads tube (1) and vortex the diluted functional beads for 5 sec.

5. Transfer 50 µL of the beads from the diluted functional beads tube (1) to the negative control tube (2) and to the test sample tube (3). The diluted functional beads tube (1) can now be discarded.

6. Add 50 µL of Wash Buffer to the negative control tube (2).

7. Add 50 µL of the appropriate PE anti-Ig Detector to the test sample tube (3).

8. Vortex negative and test sample control tubes for 5 sec.

9. Incubate tubes for 30 minutes in the dark at room temperature.

10. Add 1 mL of Wash Buffer to both tubes.

11. Centrifuge the tubes at 1,000 x g for 5 min. Aspirate and discard the supernatant – do not aspirate beads or disturb bead pellet.

12. Re-suspend bead pellets for both tubes with 150 µL of Wash Buffer.

13. Read both tubes on a flow cytometer using the appropriate BD CBA Flex Setup instrument settings (Refer to the BD CBA Instrument Setup manual for instructions).

14. For flow cytometers equipped with a plate reader, transfer beads into two free wells on a 96 well plate and read using the appropriate BD CBA Flex Setup instrument settings (Refer to the BD CBA Instrument Setup manual for instructions).

15. If the signal for the test sample is 500 MFI greater than the signal for the negative control sample then the conjugation was successful and the conjugated beads can be used in further assays.
Note: This procedure will give a qualitative result but it can be used to provide an estimate of the amount of antibody that was conjugated to the functional beads. If there is no signal difference between the negative control and the test sample then the conjugation reaction was not successful.

Immuoassay Development Hints

Identifying a Suitable Antibody Pair

To develop a bead-based immunoassay, a pair of antibodies is most often needed. One needs two antibodies that can bind to the same molecule at the same time. These antibodies should therefore recognize different epitopes. One antibody will be conjugated to the functional bead and the other antibody will be used as the detection antibody to detect the presence of the analyte of interest bound to the conjugated bead. A good starting point is to use a pair of antibodies that work optimally in an ELISA assay.

It is difficult to predict which antibody out of the pair will function best as the capture antibody. If a known ELISA pair is being used, generally the capture antibody for the ELISA will work best as the capture antibody with the functional beads. However, this is not always the case. If enough material is available, it is best to test both antibodies, each one in turn as capture antibody and detection antibody.

Confirming Bead Conjugation

After the antibody has been conjugated to the beads, researchers should confirm the conjugation reaction was successful. This can be accomplished by performing the Functional Bead Ig Detector Procedure using the appropriate PE anti-Ig Detector (see page 74).

When validating conjugation, it is recommended to use the same detection fluorochrome as when running the assay. PE can either be directly conjugated to the detection protein or a biotinylated protein can be used followed by streptavidin-PE. PE is chosen because of its brightness, which results in a broad dynamic range. Fluorochromes that emit in the red spectrum should not be used due to spectral overlap with the beads’ fluorescence.

Confirming the New Assay Measures the Correct Analyte

The conjugated functional beads and detector need to be tested against a standard, such as a recombinant protein, and against samples that contain the native protein. The samples can be tissue culture supernatant, serum, plasma or any other good source for the native protein of interest. For the measurement of intracellular proteins, detergent lysates of cells should be used.

It is important that the developed assay measures the molecule of interest in the same range that occurs physiologically. For most cytokine assays, the likely range of detection will vary from 5000 pg/ml down to 20 pg/ml or lower.

Evaluating Linearity of the Assay

Another important consideration is the linearity of the standard curve. More particularly, the standard curve and the sample titration curve must be parallel.
to each other. This will ensure that values read off of the top end of the standard curve will be comparable to values read off of the low end. If the curves are not parallel, the results obtained for sample dilutions (taking into account the dilution factor) will not be consistent across the standard curve range.

**Optimizing the Assay**

If the signal is low, consider the following:

a. One possible solution, as mentioned above is to switch the pairs. It has been seen in a number of cases that the best ELISA capture antibody may not work well as the BD CBA capture antibody.

b. The timing of the assay may be altered. An assay can be 1-step, in which beads, sample, and detector are all added at the same time or it can be a 2-step assay, in which beads and sample are pre-incubated before the addition of detector. In the case of a 2-step assay, it may be beneficial to wash before the addition of detector.

c. The timing of the various steps can also influence the total signal that is observed.

d. The detector concentration should be optimized. Generally, initial studies can be done using 200 ng of detector antibody per test. An experiment should be done in which the detector concentration is titrated down. An example would be to run a standard curve and samples and use detector concentrations from 12.5 ng/test up to 200 ng/test. Using higher detector concentrations than are necessary will use up the reagent more quickly and, more importantly, can lead to higher background.

**Multiplexing the Assay with Existing BD CBA Flex Set Assays**

If the new bead-based immunoassay is to be mixed with any existing BD CBA Flex Set assay, then experiments must be performed to determine compatibility of the assays. The new bead-based immunoassay must use the same assay procedure and buffers as the BD CBA Flex Set assay(s) it is to be mixed with. It is critical to confirm that the new bead-based assay and the BD CBA Flex Set assay(s) perform the same way as a single plex as they do in a mixture. A poor correlation between the two is usually due to cross-reactivity or non-specific binding. Researchers should make sure to verify not only how the other assays affect the newly developed assay but also how your new combination affects the existing bead sets. The following tests are recommended:

1. Make a mixture of all of the beads and all of the detectors. The standards for each assay are then added individually to different wells. A signal should only be seen with the correct bead.

2. A standard curve and a titration of a positive test sample should be tested as a single plex assay and within a multiplex assay. Quantitation of the test sample should be identical regardless of which other assays it is being run with.

*Note:* If either test gives unexpected results, it is likely that the assays cannot be mixed and should be run separately in the future.
Tips and Tricks for BD CBA Users

General

Q: How stable are BD CBA assays?

A:

- Signal can slightly decrease over time, and this can vary from one analyte to another:
  - This is not due to fading of the PE detector, but to antibody dissociation kinetics.
  - The slight decrease is not important as long as the quantitation remains the same (standard signal is also decreasing).
- To avoid this, keep samples at 4°C, or fix them.
  - When fixing samples: Do NOT fix them BEFORE performing the BD CBA experiment. Run the complete experiment, then incubate completed samples (including standards) with 1% PFA for 20 min, wash (PFA should be removed by centrifugation and samples stored in Wash Buffer), and run on the flow cytometer. Fixation will affect the overall assay signal and possibly sensitivity for various proteins. However it will not completely destroy signals and has been shown to work in other laboratories (feedback we have been given).

  Note: We do not routinely fix completed samples prior to BD CBA analysis and we recommend that researchers validate this procedure prior to implementing it on precious samples, as our development and Quality Control are based on unfixed material.
- When running a large experiment (>200-300 samples), keep plates/tubes at 4°C and transfer samples on ice – 5 samples at a time – for analysis.

Q: How should I best store my BD CBA samples prior to analysis?

A:

- At -20°C, cytokines in serum and supernatants have different half-lives, and sensitivity may decrease by up to 50% when freezing/thawing samples even only once. While IL-6 can be frozen/thawed up to 5-6 times without losing much of its activity, IFN-γ and TNF are very fragile and more prone to loss of activity.
  - If possible, do not freeze your samples.
  - If necessary, -70°C/-80°C storage is always better. When storing at -80°C, only freeze samples once (ie, aliquot them) and avoid any humidity: seal plate with parafilm and place it into a plastic bag (if assays were performed in tubes transfer samples to a plate for storage).
- When storing samples for less than a week, it is preferable to keep them at 4°C.
- Customer suggestion: add 50% glycerol to the samples when storing at
-20°C to avoid freezing

*Note:* not validated in-house at BD Biosciences.

- **Important:**
  - To allow for comparison of results, always treat samples in the same way.
  - Each cytokine will behave differently.

**Q:** How should I best store BD CBA reconstituted standards?

**A:**
- **BD CBA soluble protein standards:**
  - Reconstituted top standard for cytokines can be stored up to 12 hours at 4°C.
  - Generally, we do not recommend long-term storage of reconstituted cytokine standards.
- **BD CBA cell signaling standards:**
  - Reconstituted standards are stable up to 3 months when stored at 4°C.

**Q:** How can I best determine the dilution needed for my samples?

**A:**
- Do not perform large dilution steps: When the dilution factor is large, quantitation will be less reliable as initial pipetting errors get amplified. Also, the higher the dilution, the more difficult it becomes to distinguish a positive signal from background. We recommend diluting each time by ½.
- The ideal diluent is your culture medium.
- Serum and plasma samples: Dilution of these types of samples may lead to increased recovery, as possible inhibitors present in the sample also get diluted. To check for presence of inhibitors, perform a spike recovery assay by adding a known amount of recombinant protein to the samples, analyze and see how much analyte is detected. If values are lower than the amount of protein added, then there are most probably factors in the sample that are breaking down the analyte or otherwise inhibiting the signal.
- For spike recovery assays: use RPMI with 10% FCS, or Assay Diluent (contains less than 10% FCS).
- The software will extrapolate results for data points that fall outside of the assay range; data should thus be carefully reviewed.
  - For accurate results, only use values 2x above the background, and look at the top end: if you are already out of the linear range, do not use this value.
- R2 should be between 98 and 100; if not, change your parameter fitting equation.
Q: How many analytes can I measure simultaneously in a BD CBA Flex Set assay?

A:
- Running large plexes (e.g., 27-plex) with the BD CBA Flex Set formats may increase the background and compromise the sensitivity at the 5-20 pg/ml range so one might not be able to detect a signal in samples with lower levels of analyte. One suggestion is to split the plexes into smaller groups (e.g., of 10-12), with groups based on expected levels of analyte expression, if possible.

Q: Can I adapt the duration of the incubation step with the detector?

A:
- The 1hr incubation step with the detector reagent is critical and duration of this step should not be modified. Shortened incubation times will result in decreased signals, longer incubation times will result in increased background.

Cell signaling BD CBA

Q: How sensitive is a cell signaling BD CBA assay compared to Western blot?

A:
- Assay sensitivity of a cell signaling BD CBA is similar to that of a Western blot.

Note: Sample lysates for cell signaling assays should be denatured and centrifuged prior to performing the BD CBA assay, to remove any precipitates that could interfere with assay performance.

Q: How much protein / how many cells do I need to perform a cell signaling BD CBA assay?

A:
- Recommended cell lysate protein concentration is 1 mg/mL (or higher), or ~40.10^6 cells starting material.

Note: Protein concentration may vary with cell type, Jurkat: 100-200 μg total protein per 10^6 cells; peripheral blood lymphocytes: 25-50 μg total protein per 10^6 cells.

Q: Can BD CBA cell signaling assays and BD CBA assays for soluble proteins be combined?

A:
- Samples for cell signaling assays need to be denatured, while soluble proteins (e.g., cytokines) are assessed in their native conformation. Hence the two types of assays use different buffers and it is not advisable to analyze both cell signaling and soluble proteins in the same sample; we have not studied this in-house.
• The main issue would be a difference in kinetics: phospho-signaling happens within minutes, while cytokine synthesis секретion takes hours. It is therefore unlikely that one could accurately measure both in the same sample.

BD CBA in plates

Q: How can I best perform BD CBA assays in plates?

A:

• Use only plates from Millipore (MultiScreenHTS-BV 1.2 µm clear non-sterile filter plates, Cat. No. MSBVN1210: 10 pack or MSBVN1250: 50 pack): these feature a patented, hydrophobic membrane under the filter that prevents liquid from leaking before vacuum is applied.
• Filters must be dry after the vacuum step (for cell signaling assays this is easy to see due to the colored buffers); absorb what is left on a paper towel.
• For “partial” plates that are not completely vacuumed dry: cover unused wells.
• Do not put the plate on an absorbent surface during incubation (the medium will be wicked through the filter).
• Do not shake plates at speeds over 500 rpm to avoid cross-contamination of wells.

  Note: For cell signaling BD CBA assays, higher shaking speeds may also cause frothing due to the SDS present in the buffers.

BD CBA Functional Beads

Q: How can I best choose capture and detection antibody when setting up a BD CBA Functional Bead assay?

A:

Starting from a matched BD ELISA pair:

• The antibody with the highest affinity will be the capture antibody; the other one – with less affinity – will be the detection antibody.

BD ELISA pairs cannot always be adapted to BD CBA assays:

• Try to reverse the pair (use capture as detection, and vice-versa).
• Try to use one antibody (either capture or detection) from BD, and combine with an antibody from a different supplier.

Q: Why/how should I use directly labeled detection antibodies when creating a BD CBA assay with Functional Beads?

A:

• Directly labeled detection antibodies ensure lower background. 200 ng/test is typically the highest detection antibody concentration used. When optimizing BD CBA Functional Beads, start at 200 ng/test and titrate down.
Specific questions

Q: My basic FGF, VEGF and angiogenin standard curves are flatter at the low end, compared to those of other analytes in my assay. Why?
A:
• Basic FGF, VEGF and angiogenin BD CBA standards have a flatter curve at lower concentrations. Adjusting parameter logistics while analyzing the data with the FCAP Array software may help (ie, increasing it to 5-parameter logistics). Alternatively, mask these lower data points in the FCAP Array software so they don’t interfere with the rest of the curve (however, one will lose those points in the analysis).

Q: Why use 5-parameter logistics vs. 4-parameter logistics for generating BD CBA standard curves in the FCAP Array Software?
A:
• The 5-parameter equation gives a better fit of the standard curve at the low end and also at the top end of the curve when the curve is reaching a plateau. 4-parameter logistics work well at the low end, but not at the top end of the curve. The best is to run a standard curve using both logistic equations, in order to see which equation fits the curve better.

Q: Can I perform BD CBA assays with colloidal serum samples?
A:
• For colloidal serum samples, spin down the samples in Eppendorf tubes and use only the supernatants as the colloidal material may interfere with the assay.
References


Related BD Biosciences Literature

BD Cytometric Bead Array. Multiplexed Bead-Based Immunoassays. BD Biosciences Brochure.


Additional Technical Resource:

Our Scientific Support Team is trained to help you with technical questions related to flow cytometry and immunoassays. Just contact them at the numbers listed at the end of this manual, or e-mail: help.biosciences@europe.bd.com.
Intracellular Cytokine Detection by Flow Cytometry: BD Cytofix/Cytoperm™ Method

Introduction

Cytokine Flow Cytometry (CFC) Offers Multiplexing Combined with Single Cell Analysis

Intracellular cytokine staining and detection by flow cytometry, in short, Cytokine Flow Cytometry (CFC), has established its position as an extremely powerful tool for analysis of the nature and frequency of cells that produce cytokines, chemokines and inflammatory mediators.1-4 It has been widely adopted since the core methodology for intracellular immunostaining was established over a decade ago.5-8

The power of this technology lies in the multiparameter flow cytometric detection, in which individual cells can be analyzed for coexpression of several markers, including cell surface and intracellular molecules (as defined by fluorescently labeled antibodies), as well as cell size and granularity (Figure 1). Unlike ELISA, or BD™ CBA (Cytometric Bead Array) assays, which provide a quantitative measure for the average, net cytokine production by all cells in a sample, CFC gives you a cell-by-cell readout. While ELISPOT assays also provide largely single cell results, they are limited in their capability to multiplex.

Obtain In-Depth Analysis of Leucocyte Cell Subsets

The multiparameter analysis provided by flow cytometry poses a particular advantage when studying immune responses in mixed populations of cells, such as in whole blood or PBMCs. Thus, it is possible to analyze CD4 and CD8 responses in the same sample, or to assess expression of other phenotypic markers on the cells of interest. This potentially provides a wealth of information about the behavior of functional populations of memory T cells that respond to specific soluble antigens in short term re-stimulation assays.1,2,8 In addition to enabling highly-specific and sensitive measurements of several parameters for individual cells simultaneously, this method has the capacity for rapid analysis of large numbers of cells that are required for making statistically significant measurements of rare events, such as antigen-specific responses.

Applications: Monitor T Cell Responses to Infection or Vaccine Immunogenicity

CFC is particularly well suited for studies concerned with characterizing Th1 vs. Th2 responses to infection or in disease states.8,9 For example, activated cell populations can be stained simultaneously for cell surface CD4 and for intracellular IFN-γ and IL-4. With flow cytometric analysis, it is possible to identify and enumerate individual CD4+ cells that express these cytokines in either a restricted (eg, Th1- versus Th2-like cells) or unrestricted (eg, Th0-like cells) pattern.
Recent improvements in the standardization and convenience of the technology\textsuperscript{10,11,12} have contributed to its increasing use in the pre-clinical setting. Numerous studies have used CFC to assess the importance of monitoring immune status during disease,\textsuperscript{1-3} or as an \textit{ex vivo} monitor of cellular immune responses to vaccine candidates.\textsuperscript{13, 14} The potential relevance of multi-cytokine analyses for monitoring vaccine efficacy or disease progression has been raised in a study using 12-color analysis and up to five cytokines.\textsuperscript{16, 17} In this initial study the authors demonstrated that the functional breadth of T cell responses goes beyond those describable using a single-cytokine analysis. Other studies also point to the fact that measurement of IFN-\(\gamma\) alone may not give the best correlate with clinical parameters (see citations within 1).

**Critical Parameters for Reliable Intracellular Staining**

Staining of intracellular antigens depends on the identification of antigen-specific monoclonal antibodies that are compatible with a fixation and permeabilization procedure.\textsuperscript{18} Optimal staining of intracellular cytokines, for example, has been reported using a combination of fixation with paraformaldehyde and subsequent permeabilization of cell membranes with the detergent saponin. Paraformaldehyde fixation allows preservation of cell morphology and intracellular antigenicity, while also enabling the cells to withstand permeabilization by detergent (Figure 2). Membrane permeabilization by saponin allows the antigen-specific monoclonal antibody to penetrate the cell membrane, cytosol, and membranes of the endoplasmic reticulum and Golgi apparatus.

Critical parameters for staining intracellular effector molecules include the following: cell type and activation protocol; the time of cell harvest following activation; the inclusion of a protein transport inhibitor during cell activation (Figure 3) and the choice of antibody. These are aspects that have either been incorporated into the following protocol, or are discussed within it.
Figure 1. Analysis of IL-2 and TNF production in activated human PBMCs and lysed whole blood cells. Heparinized blood was either passed over Ficoll-Paque™ to isolate human PBMCs or treated with ammonium chloride buffer to lyse erythrocytes and obtain “lysed whole blood cells” (LWB). Both blood cell preparations were stimulated with PMA (5 ng/ml, Sigma, Cat. No. P-8139) and Ionomycin (500 ng/ml, Sigma, I-0634) in the presence of BD GolgiPlug™ (brefeldin A, 1 µg/ml, Cat. No. 555029) for 4 hrs. Following incubation the cells were harvested, fixed, and permeabilized with BD Cytofix/Cytoperm™ Solution (Cat. No. 554722). The cells were subsequently stained with FITC-anti-human-CD4 (Cat. No. 555346) and either PE-anti-human IL-2 (Cat. No. 554566) (panels C and D), or PE-anti-human TNF (Cat. No. 554513) (panels E and F). The forward and side light scatter profiles for human PBMCs and lysed whole blood are shown in panels A and B, respectively. Dot plots (panels C-F) were derived from gated events with the forward and side light scatter characteristics of mononuclear cells.
Figure 2. Effects of the BD Cytofix/Cytoperm Solution on cell light scattering properties, cell surface antigen staining, and intracellular cytokine staining. Panels A and B show the forward light scatter and side light scatter profiles for freshly-prepared, untreated mouse splenocytes and Ficoll-Hypaque™-isolated human PBMCs, respectively. Panels C and D show the forward light scatter and side light scatter profiles of the same cell populations (in Panels A and B) after they were treated with BD Cytofix/Cytoperm Solution. Panels E and F are examples of mouse and human cells, respectively, that were stained with anti-CD4 and anti-CD8 followed by incubation with the BD Cytofix/Cytoperm Solution. Panels G and H are examples of mouse and human cells, respectively, that were activated for 4 hours with PMA and ionomycin in the presence of BD GolgiStop™, and were subsequently stained with PE-anti-CD4 or BD PE-Cy5-anti-CD3. The cells were then incubated with the BD Cytofix/CytoPerm solution and then stained for intracellular IL-2 (mouse) and IFN-γ (human) respectively. Dot plots (Panels E-H) were derived from gate events with forward- and side-light scatter characteristics of mononuclear cells.
Figure 3. The effect of protein transport inhibitors on intracellular cytokine staining. Human PBMCs were isolated from heparinized whole blood by density centrifugation on Ficoll Paque™ and were either cultured for 4 hrs with no activators and no protein transport inhibitors (A, B) or were stimulated with PMA (5 ng/ml, Sigma, Cat. No. P-8139) and ionomycin (500 ng, Sigma, P-8139) in the presence of either brefeldin A (BD GolgiPlug, Cat. No. 555029) (E, F), monensin (BD GolgiStop, Cat. No. 554724) (G, H) or without any protein transport inhibitor (C, D). Following incubation the cells were harvested, fixed and permeabilized with BD Cytofix/Cytoperm reagents (Cat. No. 554722). The cells were subsequently stained with FITC-anti-human CD4 and either PE-anti-human IL-2 (Cat. No. 554566) or PE-anti-human TNF (Cat. No. 554513). Dot plots were derived from gated events with the forward- and side-light scatter characteristics of mononuclear cells.
Products for Intracellular Cytokine Staining: Choose from Two Options

BD Biosciences offers two separate ranges of reagents for Intracellular Cytokine Flow Cytometry:

- **BD FastImmune™ Cytokine System**
  Streamlined Assay for Defined Applications in Human Cells
- **BD Cytofix/Cytoperm™ Method**
  For Maximum Flexibility and Multiple Species

**BD FastImmune™ Intracellular Cytokine Detection System**

- Developed for human whole blood or PBMCs
- Streamlined protocol to reduce time and sample manipulation
  - Get results in hours, not days
- Highly reproducible detection of rare events such as antigen-specific activation

Trimmed to the needs of applied research with human samples, the BD FastImmune System is developed for use with human whole blood, thus allowing minimal sample manipulation and re-stimulation in close to *in vivo* conditions. The streamlined protocol involves a hands-on time of just two hours and gives you results in as few as eight hours.

Designed in particular for analysis of antigen-specific activation in whole blood (see the related Application Note), the FastImmune Cytokine System provides a very clean background, making it possible to detect very low frequency events in a reproducible manner. BD FastImmune is an open system for the activation antigen of your choice.

Several recent procedural developments have contributed to the convenience of the FastImmune methodology, so that it meets the needs of vaccine monitoring in clinical trials. These include the ability to interrupt the assays with the use of timed cooling and the ability to batch samples via freezing of activated cells.

This chapter covers the details for staining using the BD Cytofix/Cytoperm method. Please see Chapter 4 for more details about the BD FastImmune Protocol

**BD Cytofix/Cytoperm™ Method: For Maximum Flexibility in Intracellular Cytokine Staining**

- Compatible with a wide range of cell samples:
  - Human, Non-human Primate, Mouse, Rat and Pig
  - PBMCs, cell lines and whole blood
- Reagents for a wide range of cytokines
• More multicolor options
• More flexibility in experimental design

The BD Cytofix/Cytoperm method and related reagents have been developed with the need for flexibility of the basic researcher in mind. The protocol has been developed to work not only in human cells, but also in non-human primate, mouse and rat samples. A wide range of BD Pharmingen™ brand reagents optimized for intracellular staining in this system is offered for the species listed above. All the antibodies are offered as single-color fluorochrome conjugates, allowing you to combine these with other markers for cytokines or cell surface markers as your experimental question demands. That can mean anything from single-cytokine, 3-color analyses similar to those with the BD FastImmune System, to even more in-depth analysis of the behavior of further cell subsets.

**BD Pharmingen™ Intracellular Cytokine Antibodies: Multiple Species, Multicolor Options**

A critical factor for obtaining successful intracellular staining is the careful choice and preparation of anti-cytokine antibodies. The BD Pharmingen brand antibodies for intracellular cytokine staining have been carefully selected and stringently tested to provide optimal intracellular staining using the BD Cytofix/Cytoperm method.* Offering a wide range of specificities for a number of species (human, non-human primates, mouse and rat), this reagent range is more broadly applicable.

We offer directly fluorochrome-conjugated antibodies in a wide range of colors to give you more flexibility to design multicolor experiments and allow you to take advantage of the expanded fluorescent detection capabilities of our flow cytometers. Our conjugates include several Alexa Fluor® conjugates, which are generally brighter and more photostable than most other fluorophores. They also go beyond your typical four colors to include APC, Alexa Fluor® 647, Alexa Fluor® 700, PE-Cy7, BD™ APC-H7 and BD Horizon™ V450 conjugates for the most commonly measured cytokines.

For a product listing, please refer to the current companion brochure “Immune Function: Get the Whole Picture”.

*Note: *BD Pharmingen™ brand cytokine antibodies have been optimized using the Cytofix/Cytoperm reagents, whereas BD FastImmune™ antibodies have been developed for optimal performance using BD FACSTM Lysing Solution and BD FACS Permeabilizing Solutions. Mixing fixation, permeabilization, and staining reagents from these two product lines, or with those from other commercial systems, is not advisable.

**BD Cytofix/Cytoperm and Supporting Reagents: A Convenient Means to Consistent Results**

BD Biosciences offers three cell fixation and permeabilization kits to simplify the preparation of cells for intracellular staining of cytokines. Not only are they convenient, they also add an additional factor of standardization to intracellular staining assays: using quality control-tested fixation reagents assures lot-to-lot consistent results.
All three kits enable one-step fixation and permeabilization of cells. The BD Cytofix/Cytoperm Kit provides a fixation and permeabilization solution and an antibody diluent/wash buffer. The BD Cytofix/Cytoperm™ Plus Kits (with either BD GolgiStop™ or BD GolgiPlug™) provide these two solutions plus a protein transport inhibitor for inclusion in cell culture during cell activation. These kits provide sufficient solution for 250 tests for cell staining in tubes and significantly more tests for staining in microwell plates.

The fixation and permeabilization reagents in these kits are also available separately. In addition, we offer several support reagents, such as isotype controls specifically tested for this method, and positive control cells. More details about these support reagents and the detailed protocol can be found in the following section (Protocol), as well as in the Cytofix/Cytoperm Kit Manual, listed at the end of this chapter.

Intracellular Cytokine Staining Starter Kits

In order to make getting started even easier, we offer two Starter Kits based on the BD Cytofix/Cytoperm method, one for mouse, and one for human. The Starter Kits provide the buffers, antibodies, controls and protocols required to successfully activate and stain mouse or human lymphocytes for IL-2, IFN-γ and TNF. More details about these kits can be found in the Instruction Manuals, listed at the end of this chapter.

Customization Options

Do your studies require a unique combination we don’t offer as an off-the-shelf product? We can provide custom conjugations of our antibodies to a wide range of fluorochromes. We can also prepare customized multi-color antibody cocktails for your routine day-to-day work, or for special projects, such as multi-site studies or animal model studies (a minimum order is required).

Please contact your local BD Biosciences representative to discuss the possibilities.

Discover the Endless Possibilities…

When it comes to multicolor capabilities, our new flow cytometry instrumentation with accompanying software provides you with the means to make the most out of the expanding multicolor reagent options.

The BD FACSCanto™ II offers true 6-color detection, and is upgradeable to 8-color. It combines powerful analysis capabilities with ease of use.

The BD™ LSR II offers up to 18-color detection with digital acquisition and analysis.

To help you optimize your productivity and efficiency, BD Biosciences offers a High Throughput Sampler (HTS) option, providing fully automated high speed sample introduction, from either a 96- or 384-well microtiter plate. This option is available for both the BD FACSCanto II and the BD LSR II, and offers you the ultimate combination to get high-content information and increase your throughput.

Just contact your BD Biosciences representative to find out more!
General Methods

Stimulation of Cells

Various in vitro methods (Figure 4) have been reported for generating cytokine-producing cells. Polyclonal activators have been particularly useful for inducing and characterizing high frequencies of cells that produce cytokines (including chemokines) and other immunological effector molecules. These activators include: phorbol esters plus calcium ionophore; concanavalin A, phytohemagglutinin; Staphylococcus enterotoxin β; lipopolysaccharide; and monoclonal antibodies directed against subunits of the TCR/CD3 complex (with or without antibodies directed against costimulatory receptors, such as CD28).

Note: It has been reported that cellular activation with PMA alone causes reduced cell surface CD4 expression by human and mouse T cells. Cell activation with PMA and calcium ionophore together has been reported to cause a greater and more sustained decrease in CD4 expression, and also a decrease in cell surface CD8 expression by mouse thymocytes and by mouse and human peripheral T lymphocytes.

BD Biosciences recommends the use of an intracellular protein transport inhibitor during in vitro cell activation for intracellular cytokine staining. Use of BD GolgiStop™ (Cat. No. 554724; containing monensin) or BD GolgiPlug™ (Cat. No. 555029; containing brefeldin A) will block intracellular transport processes and result in the accumulation of most cytokine proteins in the endoplasmic reticulum/Golgi complex. This leads to an enhanced ability to detect cytokine-producing cells (see Figures 3 and 4). Since monensin and brefeldin A can have a dose- and time-dependent cytotoxic effect, the exposure of cells to these agents must be limited.

Note: Researchers should be aware that protein transport inhibitors can affect the expressed levels of cell surface markers. For example, brefeldin A may cause decreased levels of CD14 staining.
**Figure 4. The effect of various activation conditions and various protein transport inhibitors on intracellular cytokine staining.** Human PBMCs were isolated from heparinized whole blood by density gradient centrifugation (Ficoll-Paque™). The cells were stimulated with either LPS (1 µg/ml, Sigma, Cat. No. L-2654) and a protein transport inhibitor for 4 hrs, LPS and a protein transport inhibitor overnight or they were primed with recombinant human IFN-γ (20 ng/ml, Cat. No. 554617) for 2 hrs and stimulated with LPS in the presence of a protein transport inhibitor overnight. The cells were subsequently fixed and permeabilized using Cytofix and CytoPerm reagents (Cat. No. 554714) and stained with either PE-anti-human IL-6 (A-F) (Cat. No. 554697), PE-anti-human IL-10 (G-L) (Cat. No. 554706) or PE-anti-human IL-12p40/70 (M-R) (Cat. No. 554575). Dot plots were derived from gated events with the forward- and side-light scatter characteristics of monocytes.

1. Cultures for Generating Human Cytokine-Producing Cells

   a. IL-3+, IL-4+, IL-5+, IL-13+ and GM-CSF+ Human Cells: Human PBMCs or purified human CD4+ cells (especially for IL-5+ and IL-13+ cells) are stimulated with immobilized anti-human CD3 antibody (UCHT1 or HIT3a, 10 µg/ml for plate coating, Cat. No. 555329 or Cat. No. 555336 respectively), soluble anti-human CD28 antibody (CD28.2, 2 µg/ml, Cat. No. 555725), recombinant human IL-2 [[Cat. No. 554603, 10 ng/ml]] and recombinant human IL-4 [[Cat. No. 554605, 20 ng/ml]] for 2 days. The cells are washed and subsequently cultured in medium containing rhIL-2 and rhIL-4 for 3 days to promote cellular proliferation and differentiation. Finally, the cells are harvested and restimulated for 4 hr with PMA (Sigma, Cat. No. P–8139; 5 ng/ml) and ionomycin (Sigma, Cat. No. I-0634; 500 ng/ml) in the presence of a protein transport inhibitor.

   Note: Human IL-5 is produced in very low levels. The ability to detect such low levels of IL-5 in human cells with protocols such as the aforementioned procedure can be very challenging and varies among donors.
b. LT-α (TNF-β) Human Cells: Human PBMCs are stimulated with immobilized anti-human CD3 antibody (UCHT1, 10 μg/ml for plate coating, Cat. No. 555329) and recombinant human IL-2 (Cat. No. 554603, 10 ng/ml) for 2 days. The cells are washed and subsequently cultured in medium containing IL-2 for 3 days. Finally, the cells are harvested and restimulated for 6 hr with PMA (Sigma, Cat. No. P-8139; 5 ng/ml) and ionomycin (500 ng/ml; Sigma, Cat. No. I-0634) in the presence of a protein transport inhibitor.

c. IL-2+, TNF+, and IFN-γ Human Cells: Human PBMCs are stimulated for 4 hr with PMA (5 ng/ml; Sigma, Cat. No. P-8139) and ionomycin (500 ng/ml; Sigma, Cat. No. I-0634) in the presence of a protein transport inhibitor.

d. IL-1α, IL-6, IL-8, GRO-α, MCP-1, and MIP-1α Human Cells: Human PBMCs are stimulated for 4 hr with lipopolysaccharide [(LPS); 10 – 1000 ng/ml; Sigma, Cat. No. L-8274] in the presence of a protein transport inhibitor.

e. IL-10 Human Cells: Human PBMCs are stimulated for 24 hr with LPS (1 μg/ml; Sigma, Cat. No. L-8274) in the presence of the protein transport inhibitor.

f. IL-12 p40 and IL-12 p70 Human Cells: Human PBMCs are primed for 2 hr with IFN-γ (10 ng/ml; Cat. No. 554616). They are subsequently stimulated for 18 – 22 hr with IFN-γ (10 ng/ml) and LPS (1 μg/ml; Sigma, Cat. No. L-8274) in the presence of a protein transport inhibitor.

g. RANTES Human Cells: Human PBMCs are cultured for 24 hr in the presence of a protein transport inhibitor.

Note: RANTES is constitutively produced by unstimulated cells, but its intracellular expression is upregulated upon activation.

2. Cultures for Generating Mouse Cytokine-Producing Cells

a. IL-2, TNF, and IFN-γ Mouse Cells: Mouse splenocytes are treated to lyse erythrocytes, washed and then stimulated for 4 – 6 hr with PMA (5 ng/ml; Sigma, Cat. No. P-8139) and ionomycin (500 ng/ml; Sigma, Cat. No. I-0634) in the presence of a protein transport inhibitor.

b. IL-3, IL-4, IL-5, IL-10, GM-CSF Mouse Cells: Purified CD4 mouse splenocytes from BALB/c or C57BL/6 mice are stimulated with immobilized anti-mouse CD3 (145–2C11, 25 μg/ml for plate coating, Cat. No. 553057) and soluble anti-mouse CD28 (37.51, 2 μg/ml, Cat. No. 553294) in the presence of recombinant mouse IL-2 [(10 ng/ml, Cat. No. 550069) and recombinant mouse IL-4 [(50 ng/ml, Cat. No. 550067)] for 2 days. The cells are washed and subsequently cultured in medium containing IL-2 and IL-4 for 3 days to promote cellular proliferation and differentiation. Finally, the cells are harvested and restimulated for 4 – 6 hr with immobilized anti-mouse CD3 (25 μg/ml for plate coating) and anti-mouse CD28 (2 μg/ml) in the presence of a protein transport inhibitor. Alternatively, the cells
are restimulated with PMA (5 ng/ml; Sigma, Cat. No. P-8139) and ionomycin (500 ng/ml; Sigma, Cat. No. I-0634) for 4 – 6 hours in the presence of a protein transport inhibitor.

*Note:* Mouse cells produce very low levels of IL-5. It is very difficult to detect such low levels of mouse IL-5 following the aforementioned procedure.

c. **IL-1α⁺, IL-6⁺, IL-12p40⁺, TNF⁺, and MCP-1⁺ Mouse Cells:** Thioglycollate-elicited peritoneal macrophages from 6-month old BALB/c mice are primed with recombinant mouse IFN-γ (10 ng/ml, Cat. No. 554587) for approximately 2 hr. The cells are subsequently stimulated overnight with LPS (1 μg/ml; Sigma, Cat. No. L-8272) in the presence of a protein transport inhibitor. Finally, the adherent cells are washed with 1× PBS and incubated with 1× trypsin-EDTA solution at 37°C for 15 minutes. The cells are subsequently dislodged by gentle pipetting. Alternatively, the adherent cells can be gently dislodged using a rubber policeman.

d. **MCP-1⁺, IL-6⁺, TNF⁺ Mouse Cells:** Thioglycollate-elicited peritoneal macrophages from 6 month-old BALB/c mice are stimulated overnight with LPS (1 μg/ml; Sigma Cat. No. L-8274) in the presence of a protein transport inhibitor.

3. Cultures for Generating Rat Cytokine-Producing Cells

a. **IL-4⁺, IL-10⁺, GM-CSF⁺, and TNF⁺, IFN-γ⁺ Rat Cells:** Purified splenic CD4⁺ cells from an adult rat are stimulated with immobilized anti-rat CD3 (G4.18, 25 μg/ml for plate coating, Cat. No. 554829) and soluble anti-rat CD28 (JJ319, 2 μg/ml, Cat. No. 554993) in the presence of recombinant rat IL-2 [(10 ng/ml, Cat. No. 555106)] and recombinant rat IL-4[(50 ng/ml, Cat. No. 555107)] for 2 days. The cells are washed and subsequently cultured in medium containing IL-2 and IL-4 for 3 days to promote cellular proliferation and differentiation. Finally, the cells are harvested and restimulated for 4 – 6 hr with PMA (5 ng/ml; Sigma, Cat. No. P-8139) and ionomycin (500 ng/ml; Sigma, Cat. No. I-0634) in the presence of a protein transport inhibitor. Alternatively, the cells are restimulated with immobilized anti-rat CD3 and soluble anti-rat CD28 for 4 – 6 hr in the presence of a protein transport inhibitor.
Figure 5. Frequencies of detectable cytokine-producing cells are comparable when staining activated PBMCs or activated whole blood from the same donor. Ficoll-Hypaque™ purified PBMCs (left panels) and whole blood (right panels) from each of the three donors were activated with PMA (50 ng/ml) and ionophore A23187 (1 μg/ml) for 5 hr in the presence of BD GolgiStop, fixed, permeabilized, and stained with PE-anti-human IL-4 (Cat. No. 554485; 0.06 µg) and FITC-anti-human IFN-γ (Cat. No. 554700; 0.25 µg) according to BD Biosciences intracellular cytokine staining protocols (Standard or Whole Blood Method). Dot plots were derived from gated events with the forward and side light scatter characteristics of lymphocytes.
Viable, activated cell populations can be prepared from in vivo-stimulated tissues or harvested from *in vitro*-stimulated cultures that contain normal cell populations or cell lines. The cells can be suspended and distributed to plastic tubes (BD Falcon™, 12 × 75 polystyrene tubes, Cat. No. 352008) or 96-microwell plates (BD Falcon, polystyrene assay plates, Cat. No. 353910) activated with protein transport inhibitors and stained for immunofluorescent staining. Cells should be protected from light throughout staining and storage prior to flow cytometric analysis.

**Block Immunoglobulin Fc Receptors**

Reagents that block Immunoglobulin (Ig) Fc receptors may be useful for reducing nonspecific immunofluorescent staining.26

1. In the mouse and rat systems, purified 2.4G2 and D34–485 antibodies directed against FcγII/III (mouse BD Fc Block™; Cat. No. 553142 and 553141) and Fcγ receptors (rat BD Fc Block CD32 Cat. No. 550271 and 550270) respectively, can be used to block nonspecific staining caused by fluorescent antibodies that bind to Ig Fc receptors. To block mouse Ig Fc receptors with BD Fc Block, preincubate cell suspension with 1 μg BD Fc Block/10^6 cells in 100 μl of staining buffer for 15 min at 4°C.
The cells are then washed and stained with a fluorescent antibody that is specific for a cell surface antigen of interest.

2. Ig Fc receptors on human cells can be pre-blocked by incubating cells with an excess of irrelevant, purified polyclonal Ig (1 – 10 μg/10⁶ cells) from the same species and containing the same Ig isotype as the antibodies used for immunofluorescent staining. Alternatively one can use 10% normal human serum or polyclonal human IgG (Sigma Cat. No. I-4506) in PBS for 20 minutes at 4°C to block Ig Fc receptors.

Stain Cell Surface Antigens (also see Chapter 1)

1. Incubate ~10⁶ cells in 100 μl of staining buffer* with a pretitrated optimal concentration (≤ 1.0 μg) of a fluorescent monoclonal antibody specific for a cell surface antigen, such as CD3, CD4, CD8, CD14, or CD19 (15 – 30 min, 4°C). Multicolor immunofluorescent staining of different cell surface antigens can be carried out to provide controls for setting proper compensation of the brightest fluorescent signals.

   Note: Some antibodies that recognize native cell surface markers may not bind to fixed/denatured antigens. For this reason, it is recommended that the staining of cell surface antigens be done with live, unfixed cells PRIOR to fixation/permeabilization and staining of intracellular antigens. Altering the procedure such that cells are fixed prior to staining of cell surface antigens requires that suitable antibody clones be empirically identified.

2. Wash cells 2x with staining buffer* (1 ml/wash for staining in tubes), pellet by centrifugation (250 × g), and remove supernatant.

Fix and Permeabilize Cells

1. Thoroughly resuspend cells in 100 μl of BD Cytofix/Cytoperm™ Solution* for 10 – 20 min at 4°C.

   Note: Cell aggregation can be avoided by vortexing prior to the addition of the BD Cytofix/Cytoperm Solution.

2. Wash cells two times in 1x BD Perm/Wash Buffer* (1 ml/wash for staining in tubes), pellet, and remove supernatant.

   Note: BD Perm/Wash Buffer is required in washing steps to maintain cells in a permeabilized state.

Alternative Fixation and Permeabilization Protocol

Cells can be fixed and stored to continue the intracellular staining at a later time.

1. Fixation and Storage of Cells.
   a. Resuspend cells in 100 μl (or 1 ml/10⁷ cells for bulk fixing) of BD Cytofix™ Buffer at 4°C for 10 – 20 min.
   b. Wash cells 2x in staining buffer.
   c. Resuspend cells in staining buffer for storing cells at 4°C for up to 30 days or in 90% FCS + 10% dimethyl sulfoxide (DMSO) for storing at –80°C.

* a list of buffers and solutions can be found on p. 103

Unless otherwise specified, all products are for Research Use Only. Not for use in diagnostic or therapeutic procedures. Not for resale.
2. Permeabilizing Fixed Cells
   a. For frozen cells, after thawing, gently wash 2x in BD Perm/Wash Buffer to remove DMSO.
   b. For all cells resuspend in BD Perm/Wash Buffer for 15 min.
   c. Pellet by centrifugation.
   d. Stain for intracellular cytokines.

Stain for Intracellular Cytokines

1. Thoroughly resuspend fixed/permeabilized cells in 50 μl of BD Perm/Wash Buffer (100 μl for staining in tubes) containing a pre-determined optimal concentration of a fluorescent anti-cytokine antibody or appropriate negative control. Incubate at 4°C for 15 – 30 min in the dark.

2. Wash cells 2 times with 1× BD Perm/Wash Buffer (1 ml/wash for staining in tubes) and resuspend in staining buffer* prior to flow cytometric analysis.

Alternative Protocol – Activation and Intracellular Staining of Whole Blood Cells

1. Aliquot 1 ml of whole blood into sterile 15 ml conical tubes.
2. Add cell activator or mitogen to blood [eg, 2 μg of anti-CD28 (CD28.2, Cat. No. 555725), 2 μg of anti-CD49b (AK-7) and 1 – 3 μg of Staphylococcus enterotoxin β (Sigma, Cat. No. S-4881)] and incubate for 6 hr in the presence of BD GolgiPlug (Cat. No. 555029). In cases where longer incubations with either the cell activator or mitogen is desired, all reagent concentrations should be doubled except for brefeldin A.

   Note: Prolonged incubation of the cells with brefeldin A (>16 hr) can adversely affect cell viability.

3. Vortex briefly to mix. Incubate for 4 – 6 hr in 5% CO₂ at 37°C.
4. Add 100 μl of ice-cold 20 mM EDTA, vortex, and incubate for 10 min at room temperature (RT).
5. Add 10 ml of BD PharmLyse™ buffer (Cat. No. 555899), vortex, incubate for 10 min at RT in the dark.
6. Spin 5 min at 500 × g.
7. Aspirate supernatant. Wash 1× in staining buffer.* Spin 5 min at 500 × g. Aspirate supernatant.
8. Continue with staining for cell surface molecules and intracellular cytokines following the previous protocol.
**Figure 6. Comparison of the effects of BD GolgiPlug™ and BD GolgiStop™ on intracellular cytokine accumulation by restimulated purified mouse CD4+ cells.** Activated mouse CD4+ cells were restimulated with PMA (10 ng/ml) + ionomycin (250 ng/ml) for 5 hr in the presence of BD GolgiPlug or BD GolgiStop and were stained for the intracellular cytokines listed. In this case, BD GolgiPlug was more effective in allowing cells to accumulate TNF whereas BD GolgiStop was more effective in permitting the accumulation of IL-4 and IL-10. Both protein transport inhibitors allowed for similar accumulations of detectable intracellular IFN-\(\gamma\).

**Flow Cytometric Analysis**

Set PMT voltage and compensation using cell surface staining controls. Set quadrant markers based on blocking controls, Ig isotype controls, or unstained cells. See Chapter 1 for additional information.

The frequencies of cytokine-producing cells present in activated human PBMC cultures can vary widely due to donor variability. Therefore, cryopreserved cells from a single donor are useful for longitudinal studies.\(^6,21\)

For proper flow cytometric analysis, cells stained by this method should be inspected by light microscopy and/or by analysis of flow cytometric light scatter patterns to confirm that they are well dispersed. In order to make statistically-significant population frequency measurements, sufficiently large sample sizes should be acquired during flow cytometric analysis.\(^28\) Bivariate dot plots or probability contour plots can be generated upon data reanalysis to display the frequencies of and patterns by which individual cells coexpress certain levels of cell surface antigen and intracellular cytokine proteins.\(^28\)

**Staining Controls**

**Positive Staining Controls**

As described in the General Methods, in stimulation of cells and also in our Technical Data Sheets (TDS) for BD Pharmingen™ fluorescent anti-cytokine antibodies, *in vitro* culture systems can induce detectable frequencies of cytokine-
producing cells at specific time-points. Cells stimulated by these methods can be used as positive controls for experimental systems. Published reports of immunofluorescent staining and analysis can also provide useful information regarding different experimental protocols for generating cells that express a particular type and level of cytokine (or other intracellular molecules).18, 19

1. Positive control cells

To serve as positive controls for intracellular cytokine staining, BD Biosciences offers sets of activated and fixed Mouse and Human cell populations that have been screened and found to contain cells that express detectable levels of certain intracellular cytokines (aka, MiCK and HiCK Cells, respectively).

<table>
<thead>
<tr>
<th>Cell Set</th>
<th>Cytokines Measured</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mouse</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MiCK-1</td>
<td>IL-2, TNF, IFN-γ</td>
<td>554652</td>
</tr>
<tr>
<td>MiCK-2</td>
<td>IL-3, IL-4, IL-10, GM-CSF</td>
<td>554653</td>
</tr>
<tr>
<td>MiCK-3</td>
<td>IL-6, IL-12p40, TNF, MCP-1</td>
<td>554654</td>
</tr>
<tr>
<td><strong>Human</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HiCK-1</td>
<td>IL-2, TNF, IFN-γ</td>
<td>555061</td>
</tr>
<tr>
<td>HiCK-2</td>
<td>IL-3, IL-4, IL-10, IL-13, GM-CSF</td>
<td>555062</td>
</tr>
<tr>
<td>HiCK-3</td>
<td>IL-1α, IL-1β, IL-6, IL-12, TNF</td>
<td>555063</td>
</tr>
</tbody>
</table>

Negative Staining Controls

One or more of the following three controls can be used to discriminate specific staining from nonspecific staining. Researchers should choose which staining controls best meet their research needs. Intracellular cytokine staining techniques and the use of blocking controls are described in detail by C. Prussin and D. Metcalf.5

1. **Ig Isotype Control:** Stain with an Ig isotype-matched control of irrelevant specificity. Refer to list of isotype controls specifically for intracellular staining in the product listing.

   a. Resuspend cell pellet in 50 μl of BD Perm/Wash Buffer (100 μl for staining in tubes) containing the Ig isotype control antibody at the same concentration as for the anti-cytokine antibody (typically < 0.5 μg/10⁶ cells).

   b. Incubate 15 – 30 min at 4°C.

   c. Wash cells using the aforementioned procedure for intracellular staining.
2. **Ligand Blocking Control**: Pre-block anti-cytokine antibody with cognate recombinant cytokine protein (eg, 0.25 μg/test).
   
a. Preincubate fluorescent antibodies with appropriately-diluted recombinant cytokine protein in a volume ≥ 50 μl (100 μl for staining in tubes) of BD Perm/Wash Solution at 4°C for 15 – 20 min.
   
b. Resuspend fixed/permeabilized cells in 50 μl (100 μl for staining in tubes) of pre-blocked fluorescent anti-cytokine antibody (in BD Perm/Wash Solution) and incubate 15 – 20 min at 4°C.
   
c. Wash cells using the aforementioned procedure for intracellular staining.

3. **Unconjugated antibody control**: Preincubate cells with unconjugated antibody.
   
a. Resuspend fixed/permeabilized cells in 25 μl BD Perm/Wash Solution (50 μl for staining in tubes) containing purified, unconjugated anti-cytokine antibody (same clone as conjugated antibody) diluted to the appropriate concentration (> 5 μg/10^6 cells), and incubate 15 – 20 min at 4°C.
   
b. After incubation, add fluorescent anti-cytokine antibody at an optimal concentration in 25 μl BD Perm/Wash Buffer (50 μl for staining in tubes) for a final volume of 50 μl for staining in microwell plates or 100 μl for staining in tubes, and incubate 15 – 20 min at 4°C.
   
c. Wash cells using the aforementioned procedure for intracellular staining.

*Buffers and Solutions for Staining Intracellular Molecules*

**Staining Buffers**

1. Staining Buffer Recipe
   - Dulbecco’s PBS (DPBS)
   - 3% heat-inactivated FCS
   - 0.09% (w/v) sodium azide
   - Adjust buffer pH to 7.2 – 7.4, filter (0.2 μm pore membrane), and store at 4°C.

2. BD Biosciences Staining Buffers
   BD Biosciences offers two buffers, BD Pharningen™Stain Buffer (FBS) (Cat. No. 554656) and BD Pharningen™Stain Buffer (BSA) (Cat. No. 554657), that are rigorously pretested for their ability to optimize immuno-fluorescent staining and maintain cell viability.
Fixation and Permeabilization Reagents

BD Biosciences offers three cell fixation and permeabilization kits to simplify the preparation of cells for intracellular staining of cytokines. All three kits enable one-step fixation and permeabilization of cells. The BD Cytofix/Cytoperm™ Kit provides a fixation and permeabilization solution and an antibody diluent/wash buffer. The BD Cytofix/Cytoperm™ Plus Kits (with BD GolgiStop™ or BD GolgiPlug™) provide these two solutions plus a protein transport inhibitor for inclusion in cell culture during cell activation. These kits provide sufficient solution for ≥ 250 tests for cell staining in tubes and significantly more tests for staining in microwell plates.

1. BD Cytofix/Cytoperm Kit (Cat. No. 554714)

   This kit enables the one-step fixation and permeabilization of cells that is necessary prior to the staining of intracellular cytokines with fluorescent anti-cytokine antibodies. This kit provides two reagents: BD Cytofix/Cytoperm Solution and BD Perm/Wash Buffer. After the cells are fixed and permeabilized with the BD Cytofix/Cytoperm Solution, the BD Perm/Wash Buffer is used to wash the cells and to dilute the anti-cytokine antibodies for staining. It is important that the BD Perm/Wash Buffer be used for dilution of anti-cytokine antibodies, rather than a standard staining buffer, in order to maintain cells in a permeabilized state for intracellular staining.

   Kit components:
   - BD Cytofix/Cytoperm Solution
   - BD Perm/Wash Buffer
   - Detailed protocol with sample data

2. BD Cytofix/Cytoperm Plus (with BD GolgiStop) (Cat. No. 554715)

   In addition to the fixation/permeabilization and diluent/wash solutions included in the BD Cytofix/Cytoperm Kit, the BD Cytofix/Cytoperm Plus Kit provides BD GolgiStop, containing monensin, a protein transport inhibitor. Addition of BD GolgiStop to cell activation cultures blocks intracellular transport processes, thereby resulting in the accumulation of most cytokine proteins in the Golgi complex and enhancing cytokine staining signals. Sufficient BD GolgiStop reagent is provided for treating ≥ 1 liter of cultured cells.

   Kit components:
   - BD Cytofix/Cytoperm Solution
   - BD Perm/Wash Buffer
   - Detailed protocol with sample data
   - BD GolgiStop
Note: Because differential effects comparing monensin and brefeldin A have been observed for the detection of certain cytokines by intracellular cytokine staining (Figure 6), it is recommended that the researcher test both protein transport inhibitors in their experimental system to determine which one is optimal. Each inhibitor is also sold separately.

3. BD Cytofix/Cytoperm Plus (with BD GolgiPlug) (Cat. No. 555028)

In addition to the fixation/permeabilization and diluent/wash solutions included in the BD Cytofix/Cytoperm Kit, the BD Cytofix/Cytoperm Plus Kit provides BD GolgiPlug, containing brefeldin A, a protein transport inhibitor. Addition of BD GolgiPlug to cell activation cultures will block intracellular transport processes, thereby resulting in the accumulation of most cytokine proteins in the endoplasmic reticulum and enhancing cytokine staining signals. Sufficient BD GolgiPlug reagent is provided for treating ≥ 1 liter of cultured cells.

Kit components:
- BD Cytofix/Cytoperm Solution
- BD Perm/Wash Buffer
- Detailed protocol with sample data
- BD GolgiPlug

Note: Because differential effects comparing monensin and brefeldin A have been observed for the detection of certain cytokines by intracellular cytokine staining (Figure 6), it is recommended that the researcher test both transport inhibitors in their experimental system to determine which one is optimal. Each inhibitor is also sold separately.
References


15. BD Biosciences Application Note: Cytokine Detection in Antigen-Activated CD8+and CD4+ T cells. Available as a download at www.bd biosciences.com/immunocytometry_systems_/new_products/23_5195_00.pdf


**Relevant BD Biosciences Literature and Links**


**Application Notes**

1. BD Biosciences Non-Human Primate CFC Protocol [ask BD Scientific Support]

**Instruction Manuals**

1. BD Cytofix/Cytoperm™ Kit Manual [Literature Part Nr.: 00-81014-4-C]
2. BD Pharmingen™ Intracellular Cytokine Staining Starter Kit Manual - Human [Literature Part Nr.: 00-6088-559302-Ar]
3. BD Pharmingen™ Intracellular Cytokine Staining Starter Kit Manual - Mouse [Literature Part Nr.: 00-6088-559311-B]

The abovementioned product literature is available on request from your local BD Biosciences representative, or as a download at [www.bdbiosciences.com/literature](http://www.bdbiosciences.com/literature) [go to Literature Library]

For additional resources containing practical tips about multicolor flow cytometry in general, please refer to the Resources section at the end of Chapter 1, or visit [www.bdbiosciences.com/colors](http://www.bdbiosciences.com/colors).

**Additional Technical Resources**

Our Scientific Support Team is trained to help you with technical questions related to intracellular immunostaining and flow cytometry. Just contact them at the numbers listed at the end of this manual, or e-mail: help.biosciences@europe.bd.com.
Intracellular Cytokine Detection by Flow Cytometry: BD FastImmune™ System

Introduction

The quantitative and qualitative measurement of antigen-specific T cells is being used in research studies to assess the importance of the monitoring of immune status during disease and in assessing vaccine efficacy. Various methods have been developed to identify antigen-specific T-cell responses. Traditional assays have analyzed bulk populations of T cells for proliferation (by $^3$H-thymidine incorporation) or for cytotoxicity (by $^{51}$Cr release assays). These methods tend to be long and labor-intensive, and their results usually cannot be compared quantitatively. Recently, single-cell assays of antigen-specific T cells have come into use, including MHC-peptide tetramer staining,1,2 enzyme-linked immunospot (ELISPOT) assays,3,4 and intracellular cytokine assays.5-7 Each of these assays can provide truly quantitative readouts since they enumerate antigen-specific cells without lengthy in vitro restimulation, which would allow time for proliferation or apoptosis or both. Of the three assays, ELISPOT and intracellular cytokine assays measure a functional readout (cytokine production) as opposed to tetramers, which measure antigen specificity without regard to function. Since some disease states can evoke populations of anergic (non-functional) T cells,8,9 the use of tetrarams in combination with a functional assay might be warranted. Also, tetramers can only identify T cells with single peptide/MHC specificities, while cytokine assays can determine the sum total of T-cell responses to a particular protein or pathogen.

A major advantage of intracellular cytokine staining over ELISPOT is the ability to analyze multiple parameters per cell. Thus, it is possible to analyze CD4 and CD8 responses in the same sample, or to assess expression of other phenotypic markers on the cells of interest. In addition to potentially providing more information, there is greater assurance that the events being identified as cytokine-positive cells are indeed the cells of interest since they can be stained simultaneously with CD4 or CD8, for example, and an independent activation marker, such as CD69. Also, the intracellular cytokine assay can be performed in whole blood without the need for separation of peripheral blood mononuclear cells (PBMCs), or CD4 or CD8 cells, and with stimulation periods as short as 6 hours. Several recent procedural developments have also contributed to the convenience of intracellular cytokine assays. These include the ability to interrupt the assays with the use of timed cooling6 and the ability to batch samples via freezing of activated cells.5,6

Intracellular cytokine staining has been made possible by the advent of high-affinity anti-cytokine antibodies, optimized cell fixation and permeabilization protocols, and the use of secretion inhibitors such as Brefeldin A (BFA). This technique allows the detection of functional populations of memory T cells that respond to specific soluble antigens in short term restimulation assays.5,6,9-18
BD FastImmune™ Intracellular Cytokine Detection System

One of two reagent systems offered by BD Biosciences for intracellular cytokine detection, the BD FastImmune System is trimmed to the needs of applied research with human samples. It has been developed for use with human whole blood, thus allowing minimal sample manipulation and re-stimulation in close to in vivo conditions. The streamlined protocol involves a hands-on time of just two hours and gives you results in as few as eight hours.

- Developed for human whole blood or PBMCs
- Streamlined protocol to reduce time and sample manipulation
  - Get results in hours, not days
- Highly reproducible detection of rare events such as antigen-specific activation

Designed to Meet the Needs of Analyzing Antigen-Specific Responses

Identifying antigen-specific responses in these assays requires a very clean background, so that very low frequency events (0.1% or less) can still be read as positive. Designed in particular for analysis of antigen-specific activation in whole blood (see the related Application Note: Detecting Cytokines in Antigen-Activated Lymphocytes), the BD FastImmune Cytokine System provides a very clean background, making it possible to detect very low frequency events in a reproducible manner.

A number of different antigens have been tested in the development of the BD FastImmune Cytokine System, including viral lysates, recombinant viral proteins, and peptides. In principle, this technique can be applied to other antigens as well. However, the optimal antigen titer will need to be determined. Also, the expected frequency of responding T cells in the blood of immune individuals will vary with different antigens.

When using CFC as an immune monitoring platform in vaccine clinical trials, standardization is critical to allow comparison of data generated across multiple clinical sites. To help standardize CFC assays among HIV immune monitoring laboratories, a Quality Assurance Program (QAP) has been developed as a collaborative effort between BD Biosciences, the NIH/NIAID/Division of AIDS, and SeraCare BioServices, with the goal of contributing to the generation of reliable and accurate CFC data. Please refer to the article in BD Biosciences HotLines (see p. 129) for more information.

Procedural Overview

Antigen-specific activation can be done in a variety of tissues and environments. This simple method uses whole blood and provides an environment as similar as possible to that existing in vivo. PBMCs can also be used with minor modifications to the following procedure.16,18

Whole blood is stimulated with antigen and costimulatory antibodies (CD28 and CD49d) in the presence of the secretion inhibitor BFA. The inhibitor allows for intracellular accumulation of newly synthesized protein (cytokines) during sample incubation at 37ºC. After a stimulation period of 6 hours, EDTA is added to the sample in order to arrest activation and to remove adherent cells from the
activation vessel. This step is followed by the simultaneous lysis of erythrocytes and fixation of leucocytes using BD FACSTM Lysing Solution. Cells are then washed and permeabilized with BD FACS Permeabilizing Solution 2. After an additional wash, surface and intracellular staining antibodies are added in a single staining step. Finally, the cells are washed and fixed for flow cytometric analysis (Figure 1).

Several recent procedural developments have contributed to the convenience of the BD FastImmune methodology, so that it meets the needs of vaccine monitoring in clinical trials. These include the ability to interrupt the assays with the use of timed cooling and the ability to batch samples via freezing of activated cells.

The method uses a three-color staining system to identify CD4 T-cell responses (anti-cytokine FITC, CD69 PE, CD4 PerCP-Cy5.5) and a four-color staining system to identify CD8 T-cell responses (Anti-cytokine FITC, CD69 PE, CD8 PerCP-Cy5.5, CD3 APC). The most prevalent cytokine responses (to antigens that BD Biosciences has tested) include IFN-γ, IL-2, and TNF-α for CD4 T cells and IFN-γ for CD8 T cells. CD69 is an early activation antigen whose expression is induced during in vitro–antigen stimulation. The CD69 antibody is used to allow better clustering of cytokine-positive cells, and to ensure that cells defined as antigen-responsive have been stimulated to express this activation marker. The CD4 antibody is used to set an acquisition gate so that only CD4+ lymphocytes are collected for analysis. If class I–restricted peptides are used as the stimulating antigen, CD8 PerCP-Cy5.5 and CD3 APC serve to set the acquisition gate. The BD FastImmune™ CD8 Anti-Hu–IFN-γ Detection Kit includes CD3 APC to avoid misidentification of NK cell responses (CD8 dim) upon antigenic stimulus.

![Figure 1. Schematic of whole blood BD FastImmune antigen-specific assay](image)

Part A From blood draw to sample activation to flow cytometric sample processing; Part B Staining and processing of samples for flow cytometric analysis, applies to tubes 1 to 4 from Part A.
BD FastImmune™ Cytokine Detection System Products

BD FastImmune Multicolor Cytokine Reagents: Optimized Combinations

With multicolor reagent combinations tailored to measure the most prevalent cytokine responses in either CD4+ T cells (IFN-γ, IL-2, TNF) or CD8+ T cells (IFN-γ), the BD FastImmune three- and four-color cytokine reagents can be used alone or in conjunction with the BD FastImmune intracellular cytokine detection kits.* These reagents have been optimized to work together, so besides getting excellent assay performance, there is no need for tedious titrations or additional pipetting steps during your assay.

In addition to FITC-conjugated antibody to the cytokine, the 3- and 4-color reagents also include antibody to either CD4 or CD8 to set the acquisition gate, and PE-anti-CD69, to focus on the cells expressing this early activation antigen. Anti-CD4 and CD8 are conjugated to PerCP-Cy5.5 for better separation of CD4dim and CD8dim T cells from the negative cell population. The 4-color formats also include CD3 staining for optimal gating on T lymphocytes (e.g., discriminating from CD8dim NK cells).

For a product listing, please go to www.bdbiosciences.com/fastimmune

BD FastImmune Individual Reagents: For Added Flexibility of Assay Design

For added flexibility, all sample processing components and multicolor reagents found in the kits are also available individually. Additional single-color anti-cytokine reagents are also available for use in the BD FastImmune System, allowing you to extend your intracellular cytokine testing beyond the standard combinations.

Like the antibodies included in the multicolor reagents and kits, the single-color BD FastImmune antibodies are produced to high quality control standards using optimal fluorochrome-to-protein ratios, to perform consistently well in intracellular staining with minimal background.*

For a product listing, please go to www.bdbiosciences.com/fastimmune

*Note: BD FastImmune antibodies have been developed for optimal performance using BD FACS Lysing Solution and FACS Permeabilizing Solution 2, whereas BD Pharmingen™ brand cytokine antibodies have been optimized using the Cytotix/Cytoperm reagents. Mixing fixation, permeabilization, and staining reagents from these two product lines, or with those from other commercial systems, is not advisable.

Ready-to-Use BD FastImmune Cytokine Detection Kits: The Easiest Way to Get Started

BD FastImmune Cytokine Detection Kits make getting started easier. Each kit includes an optimized multicolor antibody reagent, matching isotype control, and necessary processing reagents for optimal and highly reproducible results.
Kits contain:

- BD FastImmune Anti-Human Multicolor Antibody reagent‡
  CD8 4-color Kit: cytokine FITC/CD69 PE/CD8 PerCP-Cy.5.5/CD3 APC, or
  CD4 4-color Kits: cytokine FITC/CD69 PE/CD4 PerCP-Cy5.5/CD3 APC, or
  CD4 3-color Kits: cytokine FITC/CD69 PE/CD4 PerCP-Cy5.5
- BD FastImmune matching multicolor isotype control
- Sample activation solutions:
  BD FastImmune Brefeldin A
  BD FastImmune CD28/CD49d Costimulatory Reagent
- Post-stimulation sample processing solutions
  BD FastImmune EDTA Solution
  BD FACS™ Lysing Solution
  BD FACS Permeabilizing Solution 2

Each kit provides sufficient reagents to stain 25 stimulated (specific and isotype control tube) and 25 unstimulated (specific and isotype control tube) human whole blood samples.

For more detailed information please refer to the individual Technical Data Sheets, available at www.bdbiosciences.com/fastimmune

Materials

Sample Type

Heparinized whole blood. Other anti-coagulants are not compatible with the procedure.

Antibodies and Kit Contents

Our method uses BD FastImmune CD8 and CD4 Cytokine Detection Kits. These kits contain cytokine-specific, multicolor antibody reagents, a matching multicolor isotype control, and sample processing reagents to measure antigen-specific T-cell responses. Generic or specific antigens for sample activation are not provided with the kits. Table 1 outlines the antigens that have been used in this assay by the BD Biosciences Research Department.

Our system is optimized to guarantee a streamlined, easy-to-adopt procedure while providing highly reproducible functional responses in hours.

We also offer all kit components individually to allow for more flexibility in assay design. Please contact your local BD Biosciences representative to obtain a list of these products.
**Table 1. Antigens in this assay**

<table>
<thead>
<tr>
<th>Activation Agent</th>
<th>Source</th>
<th>Stock Solution</th>
<th>Use in Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEB positive control</td>
<td>Sigma Catalog No. S4881 (1 mg)</td>
<td>Add 2 mL of sterile PBS directly to a 1-mg vial of SEB. Cap the vial and shake to dissolve all the powder. Remove the solution and dilute up to 20 mL with PBS to make a stock solution of 50 µg/mL. Store this stock solution at 4°C.</td>
<td>Use 20 µL of stock solution for stimulation of 1 mL blood at a final concentration of 1 µg/mL.</td>
</tr>
<tr>
<td>CMV Lysate</td>
<td>Advanced Biotechnologies (ABI) Catalog No. 10-144-000 (1 mg)</td>
<td>The material is diluted to a final concentration of 1 mg/20 mL (50 µg/mL) in sterile PBS, calculating from the protein concentration given in the product insert. Aliquots of 20 µL each are frozen at –80°C. NOTE: Different lots of this product might need to be titrated for optimal concentrations.</td>
<td>Use 20 µL of stock solution for stimulation of 1 mL blood at a final concentration of 1 µg/mL.</td>
</tr>
<tr>
<td>CMV pp65 protein</td>
<td>Austral Biotechnologies Catalog No. CMA-1420-4 (50 µg)</td>
<td>Fifty micrograms (50 µg) is diluted to a total of 2 mL in sterile PBS (final concentration 25 µg/mL). Aliquots of 20 µL each are frozen at –80°C.</td>
<td>Use 20 µL of stock solution for stimulation of 1 mL blood at a final concentration of 0.5 µg/mL.</td>
</tr>
<tr>
<td>Peptides</td>
<td></td>
<td>Most peptides can be dissolved in DMSO at a concentration of 2 mg/mL. Aliquots of 5 µL each are frozen at –80°C.</td>
<td>Use 5 µL of stock solution for stimulation of 1 mL blood at a final concentration of 10 µg/mL.</td>
</tr>
</tbody>
</table>

**BD FastImmune CD8 cytokine four-color kit:**

Anti-Hu–IFN-γ Kit (BD Cat. No. 346049‡)
- Anti-Hu–IFN-γ FITC/CD69 PE/CD8 PerCP-Cy5.5/CD3 APC
- IgG2a FITC/IgG1 PE/CD8 PerCP-Cy5.5/CD3 APC

**Activation and Processing Solutions**

**BD FastImmune CD4 cytokine three-color kits:**

Anti-Hu–IFN-γ Kit (BD Cat. No. 340970‡)
- Anti-Hu–IFN-γ FITC/CD69 PE/CD4 PerCP-Cy5.5
- IgG2a FITC/IgG1 PE/CD4 PerCP-Cy5.5

**Activation and Processing Solutions**

**BD FastImmune CD4 cytokine three-color kits:**

Anti-Hu–IL-2 Kit (BD Cat. No. 340971‡)
- Anti-Hu–IL-2 FITC/CD69 PE/CD4 PerCP-Cy5.5
- IgG2a FITC/IgG1 PE/CD4 PerCP-Cy5.5

**Activation and processing solutions**

Anti-Hu–TNF-α Kit (BD Cat. No. 340972‡)
- Anti-Hu–TNF-α FITC/CD69 PE/CD4 PerCP-Cy5.5
- IgG2a FITC/IgG1 PE/CD4 PerCP-Cy5.5

**Activation and processing solutions**

Unless otherwise specified, all products are for Research Use Only. Not for use in diagnostic or therapeutic procedures. Not for resale.
Activation and processing solutions (in both CD4 and CD8 kits):

Used for sample activation
- BD FastImmune CD28/CD49d costimulatory reagent
- BD FastImmune Brefeldin A (BFA) Solution

Used for sample processing post stimulation
- BD FastImmune EDTA Solution
- BD FACS Lysing Solution (10×)
- BD FACS Permeabilizing Solution 2 (10×)

Kit Working Solutions
- BD FastImmune Brefeldin A (BFA) Solution
  Upon receipt, thaw BFA, dispense into 10-μL aliquots, and store at –20°C.
- BD FACS Lysing Solution
  Dilute 10× stock to 1× with deionized (DI) water.
  Store and use 1× solution at room temperature.
- BD FACS Permeabilizing Solution 2
  Dilute 10× stock to 1× with deionized water.
  Store and use 1× solution at room temperature.

Warning: BD FACS Lysing Solution (10×) and BD FACS Permeabilizing Solution 2 (10×) each contain diethylene glycol and formaldehyde. Formaldehyde is harmful by inhalation, in contact with skin, and if swallowed (R20/21/22). It is irritating to eyes and skin (R36/38). Exposure can cause cancer. Possible risk of irreversible effects (R40). Can cause sensitization by skin contact (R43). Keep locked up and out of the reach of children (S1/2). Keep away from food, drink, and animal feedingstuff (S13). Wear suitable protective clothing and gloves (S36/37). Even small amounts of diethylene glycol can be fatal. If swallowed, seek medical advice immediately and show this container or label (S46). Dispose of according to federal, state, and local regulations.

Instrument and Instrument Set Up
- BD FACS brand flowcytometer
  The BD FastImmune CD8 Kit requires a dual-laser instrument with excitation at 488 nm and 635 nm. Refer to the appropriate instrument user’s guide for information.
- BD Calibrite™ beads (BD Cat. No. 349502; unlabeled, FITC, and PE beads); BD Calibrite PerCP-Cy5.5–labeled beads (BD Cat. No. 345036; beads plus Bead Dilution Buffer); BD Calibrite APC beads (BD Cat. No. 340487, to support the BD FastImmune CD8 Kit only). Refer to the BD Calibrite beads product inserts for instructions.
• Software
  BD FACSComp™ software, version 4.2, for instrument setup and
  BD CellQuest™ Pro or BD CellQuest™ software for acquisition and analysis.
  In addition, BD Paint-A-Gate Pro™ can be used for data analysis.5
  Refer to the appropriate software user’s guide for detailed information.

Additional Materials Required
• wash buffer: 0.5% bovine serum albumin (BSA) and 0.1% NaN₃
  in 1× PBS (Store at 4°C)
• 1% paraformaldehyde in 1× PBS (Store at 4°C)
  Refer to the paraformaldehyde product insert for warnings.
• 15-mL polypropylene tubes
  (BD Cat. No. 352096)
• 5-mL polystyrene tubes (BD Cat. No. 352058)
• micropipettor with tips (BD Electronic Pipette, BD Cat. No. 343246
  or equivalent)
• vortex mixer
• 37°C water bath or incubator
• centrifuge

Procedures
BD FastImmune CD8 Kit (Anti–IFN-γ)–peptide, peptide mixes
1. Remove an aliquot of BFA from the freezer and dilute 1:10 with sterile
  PBS.
2. Activated sample:
  Add 0.5 mL of heparinized whole blood, 5 μL of CD28/CD49d
  monoclonal antibody cocktail, 10 μL of diluted BFA stock, and antigen at
  titer (or other activation agent) to a 15-mL polypropylene tube.

Unstimulated (resting) sample:
  Add 0.5 mL of heparinized whole blood, 5 μL of CD28/CD49d
  monoclonal antibody cocktail, 10 μL of diluted BFA stock in the absence
  of antigen to a 15-mL polypropylene tube.
Vortex each tube gently and incubate 6 hours at 37°C.
Note: The 15-mL conical bottom polypropylene tube is superior to
  most other stimulation vessels that we have tested.

3. Add 50 μL of EDTA solution to each tube. Vortex vigorously and incubate
  15 minutes at room temperature. Vortex again on high setting for
  10 seconds.

4. If cells are to be stained fresh, proceed with step 4a; if cells are to be
  frozen for later staining, proceed with step 4b.
4a
- Label four 5-mL polystyrene tubes accordingly.
  Tube 1: Activated Isotype Control (AIC)
  Tube 2: Unstimulated Isotype Control (UIC)
  Tube 3: Activated Sample (AS)
  Tube 4: Unstimulated Sample (US)
- Aliquot 100 µL each of activated blood into the AIC tube and the AS tube.
- Aliquot 100 µL each of unstimulated blood into the UIC tube and the US tube.
- Proceed to step 5.

4b
- Add 5 mL of 1× BD FACS Lysing Solution (dilute 10× solution 1:10 with DI water before use) to each activated and unstimulated 0.5 mL whole blood sample.
- Vortex and incubate for 10 minutes at room temperature, and directly place the tubes in a freezer at –80°C.
- At the time of staining, thaw cells briefly in a 37°C water bath, add 7 mL of wash buffer, and centrifuge at 500 × g for 10 minutes at room temperature.
- Decant the supernatant, and resuspend the pellet in 0.5 mL of wash buffer. When ready to stain:
- Label four 5-mL polystyrene tubes and aliquot 100 µL of blood as described for activated and unstimulated fresh samples; see step 4a, Tubes 1 – 4.
- Proceed to step 7.

5. Add 1 mL of 1× BD FACS Lysing Solution (dilute 10× solution 1:10 with DI water before use) to each tube, mix gently, and incubate for 10 minutes at room temperature.

6. Add 2 mL of wash buffer to each tube, and centrifuge at 500 × g for 5 minutes at room temperature. Decant the supernatant.

7. Add 0.5 mL of 1× BD FACS Permeabilizing Solution 2 (dilute 10× solution 1:10 with DI water before use) to each tube. Vortex to resuspend the pellet. Incubate for 10 minutes at room temperature.

8. Add 2 mL of wash buffer to each tube, and centrifuge at 500 × g for 5 minutes at room temperature.
9. Decant the supernatant, and add 20 μL of the BD FastImmune cytokine-specific multicolor antibody reagent to each of the AS and US tubes. Add 20 μL of the BD FastImmune multicolor isotype control reagent to the AIS and UIS tubes. Vortex briefly. Incubate at room temperature for 30 minutes in the dark.

10. Add 2 mL of wash buffer to each tube, and centrifuge at 500 × g for 5 minutes at room temperature.

11. Decant the supernatant, and add 200 μL of 1% paraformaldehyde in PBS. Vortex to resuspend the pellet, and store at 4°C in the dark prior to flow cytometry analysis. Analyze within 24 hours.

   Note: Fixed and permeabilized cells are more buoyant than live cells, and they require higher centrifugal force to pellet. To avoid cell loss, it is recommended that decantation is used to remove the supernatant instead of aspiration.

BD FastImmune CD4 Kits (Anti–IFN-γ, Anti–IL-2 or Anti–TNF-α) – whole protein, peptide mixes

1. Activated sample:
   Add 0.5 mL of heparinized whole blood,
   5 μL of CD28/CD49d monoclonal antibody cocktail, and antigen at titer (or other activation agent) to a 15 mL polypropylene tube.

   Unstimulated (resting) sample:
   Add 0.5 mL of heparinized whole blood and 5 μL of CD28/CD49d monoclonal antibody cocktail in the absence of antigen to a 15-mL polypropylene tube. Vortex each tube gently and incubate 2 hours at 37°C.

2. Remove an aliquot of BFA from the freezer, dilute 1:10 with sterile PBS, and add 10 μL of diluted stock to each tube. Vortex and incubate an additional 4 hours at 37°C.

3. Proceed with steps 3 through 11 of the BD FastImmune CD8 Kit procedure.

Procedures
Precautions, Tips for Success, and Method Understanding
Sample Handling
Collect blood in sodium heparin since other anticoagulants severely compromise the functional capacity of lymphocytes. Store blood at room temperature to avoid platelet activation before use and use within 8 hours of collection. Antigen-presenting cell function is compromised with longer storage times, and loss of function can be compounded by shipping. All specimens and materials with which they come into contact are considered biohazards and should be handled as if capable of transmitting infection.19,20 Follow proper precautions in accordance with federal, state, and local regulations when disposing of all materials. Never pipette by mouth. Avoid specimen contact with skin and mucous membranes.
Activation Control

If you are using a specific antigen, stimulate an additional 0.5 mL of blood as a positive control with a strong activation agent, such as SEB (final concentration of 1 μg/mL of blood), and process with other tubes. This tube is used as a positive control and simplifies gating. See Figure 2 and Table 1.

Incubation Times

For CD4 responses to soluble protein antigens, optimal results are typically observed within a 6-hour incubation (the last 4 hours with BFA). Some cytokines can show a higher percentage of cells responding at time periods up to 20 hours (e.g., TNF-α and IFN-γ), but this appears to be at the expense of high fluorescence intensities. IL-2 responses are greatly diminished at longer incubation times.

CD8 responses to peptide antigens are also optimal around 6-hour incubation. Since peptide mixes do not require processing by antigen-presenting cells, BFA can be added at the same time as the antigen. BFA incubation can be increased to as long as 12 hours, if preferable, with a concomitant slight increase in numbers of responding cells. However, incubation times longer than 12 hours can result in cellular toxicity.

Recovery of Adherent Cells–EDTA

Treatment with BD FastImmune EDTA and vigorous vortexing are critical to avoid loss of activated cells adhering to the sides of the tube. For the same reason it is also essential to use polypropylene tubes for activation.

Automated Cooling of Activated Samples

Because blood samples might be collected late in the day, it is not always possible to run the entire assay in a single working day. As an alternative, cells can be cooled to 18ºC and kept at this temperature overnight after activation is completed without loss of function or increased background staining. A thermocycler or programmable water bath helps to automate this step.

Freezing of Activated, Fixed Cells

Once activated, EDTA-treated, and fixed with BD FACS Lysing Solution, cells can be directly frozen at -80°C without loss of function or increased background staining. Use of a freezing media (10% DMSO, 1% FBS in PBS) is not necessary. Freezing allows samples to be batched for parallel processing and staining at a later time or at a different site.

Centrifuging Lysed and Lysed-Permeabilized Cells

Once treated with BD FACS Lysing Solution, cells become much more buoyant than live cells. This effect is further enhanced when the cells are lysed and permeabilized. Accordingly, it is necessary to centrifuge at higher g forces (500 × g, or approximately 2,000 rpm on a Sorvall RT6000 tabletop centrifuge). Following the freezing and thawing procedure, when cell suspensions of 10 mL or more are being centrifuged, increase spin times to 10 minutes to allow for better pelleting.
Removal of Supernatant

Even with increased centrifugation speeds, fixed and fixed-permeabilized cells do not form tight pellets. Therefore, aspiration of supernatants can lead to significant cell loss unless done with great care. Accordingly, we recommend decanting supernatants with a single, gentle shake to remove most of the residual volume from the lip of the tube.

Volume of Blood per Stain

In HIV infection CD4 counts can be compromised. Consequently, 100 μL of blood per sample might not be sufficient to determine CD4 T-cell responses. In these situations staining of 200 μL or more of whole blood per sample might be needed. In few experiments, BD Biosciences investigated that the current protocol supports staining of up to 1,000 μL sample. Hereby, it is necessary to increase the volume of BD FACs Lysing Solution accordingly; other reagent volumes might not need adjusting. Modifications of the current protocol require additional validation by the user to ensure assay performance.

Selection of Staining Monoclonal Antibodies

Antibodies for intracellular staining need to have high affinity and specificity for epitopes that must not be lost under the particular fixation and permeabilization conditions used. Addition of other staining antibodies to the BD FastImmune Kits can require that these antibodies be added prior to the treatment with BD FACs Lysing Solution. CD4 and CD8 are conjugated to PerCP-Cy5.5 for better separation of CD4 dim and CD8 dim T cells from the negative cell population.

Data Acquisition and Analysis

Analyze on a BD FACS brand flow cytometer. The figures that follow show representative data performed on whole blood and analyzed on a dual-laser BD FACS brand flow cytometer with laser excitation at 488 nm and 635 nm.

Use BD Calibrite beads and appropriate software (BD FACsComp software, version 4.2, or BD AutoCOMPTM software, version 3.0.2) for setting photomultiplier tube (PMT) voltages, fluorescence compensation, and for checking instrument sensitivity before use. Refer to the BD Calibrite beads product insert and the appropriate software user’s guide for flow cytometric setup, acquisition, and analysis.

When using BD FACsComp software, the lyse/no-wash (LNW) setup should yield appropriate or nearly appropriate settings for intracellular cytokine staining. Instrument setup can also be performed manually using the multicolor isotype control tube to set PMT voltages such that CD4+ lymphocytes fall within the first decade of the FL1 and FL2 scales. Individual tubes stained with a single fluorochrome (eg, CD8 FITC, CD8 PE, CD8 PerCP-Cy5.5, and CD8 APC) can then be used to set compensation percentages. Note that any change in PMT voltages will require resetting of compensation; thus PMT voltages should always be set first. Once appropriate settings have been established for an experiment, a settings file can be saved and recalled for future experiments, with minimal adjustments.
**BD FastImmune CD8 Kit (Anti–IFN-γ)—see Figure 2**

1. Acquire data with BD CellQuest™ Pro software or BD CellQuest software, using a forward scatter (FSC) threshold. During acquisition set up, create a CD3 vs CD8 dot plot. Gate on the CD3+/CD8+ lymphocytes (R1). In addition, create an FSC vs SSC dot plot and draw a region around the lymphocytes (R2). Using the Gate List menu option, create a logical gate named G3 (G3 = R1 and R2). Collect at least 20,000 events that follow the requirements for G3.

2. Analyze data using BD CellQuest Pro software, BD CellQuest software, or BD Paint-A-Gate™ Pro software. Display data as Anti-Hu–IFN-γ vs CD69 dot plots to determine cytokine expression. The dot plots are gated with the same requirements for G3 as determined during acquisition (CD3+/CD8+ and lymphocyte scatter characteristics).

3. To obtain statistics, draw a region around the CD69 and Anti-Hu–IFN-γ double-positive events in a positive control sample (eg, SEB), and apply this region to your sample files. The % gated statistic gives frequency of cytokine-producing CD3+/CD8+ cells.

*Note:* It is important to include CD8dim/CD3dim cells for maximum detection of cytokine-positive events.

**BD FastImmune CD4 Kit (Anti–IFN-γ, Anti–IL-2, Anti–TNF-α)—see Figure 3**

1. Acquire data with BD CellQuest Pro software or BD CellQuest software, using a fluorescence or forward scatter (FSC) threshold. Collect at least 20,000 CD4+ lymphocytes. During acquisition set up a CD4 vs SSC dot plot. Gate on the CD4+ lymphocytes (R1). In addition, create an FSC vs SSC dot plot and draw a region around the lymphocytes (R2). Collect at least 20,000 events that fall in R1 and R2.

2. Display data as CD69 vs cytokine dot plots to determine cytokine expression. Analyze data using BD CellQuest Pro software, BD CellQuest software or BD Paint-A-Gate Pro software.

3. To obtain statistics, draw a region around the CD69 and cytokine double-positive events in a positive control sample, and apply this region to your sample files. A different region might be needed for each cytokine. The % gated statistic gives frequency of cytokine-producing CD4+ cells.
Figure 2. BD FastImmune CD8 Kit: 2a Gating strategy on isotype control, 2b Unstimulated and CMV-activated Anti–IFN-γ vs CD69 dot plots, 2c Importance of including CD8<sup>dim</sup>/CD3<sup>dim</sup> cells for maximum detection of cytokine-positive cells, and 2d SEB-activated positive control.

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Figure 3. BD FastImmune CD4 Kits: 3a Gating strategy on isotype control, 3b Unstimulated and CMV-activated anti-cytokine vs CD69 dot plots, 3c SEB-activated positive control, and 3d Importance of including CD4dim and SSClow cells for maximum detection of cytokine-positive cells.

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Tips for Success and Data Analysis Understanding

Gating can affect results, especially with rare-event assays. For the BD FastImmune CD8 Kit it is important to include CD3<sup>dim</sup> and CD8<sup>dim</sup> events in the CD3 APC vs CD8 PerCP-Cy5.5 gate to measure the optimal number of cytokine-positive events in a given sample (Figure 2). Similarly, when using the BD FastImmune CD4 Kit, CD4<sup>dim</sup> lymphocytes (SSC<sub>low</sub>) events need to be included in the CD4 PerCP-Cy5.5 vs SSC gate (Figure 3). Note that activated T lymphocytes will down-modulate CD8 and CD4 antigens with limitations. These cells, which might be seen as a smear coming off the main population, can include many of the responding lymphocytes in an activated sample.

Using the BD FastImmune CD4 Kits it is also important to exclude monocytes that are CD4<sup>dim</sup> but SSC<sub>high</sub>. Monocytes and activated platelets can bind nonspecifically to fluorescent-conjugated antibodies, causing background staining. In occasional donors, a reduction in nonspecific background staining might be achieved by using an exclusion channel. This refers to the use of a staining cocktail of antibodies to cell subsets that need to be eliminated from the analysis. At acquisition, a gate is set for cells that are negative for the exclusion channel reagents; this is included as part of a logical gate for acquisition. Particularly significant in terms of background for immune function assays are activated platelets and monocytes. CD33 APC for monocytes (BD Cat. No. 340474) and CD62P APC for activated platelets (available through the BD custom conjugate program) can be used as exclusion channel reagents in this assay.

Region gates, rather than quadrants, are used to define the response region. Similar results could be obtained using quadrants. We suggest setting the response region based upon where the positive population of cells is found (in a positive control sample), rather than defining it using only a negative or isotype control.

Calculating Specific Responses

The specific response of cells to any stimulus is obtained by subtracting the % positive events in the unstimulated sample from % positive events in the activated sample. Specific responses will vary by cytokine, by donor, and by antigen used.

There can be a variation of response to the same antigen among normal donors. Figure 4 shows the responses to CMV of three CMV-seropositive individuals. Note that the cytokine-producing cells for TNF-α, IFN-γ, and IL-2 always follow a hierarchy. TNF-α–producing cells are most numerous, followed closely by IFN-γ, with IL-2–producing cells a distant third. Cells producing other cytokines including IL-4, IL-5, and IL-10, are less frequent.

This is true for all antigens that we have tested to date, including recall antigens such as CMV, HIV, mumps, and TB, as well as neo-antigens such as KLH. A hierarchy also exists in terms of the relative response to different antigens. Figure 5 shows typical frequencies of IFN-γ–producing cells in seropositive individuals to three different viruses. The response to CMV is higher than that to HIV (shown in a long-term nonprogressor), and both are higher than the response to mumps. For more information on the relative number of cells responding to various herpes viruses, see reference 10. For more information on responses to HIV, refer to references 17 and 21.
Figure 4. Biological variation among CMV-seropositive donors in response to CMV.

Figure 5. Typical CD4 IFN-γ responses to three different antigens.
## Troubleshooting

The following troubleshooting matrix should help you pinpoint potential sources of problems in this assay.

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause</th>
<th>Solution</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor cell recovery</td>
<td>Inadequate centrifugation</td>
<td>Perform all spins at 500 x g for at least 5 minutes.</td>
<td>Fixed and permeabilized cells are more buoyant than live cells; therefore, they require higher centrifugal force to pellet.</td>
</tr>
<tr>
<td>Loss of pellet on aspiration</td>
<td>Decant supernatants.</td>
<td></td>
<td>Cell pellets are loose and easily disturbed by aspiration.</td>
</tr>
<tr>
<td>Low CD4 count (eg, in HIV-infected samples)</td>
<td>Stain 200μL or more blood per sample.</td>
<td>Increase volume of BD FACS Lysing Solution accordingly; other reagent volumes do not need adjusting. Validate assay performance on larger sample volumes.</td>
<td></td>
</tr>
<tr>
<td>No cytokine-positive cells</td>
<td>Inadequate activation, permeabilization, or staining as necessary</td>
<td>See Low numbers of cytokine-positive cells and Low intensity of cytokine staining in this table. Perform SEB activation on a normal donor as a positive control for these steps.</td>
<td></td>
</tr>
<tr>
<td>Lack of immune competence in the donor</td>
<td>Use a positive control, such as SEB activation, to assess the immune competence of the donor in question.</td>
<td>Calcium is required for lymphocyte activation; calcium-chelating anticoagulants prevent activation.</td>
<td></td>
</tr>
<tr>
<td>Wrong anticoagulant used for blood collection</td>
<td>Use only sodium heparin anticoagulant. Do not use ACD, EDTA, or other calcium-chelating anticoagulants.</td>
<td>Processing of complex antigens and presentation of relevant peptide epitopes on host class I-MHC molecules is inefficient when antigens are used in soluble form. Optimal class I–restricted CD8 T-cell responses are obtained by exogenous addition of peptide(s) or peptide mixtures to whole blood and PBMCs. See reference number 7 for more information on titration of antigens and kinetics of activation. See also Low intensity of cytokine staining in this table. The number of cytokine-producing cells will vary depending upon the antigen and cytokine, and the individual donor.</td>
<td></td>
</tr>
<tr>
<td>Low numbers of cytokine-positive cells</td>
<td>Inadequate activation</td>
<td>Titrates antigen to find the optimal dose for stimulation. See reference number 7 for more information on titration of antigens and kinetics of activation.</td>
<td></td>
</tr>
<tr>
<td>Low intensity of cytokine staining</td>
<td>Inadequate permeabilization or staining or both</td>
<td>Dilute BD FACS Permeabilizing Solution 2 or staining mAb. Do NOT dilute BD FACS Lysing Solution or BD FACS Permeabilizing Solution 2 in PBS or other buffers.</td>
<td>BD FACS Lysing Solution and BD FACS Permeabilizing Solution 2 should be used at room temperature, and all incubations should be at room temperature.</td>
</tr>
<tr>
<td>High background in unstimulated samples</td>
<td>Poor compensation</td>
<td>Set up using BD FACSComp software, using LNW settings, or perform manual compensation with samples individually stained for each fluorochrome. Poor compensation can result in cells appearing double-positive that are, in fact, single-positive for particular markers.</td>
<td></td>
</tr>
<tr>
<td>Imprecise gating</td>
<td>Gate carefully on FSC vs SSC to include only the small lymphocyte population. There is no need to include large blasts in the lymphocyte gate since the activation time is too short to cause increases in cell size.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gate carefully on CD4 vs SSC to include CD4 lymphocytes, but exclude monocytes, platelets, and dead lymphocytes. Gate carefully on CD8 vs CD3 to include CD8+ and CD3– lymphocytes.</td>
<td>Activated lymphocytes can down-modulate CD4 to become CD4+. Monocytes are CD4+ but have higher SSC than lymphocytes. Monocytes and platelets need to be excluded to avoid nonspecific staining. Activated lymphocytes can down-modulate CD8 to become CD8+ . Relevant to the BD FastImmune CD4 Kit assay only: Activated platelets can bind to lymphocytes and, therefore, require an additional marker to distinguish. See reference number 6 for information on exclusion channel.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Relevant to the BD FastImmune CD4 Kit assay only: Use an exclusion channel, such as CD33 APC + CD62P APC, to simplify exclusion of monocytes and activated platelets.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Long run time needed to acquire adequate number of CD4 events</td>
<td>Excessive dilution of samples in fixative before acquisition</td>
<td>Dilute cells in a minimal volume (≤200 μL) of buffer before acquisition. To avoid loss of cells when loading samples, set the cytometer to Standby, load the sample, click Acquire, and set the cytometer to Run.</td>
<td></td>
</tr>
<tr>
<td>Poor cell recovery or limited number of CD4+ cells in sample</td>
<td>See Poor cell recovery in this table.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Unless otherwise specified, all products are for Research Use Only. Not for use in diagnostic or therapeutic procedures. Not for resale.
BD Biosciences publishes this method as a service to researchers. Detailed support for non-flow cytometric aspects of this procedure might not be available from BD Biosciences.

Compatibility with BrdU Staining

With longer incubation times in isolated PBMCs, proliferation can be assessed with cytokine production. This is done using BrdU incorporation and staining with Anti-BrdU antibody. BD offers a unique reagent that combines Anti-BrdU monoclonal antibody with DNase (BD Cat. No. 340649) and that has been optimized for this procedure using PBMCs. Refer to the BD application note, *Simultaneous Detection of Proliferation and Cytokine Expression in Peripheral Blood Mononuclear Cells* and to reference number 24.

Compatibility with CD107 Staining

With a slight modification of this procedure, the release of CD107a to the cell surface during degranulation can be measured. This assay can provide information about the antigen-specific cytotoxic potential of cells. For more details on this assay and the procedure, please see Chapter 7 in this Manual.

Because of their parallel kinetics, CD107a and cytokines can be assessed at the same time in a short-term-activated blood samples.

* US Patent Nos. 4,654,312; 4,902,613; and 5,098,849
† Patents—PE and APC: US 4,520,110; 4,859,582; 5,055,556; Europe 76,695; Canada 1,179,942 PerCP: US 4,876,190
Cy: US 5,268,486; 5,486,616; 5,569,587; 5,569,766; 5,627,027
‡ Use of these products to measure activation antigens expressed on mononuclear cell subsets for the purpose of monitoring immunoregulatory status can fall under one or more claims of the following patents: US Patent Nos. 5,445,939, 5,656,446, 5,834,689; European Patent No. 319,543; Canadian Patent No. 1,296,622; Australian Patent No. 615,880; and Japanese Patent No. 2,769,156.
§ US Patent No. 5,224,058
References


References (continued)


Relevant BD Biosciences Literature and Links


Application Notes

1. Detecting Cytokines in Antigen-Activated Lymphocytes [using BD FastImmune 4-Color Kits, and peptide antigens, looking at CD8+ and CD4+ T cells; Literature Part Nr.: 23-5196-02]

2. Detecting Cytokines in Lymphocytes [Literature Part Nr.: 23-3391-03]

3. Detecting Cytokines in Monocytes [Literature Part Nr.: 23-3493-02]

4. Simultaneous Detection of Proliferation and Cytokine Expression in Peripheral Blood Mononuclear Cells [Literature Part Nr.: 23-3941-00]

User Manuals

1. BD FastImmune CFC Handbook: Performance Characteristics of Antigen-Specific Cytokine Flow Cytometry (CFC) Assays [detailed protocol for the BD FastImmune System, including comparison to other immune response assays; Literature Part Nr.: 23-6563-00]

The abovementioned product literature is available on request from your local BD Biosciences representative, or as a download at www.bdbiosciences.com/literature [go to Literature Library]

For additional resources containing practical tips about multicolor flow cytometry in general, please refer to the Resources section at the end of Chapter 1, or visit www.bdbiosciences.com/colors.

Additional Technical Resources

Our Scientific Support Team is trained to help you with technical questions related to intracellular immunostaining and flow cytometry. Just contact them at the numbers listed at the end of this manual, or e-mail: help.biosciences@europe.bd.com.
Chapter 5

Immunofluorescent Staining of Intracellular Molecules for Flow Cytometric Analysis: Immune Effectors and Signaling Molecules

Introduction

Analysis of the Nature and Frequency of Responding Cells

Flow cytometry is a powerful analytical technique in which individual cells can be simultaneously analyzed for several parameters. These include size and granularity, as well as the coexpression of cell surface and intracellular molecules, as defined by fluorescent antibodies.\(^1,2\) Intracellular cytokine staining and detection by flow cytometry (covered in Chapters 3 and 4) has been widely adopted in a variety of immune response studies in which the analysis of the nature and frequency of cells is critical. The advantages of obtaining an in-depth analysis of the response of individual leucocyte subsets have been discussed in Chapter 3.

Areas of Application

A wide variety of intracellular molecules in addition to cytokines are of interest in studies of immune responses. Here we list the ones for which BD Biosciences offers reagents and protocols. While it is not within the scope of this handbook to include all the details here, we do mention a number of other reference sources later in this chapter.

- Cytoplasmic Immunoglobulins
- Other Immune Mediators (for example, Granzyme B and Perforin)
- Cell signaling
- Apoptosis
- Cell Cycle Molecules
- DNA content
- Nuclear antigens, including FoxP3

Critical Parameters for Reliable Intracellular Staining

Staining of intracellular antigens depends on the identification of specific antibodies that are compatible with a fixation and permeabilization procedure. The empirical selection of fixation and permeabilization reagents must take into consideration factors such as retention of immunoreactivity of the antigen, retention of sub-cellular structures (and antigen location), and retention of light scattering properties of the cells.
The methods for staining of intracellular cytokines have been well worked out, and are described in detail in Chapters 3 and 4. Methods for staining other intracellular molecules such as inflammatory mediators (perforin and granzymes), cell signaling proteins, and immunoglobulins have also been developed in many laboratories. While each protocol is usually developed for a specific application, many are very similar to the BD Cytofix/Cytoperm™-based methodology.

In addition to choosing an appropriate fixation and permeabilization procedure, the availability of high quality directly fluorochrome-conjugated primary antibodies can be critical to achieve “clean” staining, with low background and high signal.

The choice of a variety of direct fluorochrome conjugates of the antibodies also offers you flexibility in designing multicolor experiments. For tips on designing multicolor experiments and choice of fluorochromes, please refer to Chapter 1.

**Phospho-Protein Profiling in Single Cells**

Protein phosphorylation is the driver for a wide variety of cell signaling pathways, from growth and cell cycle control, to apoptosis or stress response, to immune function signaling. Until recently, the study of signaling-related phosphorylation in small cell populations has been limited by the shortcomings of available techniques. Conventional methods, such as kinase assays, immunoprecipitation and Western blotting, indeed require relatively large amounts of sample, are time consuming, and are not conducive to multi-parameter analysis.

**Phospho-Specific Flow Cytometry**

BD™ Phosflow is a highly innovative flow cytometry-based technology that enables activation state analysis of multiple proteins simultaneously at single-cell levels. Using phosphorylation site-specific antibodies in combination with cell surface markers, phosphorylation events can be studied directly in small sub-populations of complex primary samples such as whole blood or peripheral blood mononuclear cells (PBMCs). By evaluation of coexpression of both surface and intracellular markers, the intracellular signaling events can be correlated with a discrete sub-population of cells, without physically separating the cells.

Understanding the differential responses of cell subsets (such as T cells, B cells, NK cells and monocytes, or naïve versus memory T cells) can be particularly useful for following signaling events during immune responses. While a significant understanding of pathological processes can be obtained through the study of phenotypic surface markers, a more comprehensive appreciation of intracellular signaling events is often required to assess subset-specific cell behavior in both normal and disease states.3-5 Single cell-based comparison of the phospho-proteome in its resting state versus how it may rearrange in response to single or multiple inputs, offers a dynamic view of phospho-signaling network functionality. Phospho-specific flow cytometry opens the way to the identification of new, clinically relevant network phenotypes, for instance allowing unique cancer network signatures to be revealed.4,6

Unless otherwise specified, all products are for Research Use Only. Not for use in diagnostic or therapeutic procedures. Not for resale.
BD™ Phosflow technology and reagents have been developed in collaboration with the laboratory of Garry P. Nolan, PhD at Stanford University. Leading the way in flow cytometry reagents and analysis, BD Biosciences has joined Dr. Nolan in a collaborative effort to make phosphospecific flow cytometry available to the Research community.

**BD Phosflow Reagents – Designed to Perform**

Development of BD Phosflow antibody reagents involves a highly stringent validation process, including extensive specificity testing and confirmation of results in multiple techniques. BD Phosflow antibody reagents are confirmed to perform well in flow cytometry using our optimized, standard staining procedure, giving a minimum signal in a model test system.

The availability of direct conjugates to a variety of fluorophores makes multi-parameter studies of phosphorylation events easier, be it in three-, four-, five- or even six-color flow cytometric experiments. Use of direct conjugates also reduces undesired background staining and improves signal-to-noise.

For an overview of our BD Phosflow reagent offering, please visit [www.bdphosflow.com](http://www.bdphosflow.com)

**Optimized Protocols and Validated Surface Markers for Streamlined Staining Procedures**

Since phosphorylation states of individual signaling elements turn over rapidly in response to environmental stimuli, fixation is necessary to suspend cellular phosphorylation events during the staining procedure. We have developed and optimized a standard protocol for multi-parameter, flow cytometric phosphorylation monitoring combining BD Phosflow reagents and cell surface antibodies (see Figure 1 for an overview of the staining procedure). With minor variations, this protocol can be applied to different types of samples, from human whole blood and PBMCs, to mouse thymocytes and splenocytes, to adherent cultured cells.

To view and download the protocols for different sample types, visit [www.bdphosflow.com](http://www.bdphosflow.com)

The protocols are also described in detail in the BD Biosciences *Techniques for Phospho Protein Analysis Application Handbook, 1st Edition.*

Our proprietary buffer system also enables phospho-protein analysis directly in whole blood samples, minimizing artifacts caused by cell isolation steps and providing more relevant information, since assays are performed in near native conditions. Studies have shown that cellular responses and underlying signaling events are influenced by external factors, of which cell-cell interactions play a key role. Assessing intracellular signaling mechanisms within the context of other contacting cells is therefore crucial.
Figure 1. Overview of the BD Phosflow staining procedure.

To help streamline experimental design of multicolor studies, BD Biosciences has done the pre-work of defining workable combinations of surface markers. We have validated a broad panel of directly labeled BD Phosflow conjugates in combination with cell surface markers for multiplexed analysis of phosphorylation events in different cell types. For more information on validated markers and tested color combinations in human and rodents, please visit www.bdphosflow.com

To meet your screening needs, BD Biosciences has adapted the BD Phosflow staining procedure to allow for staining and processing of your samples in a 96-well
plate format. With this protocol, hundreds of samples can be analyzed per day, making it possible to perform higher throughput, high content, cell-based screens of phospho-signaling events, as might be required in drug discovery and development.

A detailed protocol for performing BD Phosflow in a 96-well plate format can be found at [www.bdphosflow.com](http://www.bdphosflow.com).

To learn more about how to apply intracellular flow cytometry for phospho-protein analysis in single cells, or to view a selection of recent publications visit [www.bdphosflow.com](http://www.bdphosflow.com).

**FoxP3 Staining of Human and Mouse CD4\(^+\) Regulatory T Cells**

The FoxP3 transcription factor, also known as Scurfin, IPEX and JM2, is among the most definitive markers associated with natural CD4\(^+\) regulatory T cells.\(^{12-14}\) FoxP3 has been found to be associated with CD4\(^+\) regulatory T cells and is mutated in the human X-linked autoimmunity-allergic dysregulation syndrome (XLAAD or IPEX) and also in scurfy (sf) mice. Recent research in human disease processes have shown alterations in the numbers of regulatory T cells and in particular, those that express FoxP3.

The BD Pharmingen™ brand human FoxP3 antibody and buffer kit is a high performance reagent system for the detection of FoxP3-positive regulatory T cells. The 259D/C7 monoclonal antibody clone reacts with all currently identified isoforms of human FoxP3 and is cross-reactive with Cynomolgus, Rhesus and Baboon. Available fluorescent conjugates include Alexa Fluor® 488, Alexa Fluor® 647 and PE formats to enable maximum flexibility for design of multicolor panels in combination with any of our family of BD FACSTM brand flow cytometers. For flow cytometers equipped with violet lasers, BD offers FoxP3 conjugated to BD Horizon™ V450. Human FoxP3 detection kits containing all necessary reagents for identification of Tregs using FoxP3 are also available. The BD Pharmingen™ Human FoxP3 Buffer Set (Cat.No. 560098) fixes and permeabilizes cells in just a few simple steps.

Mouse FoxP3 antibodies are available in PE, Alexa Fluor® 488 and Alexa Fluor® 647 conjugates. The BD Pharmingen™ Mouse FoxP3 Buffer Set (Cat. No. 560409) allows fixation and permeabilization of cells in just a few steps and is developed to provide optimal staining of mouse FoxP3. Please refer to the Technical Data Sheet for the recommended staining protocol.

**Recommended Assay Procedure:**

**Cell Preparation and Staining Procedures for Conjugated Anti-Human FoxP3 Antibody**

1. Bring the buffers to RT before use. Prepare working solutions of the BD Pharmingen Human FoxP3 Buffer Set (Cat. No. 560098; for details of the buffer preparation, please see the Technical Data Sheet of the buffer set).

2. Prepare human PBMC. Dilute the cells with BD Pharmingen Stain Buffer (FBS)* to ten million cells/ml.

3. Pipette appropriate amount of surface staining reagent to bottom of each 12 x 75 mm tube.
4. Add 100μl of cells per tube, vortex, incubate for 20 minutes at RT protected from light.

5. Add 2 ml of wash buffer. Centrifuge 250 x g for 10 minutes, and remove wash buffer.

6. To fix the cells, gently re-suspend pellet in residual volume of wash buffer and then add 2ml of 1x Human FoxP3 Buffer A. Vortex. Incubate for 10 minutes at RT in the dark.

7. Centrifuge 500 x g for 5 minutes, and remove fixative. Caution: Be aware the pellet is buoyant.

8. To wash cells, re-suspend each pellet in 2ml of BD Pharmingen Stain Buffer (FBS)*, and centrifuge 500 x g for 5 minutes. Remove wash buffer.

9. To permeabilize the cells, gently re-suspend pellet in residual volume of wash buffer and then add 0.5 ml of 1x working solution Human FoxP3 Buffer C to each tube. Vortex. Incubate for 30 minutes at RT protected from light.

10. To wash cells, add 2 ml of BD Pharmingen Stain Buffer (FBS)* to each tube, centrifuge 500 x g for 5 minutes at RT. Remove buffer and repeat wash step. Remove buffer.

11. Add conjugated FoxP3 antibody at appropriate concentrations to re-suspend the pellet. Gently shake or vortex.

12. Incubate for 30 minutes in the dark at RT.


14. Resuspend in wash buffer and analyze immediately.**

**Optional** Add 300μl of 1% formaldehyde in 1x PBS and store at 4°C. Analyze cells within 24 hours.

*Note: We recommend using the BD Pharmingen Stain Buffer (FBS; Cat No. 554656) for all wash steps and covering tubes during incubation steps with caps or parafilm.

**Acquisition Tip:** We recommend optimizing forward scatter and side scatter voltages to visualize lymphocytes as separate from debris, red cell ghosts and/or platelets before acquisition. Acquire at least 15,000 to 25,000 CD4 positive lymphocytes.

### Suggested Companion Products

<table>
<thead>
<tr>
<th>Catalog Number</th>
<th>Name</th>
<th>Size</th>
<th>Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>554656</td>
<td>BD Pharmingen™ Stain Buffer (FBS)</td>
<td>500 ml</td>
<td>(none)</td>
</tr>
<tr>
<td>560098</td>
<td>Human FoxP3 Buffer Set</td>
<td>100 tests</td>
<td>(none)</td>
</tr>
<tr>
<td>560249</td>
<td>Human Regulatory T Cell Cocktail</td>
<td>50 tests</td>
<td>SK3, ZA3,</td>
</tr>
<tr>
<td></td>
<td>(CD4, CD25 and CD127)</td>
<td></td>
<td>hIL_7R_M21</td>
</tr>
</tbody>
</table>

To find more products for regulatory T cell research please visit www.bdbiosciences.com/treg
Figure 2. Flow cytometric analysis of Alexa Fluor® 488 anti-human FoxP3 on resting PBMC. Human PBMC were stained with APC anti-human CD4 (clone RPA-T4, Cat. No. 555349) and PE anti-human CD25 (Clone M-A251, Cat. No. 555432) simultaneously. Cells were fixed and permeabilized (see recommended assay procedure) followed by intracellular staining with Alexa Fluor® 488 anti-Human FoxP3 (clone 259D/C7; Cat No. 560047). The dot plots were derived from the gated events based on light scattering characteristics of lymphocytes and fluorescence characteristics of CD4⁺ or CD25⁺ respectively, shown as either FoxP3 vs CD25 (left panel) or FoxP3 vs CD4 (right panel). Flow cytometry was performed on a BD FACSCalibur™ System.

Other Areas of Application of Intracellular Staining

A wide variety of intracellular molecules in addition to cytokines are of interest in studies of immune responses. Here we list the ones for which BD Biosciences offers reagents and protocols. While it is not within the scope of this handbook to include all the details here, we can offer a number of other reference sources.

<table>
<thead>
<tr>
<th>Intracellular Measure</th>
<th>Protocol source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasmic Immunoglobulins</td>
<td>see protocol on the product Technical Data Sheets, available on our web</td>
</tr>
<tr>
<td>Other Immune Mediators (e.g., Granzyme B and Perforin)</td>
<td>see BD Biosciences Hotlines 8(3):15-16 or product Technical Data Sheets</td>
</tr>
<tr>
<td>Cell signaling</td>
<td>see Chapter 2 in <em>Techniques for Phospho Protein Analysis: Applications Handbook, 1st Edition</em></td>
</tr>
<tr>
<td>Apoptosis</td>
<td>see our brochure <em>Life, Death and Cell Proliferation</em></td>
</tr>
<tr>
<td>Cyclins</td>
<td>see protocol on our web: <a href="http://www.bdbiosciences.com/pharmingen/protocols">www.bdbiosciences.com/pharmingen/protocols</a></td>
</tr>
<tr>
<td>Proliferation / BrdU</td>
<td>see <em>Chapter 8</em></td>
</tr>
<tr>
<td>DNA content</td>
<td>see <em>Chapter 8</em></td>
</tr>
<tr>
<td>Nuclear antigens</td>
<td>see protocol on our web for Ki-67: <a href="http://www.bdbiosciences.com/pharmingen/protocols">www.bdbiosciences.com/pharmingen/protocols</a> this protocol is also suitable for staining of a variety of other intracellular molecules, such as p53, PCNA, or p16 INK4a.</td>
</tr>
<tr>
<td>FoxP3</td>
<td>see protocol above</td>
</tr>
</tbody>
</table>
References

Relevant BD Biosciences Literature and Links
For additional resources containing practical tips about multicolor flow cytometry in general, please refer to the Resources section at the end of Chapter 1, or visit www.bdbiosciences.com/colors.

Application Manual
Techniques for Phospho Protein Analysis Application Handbook – provides detailed protocols and valuable guidelines for designing your phospho-protein profiling experiments. This is available on request from your local BD Biosciences representative, or as a download at www.bdbiosciences.com/literature [go to Literature Library]
Tutorial Video

A tutorial video illustrating the use of BD Phosflow technology can be found at www.bdphosflow.com

Web

www.bdphosflow.com – offers an up-to-date source of information related to the use of BD Phosflow technology. Use it for direct access to additional sample data, recommended color combinations and protocols, comprehensive information on validated cell surface markers for human and mouse, as well as key publications on phospho-specific flow cytometry.

Additional Technical Resources

Our Scientific Support Team is trained to help you with technical questions related to intracellular immunostaining and flow cytometry. Just contact them at the numbers listed at the end of this manual, or e-mail: help.biosciences@europe.bd.com.
Chapter 6

BD™ ELISPOT Assays for Cells That Secrete Biological Response Modifiers

Introduction

The enzyme-linked immunospot (ELISPOT) assay is a powerful tool for analyzing the immunological functions of peripheral blood and lymphoid cell populations. The ELISPOT assay allows for the detection, enumeration, and characterization of individual antibody- or cytokine-secreting cells within cultured cell populations.\(^1\) The ELISPOT assay was derived from the sandwich enzyme-linked immunosorbent assay (ELISA).\(^2\) The ELISPOT method developed by BD Biosciences uses purified NA/LE (no sodium azide/low endotoxin) antibodies adsorbed onto polyvinylidene difluoride (PVDF) membrane-coated microwell culture plates. The immobilized antibody specifically captures proteins that are secreted or released from cultured cells that are applied to the plate. After removing the cells and washing, the captured proteins are specifically detected by biotin-conjugated antibodies followed by enzyme-labeled avidin. The application of substrate generates colored spots that can be enumerated by conventional means, (ie, with a magnifying glass or dissecting microscope), or in a high-throughput manner by image analysis, (eg, with an ELISPOT plate reader).

Although originally developed for analyzing specific antibody-secreting cells,\(^3,4\) the ELISPOT assay has been adapted for measuring the frequencies of cells that produce and secrete other effector molecules, such as cytokines, chemokines and granzymes.\(^5\)\(^\text{-}^7\) The capacity to measure robust (eg, mitogen-driven), intermediate, and even low (eg, antigen-stimulated) cell frequency responses is an attractive feature of the ELISPOT assay method. The high sensitivity of the assay lends itself to the measurement of even very low frequencies of cytokine-producing cells (eg, 1/300,000).\(^1\)

Recent developments in assay plate design and in ELISPOT plate-reader instrumentation have significantly contributed to the utility and quality of the ELISPOT method for rapid analyses of cytokine producing-cell frequencies and the relative levels of cytokine produced per cell (ie, spot size).\(^1\) The use of PVDF ELISPOT plates has significantly improved the signal-to-noise ratio for counting cytokine spots.\(^1\) PVDF membranes provide a significantly greater surface area for capture than do other previously-used standard membranes. Scientifically-validated, computer-based image acquisition and ELISPOT analysis software have also dramatically improved the ELISPOT assay’s capacity for the objective quantification of large numbers of samples in a relatively short period of time.\(^2\)

BD Biosciences has identified a panel of novel, optimized antibody pair combinations that are specially formulated for performing ELISPOT assays. In the course of screening many antibody clones for the ELISPOT application, we determined that some antibody pairs that perform very well for ELISAs were...
not necessarily optimal for use in the ELISOT assay. ELISOT-compatible antibody clones (ie, paired capture and detection antibodies) were selected based on their ability to generate discreet, densely-colored “spots” (ie, ELISOTs). In some cases, the best performing ELISOT antibody combinations were developed by mixing multiple capture antibody clones together. To avoid the undesirable effects of sodium azide and endotoxin on responses made by cultured cells, the BD Biosciences ELISOT capture antibodies are specially formulated. They contain no sodium azide and minimal endotoxin (< 0.01 ng/μg antibody) (NA/LE). Moreover, our detection antibodies are specially-formulated to avoid the development of nonspecific spots.

Image analysis-assisted cytokine ELISOT assays have recently emerged as one of the most sensitive and robust techniques for analyzing and monitoring cells that mediate immunological functions. Some advantages and unique strengths of the ELISOT assay include its high sensitivity, its capacity for high-throughput analyses, its minimal cell number requirement, and its ability to analyze cryopreserved lymphocytes. ELISOT analysis is also compatible with other assays. For example, cells characterized by ELISOT analysis can be subsequently transferred for cloning, proliferation assays, flow cytometric analysis, or other methods of analysis.

Granzyme B ELISOT assay

Granzyme B ELISOT assay is a non-radioactive alternative to Chromium-release assays. Detection of Granzyme B-secreting cells in ELISOT assays correlates with cytolytic responses measured by classic radioactive 51Cr-release assays. Granzyme B is a neutral serine protease that induces apoptosis by cleaving and activating members of the caspase family. Granzyme B is secreted by cytolytic effector cells. The effector cells target cells through transmembrane pores formed by perforin. The Granzyme B ELISOT assay directly measures the frequencies of Granzyme-B-producing cells. See Chapter 7 - Cytotoxicity and Degranulation Assays for more details and sample data.
Overview of the ELISPOT Assay Protocol

1 Capture Antibody
   For Sets and Pairs: Coat microwells with capture antibody. For Kits: Go to Step 3; Steps 1 and 2 are not necessary.

2 Blocking
   Block unoccupied sites with protein

3 Add Cells
   Culture cells in well with antigen, mitogen, etc.

4 Wash
   Cells are washed off; secreted analyte remains bound to capture antibodies.

5 Detection Antibody
   Add biotinylated detection antibody

6 Enzyme-Conjugate
   Add Streptavidin-HRP (SAv-HRP)

7 Develop With Substrate
   Add substrate and monitor formation of colored spots

8 Topview of ELISPOT plate microwell with colored ELISPOTS
BD™ ELISPOT Assay Protocol

The following protocol is for using BD ELISPOT Antibody Pairs and Sets.
The protocol for using BD ELISPOT Kits starts with Step 3.

Note: Use ELISPOT plates and reagents under aseptic conditions (e.g., in laminar flow hood) for Steps 1 – 3. Solutions, buffers, and media that are noted with an asterisk (*) are described in the Buffers, Media, and Other Reagents Section on page 146.

1. Coating Antibody:
   a. Dilute the Capture Antibody to the recommended concentration with coating buffer* (refer to the Certificate of Analysis included with the product). Add 100 μl of diluted antibody solution to each well of an ELISPOT plate. When using the ELISPOT Kit format, the plates are pre-coated and steps 1 and 2 should therefore be omitted. The kit protocol starts with step 3.
   b. Replace the ELISPOT plate lid and store plates at 4°C overnight.

2. Blocking:
   a. Discard the coating antibody. Wash the wells 1× with 200 μl/well of complete tissue culture medium* that contains 10% fetal bovine serum.
   b. Add 200 μl/well of complete tissue culture medium*, replace the ELISPOT plate lid and allow blocking for 2 hours at room temperature.

3. Cell Activation: (Note: Kit Protocol begins here) Specific activation protocols including cell concentrations and incubation times will vary depending on the cell type, choice of stimulus, and target analyte of interest. For general methods of cell stimulation, please refer to the section on Intracellular Cytokine Detection by Flow Cytometry (see Chapter 3). Please note that protein transport inhibitors should not be used for ELISPOT cultures.
   a. Discard the complete tissue culture media. (Not necessary for the ELISPOT Kits.)
   b. Prepare mitogen or antigen diluted in complete tissue culture medium. Add 100 μl/well to ELISPOT plate.
   c. Prepare cell suspensions at different densities, (e.g., ranging from 1 × 10^5 cells/ml to 2 × 10^6 cells/ml). Note that appropriate negative controls should be prepared by adding cells to wells without the particular stimulus and by establishing background wells without cells, (i.e., wells that just receive the complete tissue culture media). Cell titrations can be performed either in another cell culture plate or in tubes and then transferred to the ELISPOT plate microwells, or performed directly in the ELISPOT plate. Care should be taken not to touch or damage the coated microwell surface. Cells should be added in 100 μl volumes to ELISPOT plate microwells. Conditions for generating cells that secrete a particular analyte to serve as a positive control are included in the Certificate of Analysis that is supplied with the ELISPOT product.
d. After adding the cells, replace the ELISPOT plate lid and incubate the plate at 37°C, 5% CO₂, and 99% humidity. The duration of the incubation time will vary depending on the analyte of interest (eg, cultures are usually established for 2 – 48 hr).

4. Detection Antibody:
   a. Aspirate the cultured cell suspensions from the ELISPOT plate microwells. After step 3, aseptic conditions are not required. Wash and soak the wells 2x with 200 μl/well of distilled water (dH₂O). Allow wells to soak for 3 – 4 min at each wash step.
   b. Wash wells 3x with 200 μl of PBS-Tween* per well. Discard Wash Buffer.
   c. Dilute Detection Antibody in Dilution Buffer*. Add 100 μl per well.
   d. Replace the ELISPOT plate lid and incubate for 2 hr at room temperature.

5. Streptavidin-Horseradish Peroxidase (SAv-HRP) (Cat. No. 557630):
   a. Discard Detection Antibody solution. Wash wells 3x with 200 μl/well of PBS-Tween. Allow wells to soak for 1 – 2 min at each wash step.
   b. Dilute SAv-HRP in Dilution Buffer. Add 100 μl of diluted SAv-HRP per well.
   c. Replace the ELISPOT plate lid; incubate for 1 hr at room temperature.

6. Substrate:
   a. Discard SAv-HRP solution. Wash wells 4x with 200 μl of PBS-Tween per well. Allow wells to soak for 1–2 min at each wash step.
   b. Wash and soak wells 2x with 200 μl of PBS per well.
   c. Add 100 μl of AEC Substrate Solution* (Cat. No. 551951) to each well. Monitor spot development at room temperature from 5 – 60 min. Do not let color overdevelop. This will lead to high background.
   d. Stop the substrate reaction by rinsing wells thoroughly with dH₂O.
   e. Air-dry plate for 2 hr or overnight in the dark until the plate is completely dried. Removal of plastic tray under 96-well plate facilitates drying. Store the plate in a sealed plastic bag, in the dark, prior to analysis.
   f. Enumerate spots manually by inspection under a dissecting microscope (or stationary magnifying glass) or automatically using an image analyzer.
* Buffers, Media, and Other Reagents for ELISPOT Assays

a. Coating Buffer. Dulbecco’s Phosphate Buffered Saline (PBS): 8 g NaCl; 0.2 g KCl; 1.44 g Na$_2$HPO$_4$•7H$_2$O; 0.24 g KH$_2$PO$_4$; add dH$_2$O to 1 liter. Adjust pH to 7.2, autoclave or sterile filter (0.2 μm-pore) and store at 4°C.

b. Complete Tissue Culture Medium: A medium consisting of RPMI 1640 (Bio-Whittaker, Cat. No. 12-167Q) or other suitable medium containing 10% FBS, 1% Penicillin-Streptomycin-L-Glutamine (Gibco-BRL Cat. No. 10378-016), and $5 \times 10^{-5}$ M 2-mercaptoethanol is often used for culturing human, non-human primate, and rodent cells.

c. PBS-Tween: PBS containing 0.05% Tween-20 (Sigma, P-1379; 0.5 ml Tween-20 per 1 L PBS).

d. Dilution Buffer: PBS containing 10% FBS.

e. Substrate Solution can be prepared or can be purchased (Cat. No. 551951) for convenience from BD Biosciences.

To Prepare AEC Substrate Solution:

1. Prepare AEC (3-amino-9-ethyl-carbazole; Sigma A-5754) stock solution: 100 mg AEC in 10 ml DMF (N,N-Dimethylformamide; Sigma D-4551). Caution: dispense DMF in fume hood. Store solution in glassware.

2. Prepare 0.1 M Acetate Solution: add 148 ml of 0.2 M acetic acid to 352 ml of 0.2 M sodium acetate. Adjust volume to 1 L with distilled water; adjust pH to 5.0.

3. For Final Substrate Solution, add 333.3 μl of AEC stock solution to 10 ml 0.1 M Acetate Solution. Filter through 0.45 μm-pore filter. Add 5 μl of H$_2$O$_2$ (30%) and use immediately.

Cytokine ELISPOT Troubleshooting Tips

1. Take care not to puncture the membrane on the bottom of the ELISPOT plate wells. The membranes in the ELISPOT microwell plates are fragile; do not touch the bottom of the wells with the ends of the pipette tips when adding cells or reagents and washing plates.

2. To identify the optimal cell concentrations for ELISPOT analysis, use a wide range of cell concentrations (eg, $10^3 – 10^6$ cells per microwell) in the first experiment.

3. Do not disturb the incubator or ELISPOT plate during the cell culture process to avoid streaks and ambiguous spots.

4. Do not stack the plates in the incubator. Place each ELISPOT plate individually on the shelf to allow an even distribution of heat to each microwell and to avoid edge effects.
5. High backgrounds in blank wells (ie, strong red color) can sometimes be overcome by performing the following steps properly:

- Stringency of washes with PBS-Tween—follow washing instructions carefully. One or more additional washes may be necessary.
- Soaking and washing the plate with PBS prior to adding substrate. Tween–20 from the wash buffer can interfere with the substrate development and it can cause high background.
- If using a substrate other than the one recommended and optimized for BD™ ELISPOT reagents, the detection antibody and avidin-HRP concentrations must be optimized by the researcher for best results.
- Dry the plate longer if necessary. The speed at which the plate completely dries depends on the relative humidity in the environment.
- Wash cells thoroughly prior to the experiment to avoid the carryover of natural cytokines made by the cells in a preliminary culture or of recombinant cytokines that have been added exogenously.
- Monitor the substrate development carefully. Do not overdevelop, as this will lead to high background.

6. After completion of the experiment, do not dry the microplate at a temperature higher than 37°C; this may cause cracking of the membrane filters.

7. Store color-developed, dried plates in a sealed plastic bag protected from light to avoid color reduction that can be caused by air or light.
Available Formats for BD™ ELISPOT Products

A broad range of reagents is available to support the various needs of researchers:

**BD ELISPOT Reagent Pairs**
- Unlabeled Capture Antibody (BD NA/LE™ format); sufficient reagent for coating 5 plates
- Biotinylated Detection Antibody; sufficient reagent for 5 plates
- Certificate of Analysis, providing lot-specific optimal reagent concentrations

**BD ELISPOT Reagent Sets**
- 10 uncoated ELISPOT plates
- Unlabeled Capture Antibody (BD NA/LE format); sufficient reagent for coating 10 plates
- Biotinylated Detection Antibody; sufficient reagent for 10 plates
- Streptavidin horseradish peroxidase; sufficient for 10 plates
- Certificate of Analysis, providing lot-specific optimal reagent concentrations

**BD ELISPOT Kits**
- 2 pre-coated BD™ ELISPOT plates
- Biotinylated detection antibody; sufficient reagent for 2 plates
- Streptavidin horseradish peroxidase; sufficient for 2 plates
- Assay diluent
- Wash buffers
- AEC substrate reagents
- Certificate of Analysis, providing lot-specific optimal reagent concentrations

**Available Products**
For a complete listing of BD ELISPOT reagents currently available, please visit the BD ELISPOT Homepage at [www.bdbiosciences.com/pharmingen/ELISPOT](http://www.bdbiosciences.com/pharmingen/ELISPOT) and click on the "Product List" tab.
Key Features of BD™ ELISPOT

Optimized Antibody Pairs that Provide Crisp, Clear Spots

Figure 1. Superior human IL-2 ELISPOTs are obtained using BD ELISPOT Set with a cocktail of capture antibodies. Human PBMCs were stimulated (overnight) with PMA (5 ng/ml; Sigma, Cat. No. P-8139) and ionomycin (500 ng/ml; Sigma, Cat. No. I-0634) in the microwell of a BD ELISPOT plate pre-coated with a cocktail of NA/LE anti-human IL-2 antibodies. Biotinylated anti-human IL-2 antibody was used to detect the captured IL-2 produced and secreted by individual cells within the activated cell population. Spots were visualized using avidin-HRP enzyme and AEC substrate. Image analysis and spot enumeration were carried out using the ImmunoSpot® Series 2 Analyzer (CTL Analyzers LLC, Cleveland, OH). Panels A and B were derived from experiments conducted using the same activated cells in the same ELISPOT plate.
Panel A: BD ELISPOT Human IL-2 Set (Cat. No. 551282).
Panel B: Suboptimal human IL-2 ELISPOT using other antibodies.

BD ELISPOT Kits for Convenient and Consistent Results

Figure 2. Detection of IFN-γ-secreting cells with a BD ELISPOT Human IFN-γ Kit. (Cat. No. 552138). Human PBMCs were stimulated with PMA and ionomycin for 18 hours. (blank controls: microwells G 1 – 9: cells without stimuli; microwells H 1 – 9: stimuli without cells)
Consistent Performance of BD ELISPOT Human IFN-γ Kits (with pre-coated plates)

<table>
<thead>
<tr>
<th>Plate No.</th>
<th>No. of Spots/Well (Mean of 36 wells)</th>
<th>SD</th>
<th>%CV</th>
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<tbody>
<tr>
<td>Exp. 1 1</td>
<td>432</td>
<td>19</td>
<td>4.4</td>
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<tr>
<td>Exp. 2 2</td>
<td>466</td>
<td>18</td>
<td>3.8</td>
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<tr>
<td>Exp. 3 3</td>
<td>432</td>
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<td>Exp. 4 4</td>
<td>338</td>
<td>26</td>
<td>7.8</td>
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<tr>
<td>Exp. 5 5</td>
<td>365</td>
<td>19</td>
<td>5.2</td>
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<tr>
<td>Exp. 6 6</td>
<td>363</td>
<td>20</td>
<td>5.6</td>
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<tr>
<td>Exp. 7 7</td>
<td>303</td>
<td>13</td>
<td>4.3</td>
</tr>
<tr>
<td>Exp. 8 8</td>
<td>311</td>
<td>13</td>
<td>4.2</td>
</tr>
<tr>
<td>Exp. 9 9</td>
<td>301</td>
<td>17</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Average: 4.9

Note: Results of three independent experiments performed at same cell concentration (8 × 10⁴ cells/ml) and culture conditions.

**Table 1.** Consistent performance of BD ELISPOT Human IFN-γ Kits.

### Key Features and Benefits of the BD ELISPOT Human IFN-γ Kit

<table>
<thead>
<tr>
<th>Features</th>
<th>Benefits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Automated BD BioCoat™ coating process</td>
<td>Consistent results (Table 1)</td>
</tr>
<tr>
<td>Pre-coated plates</td>
<td>Minimal well-to-well and plate-to-plate variation</td>
</tr>
<tr>
<td>High quality antibody pairs (NALE™ capture Ab and specially formulated detection Ab)</td>
<td>Crisp, clearly-defined immunospots with low background (see cover photo and at right)</td>
</tr>
<tr>
<td>Cross-reactive with NHP*</td>
<td>Broad application including NHP studies</td>
</tr>
<tr>
<td>Contains all the necessary reagents</td>
<td>Convenient</td>
</tr>
</tbody>
</table>

*The BD ELISPOT Human IFN-γ Kit detects (i.e., crossreacts with) activated IFN-γ-producing cells prepared from Non-human Primates including Rhesus and Cynomolgus Macaques, Baboons, Chimpanzees, and Pigtail Monkeys.

**Table 2.** Key features and benefits of the BD ELISPOT Human IFN-γ Kit.
ELISPOT Results for Functional Assays: BD™ ELISPOT Human IFN-γ Assay Detects Bioactivity of Human IL-18

**Figure 3: ELISPOT analysis of IL-18-mediated effects on human IFN-γ-producing cells.**

Human PBMCs were prepared at $2 \times 10^6$ cells/ml in RPMI 1640 complete medium. Recombinant human IL-12 p70 (Cat. No. 554613) was added to the cell suspension at a final concentration of 400 pg/ml. The cell suspension was aliquoted and serially diluted. Recombinant human IL-18, ranging from 200 ng/ml to 1.5 ng/ml, was added to each aliquot in a 1:1 ratio and mixed. A fraction of these cells was used in the BD ELISPOT Assay, whereas the remaining fraction was cultured under standard conditions to generate supernatants destined for human IFN-γ ELISA measurement. 100 µl aliquots of the cell suspensions with IL-12, with or without IL-18, were added to pre-coated BD ELISPOT Human IFN-γ plates from the Kit (Cat. No. 552138). The plates were then cultured for 24 hours (optimal culture period). Thereafter, the plates were developed according to the BD ELISPOT Kit Manual.

Using the BD ELISPOT Human IFN-γ Kit, human IL-18 was found to synergize with human IL-12 by inducing increased numbers of IFN-γ-producing cells as shown by the formation of clear spots in the BD ELISPOT plate wells (panels A and B). The spot number in the BD ELISPOT Human IFN-γ Assay was proportional to the dose of recombinant human IL-18 that was added (panel B). The specificity of this assay was controlled by the BD ELISPOT wells that received cells with no IL-12 and IL-18 and did not generate spots (data not shown). The effective doses for IL-18 in generating responses in the BD ELISPOT Human IFN-γ Assay (●) and Human IFN-γ ELISA (■) were highly correlated (panel B).

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**Figure 4. ELISPOT analysis of co-stimulated mouse IL-2 secreting cells.** BALB/c mouse spleen cells were incubated in an ELISPOT plate that was hand coated with 5 µg/ml of anti-mouse IL-2 capture antibody (Component No. 51-1816KC in the BD ELISPOT Mouse IL-2 Set, Cat. No. 551076) and 1 µg/ml anti-mouse CD3 (Cat. No. 553057), with or without 2 µg/ml soluble anti-mouse CD28 (Cat. No. 553294) overnight. Biotinylated anti-mouse IL-2 detection antibody was added at 2 µg/ml (Component No. 51-1817KC in the BD ELISPOT Mouse IL-2 Set, Cat. No. 551076). Thereafter, the plates were developed according to the BD ELISPOT assay protocol. Panel A shows the image of spots and panel B shows the spot size distribution (determined by the ImmunoSpot® Series 2 Analyzer, CTL Analyzers LLC, Cleveland, OH.) from the plate well wherein cells were stimulated with plate-bound anti-mouse CD3 and soluble anti-mouse CD28. Panel C and panel D show the spots' image and size distribution in a plate microwell wherein cells were stimulated with plate-bound anti-mouse CD3 only.
References


Related BD Biosciences Literature and Internet Links


4. *BD ELISPOT Reagents website (including product list): www.bdbiosciences.com/pharmingen/elispot*

Cytotoxicity and Degranulation Assays

Introduction

Secretion of the cytotoxic granule contents by cytolytic effector cells is part of the mechanism that protects organisms from virus-infected cells and tumor cells. Both cytolytic T lymphocytes (CTL) and natural killer (NK) cells can lyse appropriate target cells through activation of granule exocytosis and release of cytotoxic mediators, such as perforin and granzymes. These then mediate entry to the target cell (perforin), and initiate apoptosis via the caspase- or mitochondrial-death pathway (granzymes).

The detection of specifically activated cytotoxic cells is traditionally carried out using a “killing”, or $^{51}$Cr-release assay. Assays that detect secretion of cytolytic granule contents provide an alternative functional measure of specifically activated and degranulating cells. Immunostaining of intracellular Granzyme B can be used as a phenotypic marker of cells that might have a potential to kill.\(^1\) This measure alone, however, gives no indication of the specificity of those cells.

Measurement of secreted Granzyme B using an ELISPOT assay gives a readout of cytotoxic effector function in response to a particular antigen. In addition, the Granzyme B ELISPOT is quantitative, reproducible and easy.

With new product developments we extend this functional readout to a flow cytometry-based assay for degranulation that employs CD107a, thus opening up the possibility for all the advantages of subset analysis offered by multiparameter flow assays.

BD\(^\text{TM}\) ELISPOT Human Granzyme B Kits and Sets

A Non-Radioactive Alternative to Chromium-Release Assays for the Detection of Cytolytic Cells

The Granzyme B ELISPOT assay is a high-throughput assay to monitor cytotoxic T cell function. It has been validated as a non-radioactive alternative to chromium-release assays, thus avoiding the use of potentially hazardous and difficult-to-dispose-of radioactive labels.\(^3\) Importantly, the results in the ELISPOT assay correlate with cytolytic responses measured by the classic radioactive $^{51}$Cr-release assay.\(^2\) In addition, ELISPOT Granzyme B assays typically yield superior signal-to-noise ratios when analyzing responses by human peripheral blood mononuclear cells (PBMCs). Furthermore, it is especially suitable for the large-scale testing of antigen/peptide reactivity in freshly isolated human cells.

BD ELISPOT Assay Protocol

An overview of the general BD ELISPOT assay protocol is shown in Chapter 6. The detailed protocols for the Granzyme B ELISPOT Set (Cat. No. 552572) and Kit (Cat. No. 552573) are the same as the standard protocols for our other BD ELISPOT Sets and Kits. A detailed User Manual can be found on our website (www.bdbiosciences.com) by doing a search for the catalog number.
BD ELISPOT Granzyme B Kits Ensure Consistent Results

BD Biosciences is pleased to offer BD ELISPOT Human Granzyme B assays either as a Reagent Set (Cat. No. 552572), or in a Kit format with pre-coated plates (Cat. No. 552573). The ELISPOT plates included in BD ELISPOT Human Granzyme B Kits are coated using an automated coating process to ensure consistent results with minimal variation. Containing all required reagents, the kit is designed to provide optimal spot quality, maximum time savings and convenience.

### ELISPOT Analysis of Granzyme B-secreting cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>human PBMCs at 3 x 10^4 cells/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulation</td>
<td>pre-stimulated with immobilized anti-human CD3 antibody, soluble anti-human CD28, rhIL-2, rhIL-4 for 48 hours; then with rhIL-2 and rhIL-4 for another 48 hours, then re-stimulated with 5 ng/ml PMA, and 500 ng/ml ionomycin for 15 hours.</td>
</tr>
<tr>
<td>Assay System</td>
<td>Human Granzyme B ELISPOT Kit (Cat. No. 552573)</td>
</tr>
</tbody>
</table>

**Selected Publications Using BD Granzyme B ELISPOT Assays**


References


Related BD Biosciences Literature

BD ELISPOT Set Instruction Manual (literature part no. 00-81014-6F). Available as a download from our website (quick search for the product number).

BD ELISPOT Set Instruction Manual (literature part no. 02-8100055-8E). Available as a download from our website (quick search for the product number).


Additional Technical Resources

Should you wish to have more detailed information about the use of BD Granzyme B ELISPOT assays, please contact our Scientific Support Team (contact numbers listed on the back pages, or e-mail: help.biosciences@europe.bd.com).
Cytotoxic Cell Assays Taken One Step Further: NEW
BD FastImmune™ CD107a Flow Cytometric Degranulation Assay

This new addition to our Immune Function assay portfolio takes cytotoxic cell analysis one step further. Based on the capture of CD107a, transiently expressed and released to the cell surface upon stimulation, this assay, like the Granzyme B ELISPOT, provides a functional readout. Moving to a flow cytometry-based assay, however, allows the addition of a multiparameter dimension. Combination with other measures, for example staining of other cell surface markers, Granzyme B or perforin, or IFN-γ staining (using the BD FastImmune™ system, see section on Intracellular Cytokine Detection by Flow Cytometry) opens the possibility for obtaining greater subset information on your cytotoxic cells. The CD107a detection assay also provides an alternative to ⁵¹Cr release assays.¹²⁴

CD107a: A Marker for Cytotoxicity

The BD FastImmune CD107a APC reagent is designed for the detection of cytotoxic CD⁸⁺ T lymphocytes directly in activated whole blood. The assay measures degranulation – a prerequisite for cytolysis¹ – upon stimulation with an antigen, thus providing information about the antigen-specific cytotoxic potential of cells.

Degranulating cells are identified by their surface expression of CD107a, which is a lysosomal associated membrane protein (LAMP-1) residing in cytolytic granule membranes located within the cytoplasm.¹⁷ CD107a is mobilized to the cell surface following activation-induced granule exocytosis.¹⁸⁹

Multiparameter Cytotoxic T Cell Response Phenotyping

Because of their parallel kinetics, CD107a and intracellular cytokines can be assessed at the same time in short-term-activated blood samples. Applications of this assay include studies of cytotoxic CD⁸⁺ T-cell responses to viral and tumor antigens,¹⁶ correlation of T-cell cytolytic potential and cytokine expression,¹ and live-cell sorting of functional antigen-specific CD⁸⁺ T cells.²

In-Situ Detection of Degranulating Cells

In the degranulation assay employing CD107a, whole blood is stimulated with antigen in the presence of the secretion inhibitors monensin and brefeldin A (BFA) (Figure 1). As a result of degranulation, CD107a is transiently expressed on the cell surface and rapidly re-internalized by the endocytic pathway.⁹ For this reason CD107a detection is maximized by performing antibody staining during cell stimulation. The addition of monensin prevents acidification and subsequent degradation of endocytosed CD107a antibody complexes, and BFA is required for optimal cytokine detection.
Figure 1. Overview of the BD FastImmune CD107a Flow Cytometric Degranulation Assay staining procedure
Protocol: BD FastImmune™ CD107a Flow Cytometric Degranulation Assay

Materials

Reagent

BD FastImmune CD107a APC (Cat. No. 641581), sufficient for 100 tests

For storage and handling recommendations, refer to the package insert.

Specimen Collection and Preparation

Blood should be collected in sodium heparin as other anticoagulants severely compromise the functional capacity of lymphocytes. It should be stored at room temperature to avoid platelet activation prior to use but should be used within 8 hours of collection. Antigen-presenting cell function is compromised with longer storage times, and loss of function can be compounded by shipping.

Reagents and Materials Required but Not Provided

- heparinized whole blood
- activation agent
  This kit is optimized for activation by a superantigen, such as SEB.
- BD FastImmune™ brefeldin A (Catalog No. 347688)
  Store in aliquots at –20°C. BD FastImmune brefeldin A also contains dimethyl sulfoxide (DMSO). Please refer to the product insert for warnings.
- BD GolgiStop™ protein transport inhibitor (containing monensin, Catalog No. 554724)
  Store at 2° to 8°C. Refer to the product insert for any warnings.
- BD FastImmune™ EDTA solution (Catalog No. 347689)
- BD FACSTM lysing solution (10X) (Catalog No. 349202)
  For dilution instructions and warnings, refer to the product insert.
- BD FACS permeabilizing solution 2 (Catalog No. 340973 (25 mL) or 347692 (10 mL)
  For dilution instructions and warnings, refer to the product insert.
- wash buffer
  First prepare stock solutions of 5% bovine serum albumin (BSA) in 1X phosphate-buffered saline (PBS) (filter sterilize) and 10% NaN3 in 1X PBS. Then prepare 500 mL of wash buffer by adding 50 mL of 5% BSA stock solution and 5 mL of 10% NaN3 stock solution to 445 mL of 1X sterile PBS. This represents final concentrations of 0.5% BSA and 0.1% NaN3 in PBS. Store at 4°C.
- 1% paraformaldehyde solution prepared in PBS containing 0.1% sodium azide

Unless otherwise specified, all products are for Research Use Only. Not for use in diagnostic or therapeutic procedures. Not for resale.
Store at 2° to 8°C in amber glass for up to 1 week. Refer to the product insert for warnings.

- 15-mL polypropylene tubes (Catalog No. 352096)
- 5-mL polystyrene tubes (Catalog No. 352058)
- vortex mixer
- micropipettor with tips
- 37°C water bath or incubator
- centrifuge
- BD FACS™ brand flow cytometer
  
  Refer to the appropriate cytometer user’s guide for information.
- BD Calibrite™ 3 beads (Catalog No. 340486) and BD Calibrite™ APC beads (Catalog No. 340487)
  
  Refer to the BD Calibrite beads product insert for instructions.
- BD FACSCComp™ software, version 4.2 or later, for cytometer setup
- BD FACSDiva™, BD CellQuest™ Pro, or BD CellQuest™ software for acquisition and analysis
  
  Refer to the appropriate software user’s guide for detailed information.
- BD FACS™ 7-color setup beads (Catalog No. 335775)
  
  Refer to the BD FACS 7-color beads product insert for instructions.

Staining Procedure

### Cell Activation and Staining

Because the staining for CD107a is carried out during cell activation in this assay, CD107a APC reagent is formulated with low azide concentration to minimize the possible compromising of the functional capacity of lymphocytes. Use sterile PBS for dilution and take care to avoid microbial contamination.

1. Dilute an aliquot each of BFA and BD GolgiStop protein transport inhibitor 1:10 with sterile PBS.
2. Label two 15-mL polypropylene tubes accordingly.
   - Tube 1: *Activated*
   - Tube 2: *Unstimulated*
3. Add the following to the *Activated* tube.
   - 0.5 mL of heparinized whole blood
   - antigen at titer (or other activation agent)
   - 5 µL of CD107a APC
   - 5 µL each of the diluted BFA and diluted BD GolgiStop protein transport inhibitor

Unless otherwise specified, all products are for Research Use Only.
Not for use in diagnostic or therapeutic procedures. Not for resale.
4. Add the following to the Unstimulated tube.
   • 0.5 mL of heparinized whole blood
   • 5 µL of CD107a APC
   • 5 µL each of the diluted BFA and diluted BD GolgiStop protein transport inhibitor

5. Vortex each tube gently and incubate for 4 to 6 hours at 37°C.
   
   **Note:** If you are using a specific antigen for the activation agent, you should activate an additional 0.5 mL of blood with a strong activation agent, such as SEB (final concentration of 1 µg/mL of blood). This tube is used as a positive control and simplifies gating.

6. Add 50 µL of EDTA solution in PBS to each tube.

7. Vortex vigorously and incubate for 15 minutes in the dark at room temperature.

8. Vortex again on high setting for 10 seconds.

If cells are to be **stained fresh**, proceed with the next section, *Preparing Fresh Cells*; if cells are to be **frozen** for later staining, go to the section on *Preparing Frozen Cells*.

**Preparing Fresh Cells**

1. Label two 5-mL polystyrene tubes accordingly.
   • Tube 1: Activated Sample (AS)
   • Tube 2: Unstimulated Sample (US)

2. Aliquot 200 µL of activated blood into the AS tube.

3. Aliquot 200 µL of unstimulated blood into the US tube.

4. Add 2 mL of 1X BD FACS lysing solution to each tube.
   Dilute 10X solution 1:10 with DI water before use.

5. Mix gently, and incubate for 10 minutes in the dark at room temperature.

6. Add 1 mL of wash buffer to each tube.

7. Centrifuge at 500 x g for 5 minutes at room temperature.
   Decant the supernatant.

Proceed to the section *Permeabilizing and Staining the Cells*.

**Preparing Frozen Cells**

1. Add 5 mL of 1X BD FACS lysing solution (dilute 10X solution 1:10 with DI water before use) to each activated and unstimulated 0.5-mL whole blood sample.

2. Vortex and incubate for 10 minutes in the dark at room temperature, and immediately place the tubes in a freezer at −80°C.
3. At the time of staining, thaw cells briefly in a 37°C water bath.
4. Add 7 mL of wash buffer, and centrifuge at 500 x g for 10 minutes at room temperature.
5. Decant the supernatant, and resuspend the pellet in 0.5 mL of wash buffer.

When ready to stain, label two 5-mL polystyrene tubes and aliquot 200 µL of blood as described in step 1 of the previous section, Preparing Fresh Cells.

Proceed to step 1 in the next section, Permeabilizing and Staining the Cells.

**Permeabilizing and Staining the Cells**

1. Add 1 mL of 1X BD FACS permeabilizing solution 2 to each tube.
   Dilute 10X solution 1:10 with DI water before use.
2. Vortex to resuspend the pellet, and incubate for 10 minutes in the dark at room temperature.
3. Add 2 mL of wash buffer to each tube, and centrifuge at 500 x g for 5 minutes at room temperature.
   Decant the supernatant.
4. Wash one more time by adding 2 mL of wash buffer to each tube and centrifuging at 500 x g for 5 minutes at room temperature.
   Decant the supernatant.
5. Add appropriate gating and intracellular cytokine reagents to th AS and US tubes.
   Incubate for 60 minutes in the dark at room temperature.
6. Add 2 mL of wash buffer to each tube, and centrifuge at 500 x g for 5 minutes at room temperature.
   Decant the supernatant.
7. Wash one more time by adding 2 mL of wash buffer to each tube and centrifuging at 500 x g for 5 minutes at room temperature.
   Decant the supernatant.
8. Add 300 µL of 1% paraformaldehyde in PBS.
   Vortex to resuspend the pellet, and store at 4°C in the dark before flow cytometry analysis.
   Analyze within 24 hours.

*Note:* Fixed and permeabilized cells are more buoyant than live cells and require higher centrifugal force to pellet. Therefore we recommend that you decant to remove the supernatant instead of performing the typical aspiration.
Data Acquisition and Analysis

Analyze on a BD FACSTM brand flow cytometer with laser excitation at 488 nm and 635 nm, such as the BD FACSCanto™ II, the BD FACSCanto™, the BD FACSCalibur™, or the BD™ LSR II flow cytometer. For cytometer setup instructions, refer to the appropriate instrument user’s guide.

- For BD FACSCanto II or BD FACSCanto cytometer setup, use BD FACS 7-color setup beads.
  Refer to the product insert for instructions.
- For BD FACSCalibur cytometer setup, use BD Calibrite beads and appropriate software. Use the 4-color lyse/no-wash (LNW) setting; minor adjustment of PMT settings and compensation might be required. Refer to the instructions for use for the beads and the software.
- For BD LSR II cytometer setup, refer to the application note, **BD Digital Flow Cytometer**.
  Refer to our website (bdbiosciences.com) or contact your local BD representative.

See **Figure 2** and **Figure 3** for representative data from experiments performed on normal heparinized whole blood, activated with SEB, stained with CD3 PE and CD8 PerCP-Cy™5.5 as gating reagents, IFN-γFITC for detecting intracellular cytokine, and analyzed on a BD FACSCalibur™ flow cytometer.

1. Acquire data with the appropriate software, using a forward scatter (FSC) threshold.
   - Collect at least 30,000, preferably 40,000, CD3+ lymphocytes.
   - During acquisition, set up an FSC vs SSC dot plot (**Figure 2**).
   - Gate on the lymphocytes (R1).
   - In addition, create a CD3 vs SSC dot plot with R1-gated lymphocytes, and draw a region around CD3+ cells (R2).
   - Collect at least 30,000 events, preferably 40,000 events, that fall in R1 and R2.

**Figure 2.** CD107a Assay Gating Strategy. Representative data from experiments performed on normal heparinized whole blood. Samples were activated with SEB, stained with CD3 PE and CD8 PerCP-Cy5.5 as gating reagents, IFN-γFITC for detecting intracellular cytokine production and CD107a as a degranulation marker, and analyzed on a BD FACSCalibur™ flow cytometer. At least 30,000 events falling into R1 and R2 were collected.
2. Create a CD3 vs CD8 dot plot to obtain double positive cells (R3). Display data as cytokine vs CD107a dot plots to determine CD107a and cytokine expression (Figure 3).

3. Analyze data using BD FACSDiva, BD CellQuest Pro, BD CellQuest, or BD Paint-A-Gate™ Pro software.

4. To obtain statistics, draw a quadrant region based on the unstimulated sample and apply the region to the activated sample files (Figure 3).

The %Gated statistic gives frequency of CD107a and cytokine-producing CD3+CD8+ cells.
- For CD107a expression: obtain the sum of %Gated statistics from the UR and UL region
- For cytokine expression: obtain the sum of %Gated statistics from the UR and LR region

Figure 3. CD107a Assay Gating Strategy (continued) and Results. The %Gated statistic gives the frequency of CD107a-expressing and cytokine-producing CD3+CD8+ cells.
- CD107a expression: sum of %Gated statistics from UR and UL regions
- Cytokine expression: sum of %Gated statistics from UR and LR regions

Calculating the Specific Response

The specific response of cells to any stimulus is obtained by subtracting the percent positive events in the unstimulated sample from the percent positive events in the activated sample.

Please also refer to Chapter 4 “BD FastImmune™ Cytokine Flow Cytometry” for more detailed tips on staining and analysis

Compatibility with Cytokine Staining

To see available options for combining the BD FastImmune CD107a Flow Cytometric Degranulation Assay with cytokine detection, please refer to Chapter 4, “BD FastImmune Cytokine Flow Cytometry”.
References


Additional Technical Resources

Our Scientific Support Team is trained to help you with technical questions related to these assays. Just contact them at the numbers listed at the end of this manual, or e-mail: help.biosciences@europe.bd.com.
Chapter 8

Cell Cycle Analysis and BrdU-Based Proliferation Assays for Flow Cytometry, Image Analysis and In Situ Applications

Introduction

Somatic cells proliferate to support tissue and organismal growth and to replace damaged cells. In the case of adaptive immunity, T and B lymphocytes proliferate (clonal expansion) in response to foreign antigenic stimulation. This hallmark response (along with the process of differentiation) ensures that sufficient numbers of antigen-specific effector and memory lymphocytes arise to successfully deal with the offending antigen (e.g., pathogenic microbes, viruses, toxins, and other foreign substances). Determination of the frequency and the nature of cells that respond to stimuli (and the type and magnitude of response measured at the single cell level) is crucial for better understanding the cellular basis of immunological and inflammatory responses in health and disease. For this reason, multiparameter flow cytometric analyses of lymphocyte activation and proliferation (cell cycle entry and progression) are featured in many immune function studies.

The eukaryotic Cell Cycle (aka, Cell Division Cycle) consists of a series of events that are involved in the growth, replication, and division of cells. The cell cycle can be subdivided into two major stages, interphase (a phase between mitotic events) and mitosis (Figure 1). There are three distinct, successive stages within interphase, called G1, S, and G2 phases. During G1 (first gap), cells “monitor” their environment and upon receipt of requisite signals, they induce growth (synthesize RNA and proteins). If conditions are right, cells “commit” to DNA synthesis (S phase) and replicate their chromosomal DNA. A G2 phase (second gap) follows in which cells continue to grow and prepare for mitosis. The G2 gap allows time for the cell to ensure DNA replication is complete before initiating mitosis. In mitosis (division), there are four successive phases called prophase, metaphase, anaphase, and telophase that are accompanied by cytoplasmic division (cytokinesis) giving rise to two daughter cells. For the most part, upon completion of the process, each daughter cell contains the same genetic material as the original parent cell and, in the case of lymphocytes, roughly half of its G2 level of cytoplasm.
In addition to these specific stages, the G0 phase has been described for cells that exit from the cell cycle and enter a quiescent, nondividing state. In response to external stimuli, some quiescent cells may undergo reactivation and express early response genes. Resting lymphocytes, for example, can leave G0 and enter the G1 phase of the cell cycle. The G0–G1 transition is marked by cell growth with measurable increases in newly-synthesized RNAs and proteins. This transition is reflected by the increased forward-scattered light signals (blast transformation) and by the expression of early cell-surface activation antigens (e.g., CD69 and IL-2Rα/CD25, see Chapter 4) on cells as detected by immunofluorescent staining and flow cytometric analysis. Another consequence of cellular activation may be the induction of programmed cell death (apoptosis). Together, through the counterbalancing processes of cell proliferation and apoptosis, and the establishment of quiescent or nondividing states, appropriate numbers and various types of somatic cells (including cells of the Immune System) are dynamically maintained (homeostasis) throughout the body’s lifespan.

**Flow Cytometric Analysis of Cycling Cell Populations**

A number of fluorescent probes have been developed for the flow cytometric analysis of cycling cells. The prototype for single-color flow cytometric analysis of cycling cells uses propidium iodide staining of the total cellular DNA content expressed by individual cells within activated cell populations. Further discrimination of the cycling status of cells can be achieved using multicolor flow cytometric analyses with two or more fluorescent probes. For example, cells can be analyzed by immunofluorescent staining of incorporated bromodeoxyuridine (BrdU) and staining with a fluorescent DNA-specific dye such as 7-aminoactinomycin D (7-AAD). This method enables determination of the frequency of cells that have synthesized particular levels of DNA (i.e., during the time interval that they were exposed to and incorporated the thymidine analog, BrdU) in the context of the G0/G1, S, and G2/M phases defined by total cellular DNA content.
DNA staining (ie, determined at the point in time when cells were stained for their total DNA levels). In addition, the fluorescent nucleic acid stain, Pyronin Y (PY), can be used to selectively stain RNA in the presence of a DNA specific dye (such as 7-AAD, DAPI, or Hoechst). Two-color flow cytometric analysis of RNA and DNA coexpression patterns permits further discrimination of cells within either G0 or G1 cell cycle phases based on their distinctive RNA contents. Multicolor flow cytometric analyses of cycling cell populations, such as with the combined use of 7-AAD and PY and immunofluorescent staining of incorporated BrdU or other markers (ie, three-color analyses and beyond), provide even higher resolution analyses of cells within different cell cycle compartments.

The combined use of immunofluorescence and fluorescent cell cycle probes with multiparameter flow cytometry provides an extremely important tool for analyzing the complex behaviors of individual cells within cell populations that mediate immunological responses. Detailed information can be obtained concerning the correlated expression patterns of cellular events that lead from cellular activation, growth, proliferation and differentiation to generate cells that play particular roles in immunological and inflammatory responses. Information of this type is crucial for better understanding how the Immune System works and thus how it can be manipulated to promote health.

Flow Cytometric Analysis of Cellular DNA Content

![Figure 2. Analysis of relative cellular DNA content using a fluorescent DNA dye and flow cytometry.](image)

This illustration depicts a typical data histogram that can be obtained from the relative DNA content analysis of cells within an actively-cycling cell population. The amount of DNA dye that is bound and the strength of the fluorescence signal it gives upon flow cytometric analysis is proportional to each cell's DNA content. The measurement of relative cellular DNA content is useful for identifying cells within G0/G1, S, and G2/M phases of the cell cycle.

Using fluorescent nucleic acid dyes, it is possible to identify the proportions of cells that are in one of the three interphase stages of the cell cycle by using flow cytometry to measure their relative DNA content (see Figure 2 and Table 1). Flow cytometric analyses of activated cell populations that are stained with a DNA stain, such as propidium iodide (PI), lead to the generation of characteristic cellular DNA content profiles as shown in Figure 2. These histograms can be separated into regions that represent cells within G0/G1, S, and G2/M phases of the cell cycle. Cells that are in the G0/G1 phase (before DNA synthesis) have a defined amount (1×) of DNA (ie, a diploid chromosomal DNA content).
During S phase (DNA synthesis), cells contain between $1\times$ and $2\times$ DNA levels. Within the G2 or M phases (G2/M), cells have a $2\times$ amount of DNA (i.e., a tetraploid chromosomal DNA content).

### Table 1. Commonly-used Fluorescent Dyes that Stain DNA for Cell Cycle Analysis by Flow Cytometry

<table>
<thead>
<tr>
<th>Dyes</th>
<th>Excitation Wavelength</th>
<th>Compatibility-Viable Cells</th>
<th>Compatibility-Fixed Cells</th>
<th>DNA Profile</th>
<th>Multicolor Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propidium Iodide</td>
<td>488 nm</td>
<td>No</td>
<td>Yes</td>
<td>Good</td>
<td>Yes*</td>
</tr>
<tr>
<td>7-AAD</td>
<td>488 nm</td>
<td>No</td>
<td>Yes</td>
<td>High CV</td>
<td>Yes</td>
</tr>
<tr>
<td>DAPI</td>
<td>350 nm</td>
<td>No</td>
<td>Yes</td>
<td>Good</td>
<td>Yes</td>
</tr>
<tr>
<td>Hoechst 33342</td>
<td>350 nm</td>
<td>Yes</td>
<td>Yes</td>
<td>Good</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* Propidium Iodide can be combined with FITC conjugates.

### Propidium Iodide

Propidium Iodide (PI)$^{2,3}$ is the most widely-used fluorescent dye for staining DNA in whole cells (or isolated nuclei). PI intercalates into the DNA helix of fixed and permeabilized cells. Because PI can stain both double-stranded RNA (dsRNA) and DNA (dsDNA), cells must be treated with RNase to ensure that PI staining is DNA specific. BD Biosciences offers PI/RNase staining buffer suited for this purpose (Cat. No. 550825) PI can be excited with the 488 nm wavelength of light typically generated by single-laser, benchtop flow cytometers. Since PI fluoresces strongly in both the orange and red regions (broad emission centered around 617 nm), it is often limited to use with fluorescein-conjugated antibodies (~525 nm peak emission) in single-laser, two-color flow cytometric analyses.

PI does not cross the intact plasma membrane of viable cells. However, PI can readily enter dead cells (and cells in late stages of apoptosis or that are fixed) that have damaged plasma membranes and can stain their dsRNA and dsDNA. For this reason, PI is also widely used as a discriminator of live and dead cells in experiments using immunofluorescent staining of unfixed cells with flow cytometric analyses. BD Biosciences offers a Propidium Iodide Staining Solution (Cat. No. 556463) that can be used for this purpose.

a. **Protocol for Staining DNA with Propidium Iodide for Cell Cycle Analysis$^{2,3}$**

1. Fix cells with ice-cold 70% ethanol ($\geq$ 1 hr, 4°C). Make sure that the cell suspension is thoroughly resuspended. Ethanol should be added dropwise while vortexing the cells to ensure fixation of all cells and to minimize clumping. Once fixed, cells may be stored for months in 70% ethanol at 4°C prior to PI staining and flow cytometric analysis.

2. Pellet $\sim 10^6$ cells (400 x g, 5 min) in tubes and wash 1x in staining buffer [Dulbecco’s PBS (DPBS) with 2% FCS and 0.01% NaN₃, 0.2 μm-pore filtered]. Ethanol-fixed cells may require higher centrifugal speeds to be pelleted tightly since they become more buoyant upon fixation than freshly-isolated or cultured cells. Care should be taken when aspirating off supernatants after centrifugation steps (especially after spinning cells out of ethanol) so that the cell pellet is not disturbed and cells are not lost.
3. Treat cells with ribonuclease A (RNase A) (Sigma, Cat. No. R5500; 100 Kunitz units/mg protein). The RNase A can be dissolved in DPBS at a concentration of 1 mg/ml, aliquoted, and stored frozen (–80°C). Add 50–100 μl of RNase A to each cell sample and incubate (30 min, 37°C).

4. Stain cells with 5–20 μg of PI (Sigma, Cat. No. P4170; Stock PI is at 1 mg/ml in distilled H₂O) added to 1 ml of staining buffer. Incubate for ≥ 30 min (room temperature) and then analyze samples by flow cytometry using linear amplification. Store samples protected from light at 4°C until flow cytometric analysis (ie, within 24 hours). When analyzing, keep the flow rate under 400 events/second.

Representative data from the flow cytometric analysis of PI-stained cells is shown in Figure 3.

Note: PI is a potential carcinogen and must be handled with extreme care.

![Figure 3](image-url)

**Figure 3. DNA content histograms: PI versus 7-AAD.** Cells from two mouse T cell lines, MGG3 (panels A, C, and G) and C20.4 (panels B, D, and F), were harvested, washed 1x with staining buffer, and fixed with ice-cold 70% ethanol (4°C, 1 hr). Cells were then washed to remove the ethanol. Panels A and B: Cells were treated with 100 µg of RNase A (30 min, 37°C) and were stained with 10 µg/ml of PI. Panels C and D: Cells were not treated with RNase A before PI staining. Panels E and F: Cells were stained with 10 µg/ml of 7-AAD. Notice that PI staining with RNase treatment (Panels A and B) gives a very clean DNA profile compared to no RNase treatment (Panels C and D). The DNA profiles obtained by 7-AAD staining of these same cells can be more variable with either higher (Panel E) or similar (Panel F) CV's when compared with the corresponding patterns for RNase-treated, PI-stained cells. Because of this variability, 7-AAD is not recommended as the first choice for single-color DNA content analysis.
Optional Protocol for 2-color Analysis with PI: First stain cells by direct or indirect immunofluorescence using fluorescein-conjugated reagents and then fix and stain cells as directed above. The DNA profiles for the cell subpopulations defined by the differential expression of a cell surface antigen can then be determined. Note that fixation of cells with 70% ethanol may interfere with the detection of some antigens even if they are stained prior to fixation.

Note: An alternative procedure is to incubate cells (30 min, 37°C) with a solution of 5 – 20 μg PI/ml in DPBS containing 50–100 μg/ml of RNase A (Cat. No. 550825).

7-aminoactinomycin D (7-AAD)

7-AAD is a DNA-specific dye that can be used for staining fixed and permeabilized cells to determine the DNA content profiles of cell populations in multicolor flow cytometric analyses.\textsuperscript{2,4} It is excited by the 488 nm wavelength (although excited better at 530 nm) of light typically provided by single laser flow cytometers. 7-AAD yields fluorescence emissions (emission peak ~ 650 nm) farther into the red light spectrum than PI and has very little spectral overlap with R-phycoerythrin (PE; emission peak ~578) and fluorescein (emission peak ~ 525).\textsuperscript{2} For this reason, 7-AAD can be used in the simultaneous, single-laser analysis of cellular DNA content (cell cycle position) and the coexpressed levels of two other cell-associated molecules detected by fluorescein- and phycoerythrin-conjugated antibodies (three-color fluorescence analyses). Additional parameters can be included in 7-AAD-based, cell cycle studies with the use of an additional laser(s) and fluorescent probes (eg, nucleic acid dyes and antibodies) for flow cytometric analyses of the nature of cells that transit through the cell cycle.

Although 7-AAD is useful for multicolor cell cycle analyses, it’s coefficient of variation (CV) of DNA fluorescence is larger (ie, broader G1 peak) than that obtained with PI. This may in part be explained by the fact that 7-AAD staining is more affected by chromatin structure (eg, the decondensation of chromatin upon cellular activation) than is PI or other DNA dyes. It should also be noted that 7-AAD may not give adequately strong fluorescence signals when using low powered (air cooled) 488 nm laser-based flow cytometers. This can result in DNA profiles that are not as well defined as those generated with other DNA stains. Because of this, PI is normally recommended for single-color DNA-content profiling whereas 7-AAD can be used for multicolor staining, (eg, DNA/RNA [7-AAD/PY] and BrdU/DNA [BrdU/7-AAD] staining). 7-AAD, like PI, will not enter live cells but will readily stain dead cells. For this reason, 7-AAD is also used as a live–dead cell discriminator for flow cytometric analyses. A solution of 7-AAD for viability staining is available from BD Biosciences as BD Via-Probe™ (Cat. No. 555815).

Please see the BrdU Flow Kit Staining Protocol (below) for the optional use of 7-AAD as a DNA stain in multicolor flow cytometric analyses. Representative data showing 7-AAD staining and flow cytometric analysis of cells is shown in Figure 3.
DAPI (4’-6-diamidino-phenylindole-2HCl) and Hoechst 33342 (HO33342)

Both of these dyes have a high specificity for DNA and bind preferentially to the A-T base pairs. These dyes can be excited at ~355nm by a UV light source (UV laser beam or a mercury arc-lamp). Since they are specific for binding to DNA, ribonuclease (RNase) treatment is not needed. DNA profiles of fixed cells are very similar to that of RNase-treated, PI-stained cells as shown in Figure 4. An advantage of these dyes is that they can also be used to stain viable cells for cell cycle analyses. Of the two dyes, HO33342 is the preferred dye for maintaining the viability of stained cells because it is less toxic than DAPI. Viable cell staining is performed by directly adding HO33342 to cells in culture and incubating for 30 – 90 minutes depending on the cells being labeled.

a. Protocol for Staining DNA with DAPI or Hoechst 33342 for Cell Cycle Analysis

1. Fix cells with 70% ice-cold ethanol (≥ 1 hr, 4°C). Make sure that the cell suspension is thoroughly resuspended. Cells may be stored for months in 70% ethanol at 4°C.
2. Centrifuge cells at 400 × g (5 min) and wash 1× in staining buffer (DPBS with 2% FCS and 0.01% NaN₃, 0.2 μm-pore filtered).
3. Resuspend in staining buffer with 0.5–1 μg/ml DAPI (Sigma Cat. No. D8417) or (0.5 – 1 μg) Hoechst 33342 (Sigma Cat. No. B2261). Incubate for ≥ 30 min (room temperature) and then analyze samples on a flow cytometer. Store samples protected from light at 4°C until flow cytometric analysis (ie, within 24 hours). When analyzing, keep the flow rate under 400 events/second. Stock solutions of DAPI or HO33342 can be made at a concentration of 1 mg/ml in distilled H₂O.

Representative data showing DAPI or PI staining and flow cytometric analysis of cells is shown in Figure 4.

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**Figure 4. Comparison of DNA staining profiles using DAPI or PI.** MGG3 T cells were fixed with ice-cold 70% ethanol for 1 hour at 4°C. The cells were either stained with DAPI (Panel A) or they were RNase A-treated and then stained with PI (Panel B). Cells were then analyzed on a BD LSR cytometer. The DNA profiles of cells stained with DAPI and PI are similar.
Flow Cytometric Analysis of Cellular RNA Content

Based on cellular DNA content alone, flow cytometric analysis can only reveal cells within the broadly-defined G0/G1, S, and G2/M phases. When staining cells for DNA content alone, cells within the G0 and G1 compartments cannot be distinguished. However, cells within G0 and G1 phases (and different stages within G1) can be distinguished by measurements of cellular RNA (and DNA) contents.2,5,6 The RNA levels measured in cells are mostly attributable to ribosomal RNA contents that can make up as much as ~80% of total cellular RNA content. Cellular RNA content increases as cells progress through G1, S, G2, and M phases of the cell cycle.

G0 cells are defined as resting or quiescent cells that have relatively low RNA content and a diploid chromosomal DNA content. Some quiescent cell types can be activated with an appropriate stimulus to enter the G1 phase and progress through cell cycle. The G1 phase is described as the phase wherein cells of exponentially growing populations increase their RNA and protein content (cell growth) to a level that may ultimately support their “competence” to enter S phase. Early (G1a) and late (G1b) compartments of the G1 phase can be distinguished by the flow cytometric analysis of the DNA and increasing RNA levels coexpressed by activated cell populations. Acridine Orange and PY (in combination with a DNA-specific dye) are dyes that can be used to differentially stain RNA and are described briefly below.

1. Acridine Orange [3,6-bis-(Dimethyamino)acridinium chloride hemi (zinc chloride salt)]2, 5

Acridine Orange (AO) is a metachromatic nucleic acid dye that can be efficiently excited with a 488 nm wavelength of light. It can emit strong fluorescence signals at both 530 nm and 640 nm. The unique binding and corresponding fluorescent emission characteristics of AO allow for the distinction of RNA and DNA level measurements in permeabilized cells. When AO is used for differential DNA versus RNA staining, it intercalates into double-stranded DNA as a monomer and fluoresces green (530 nm). In contrast, AO can bind to the phosphate groups of single-stranded RNA in an aggregated or stacking pattern that causes it to fluoresce red (640 nm). To obtain differential staining of DNA and RNA in cells, their RNA must be selectively denatured (ie, cells are treated with EDTA at low pH) to ensure that it is all in a single-stranded form. Under appropriate conditions, AO staining can be used to discriminate cells within different stages of the G1 phase of the cell cycle. A detailed AO staining protocol is found in reference 5.

A major disadvantage of using this dye is that it sticks to the plastic tubing in cytometers. For this reason, some researchers have found this dye too problematic to work with. If AO-stained samples are run on FACScan or FACS Calibur Flow Cytometers, then bleach must be run through the system for 10 minutes after its use. The completeness of AO removal can be monitored by running unlabeled viable cells through the system to see if there is any evidence of residual AO leaching from the tubing that can
stain cells. If AO remains, the cells will start to fluoresce green. If the lines are clear, ethanol should be run through the lines for 5 minutes. For cell sorters, it is recommended that the sample tubing be replaced.

2. Pyronin Y\textsuperscript{2,6–8}

Pyronin Y (PY) is the xanthene homologue of acridine orange. PY can be excited with the 488 nm wavelength of light typically generated by single-laser, benchtop flow cytometers and read at \~575 nm. At low concentrations, PY preferentially binds to dsRNA and fluoresces. High concentrations of PY can denature dsRNA; PY’s fluorescence is quenched when it is bound to ssRNA. At high concentrations, PY intercalates into dsDNA and fluoresces. Therefore, the dose of PY used for staining cellular RNA is crucial. PY should be used at a concentrations of 2 μM or less to avoid nonspecific staining. PY staining of cellular DNA can be blocked by co-staining cells with a DNA-specific dye. PY can be combined with 7-AAD or any of the UV-excited dyes (DAPI and Hoechst 33342) for the correlated analysis of RNA and DNA levels expressed by cells\textsuperscript{2,6–8}

High-resolution, cell cycle analyses are possible with PY and 7-AAD staining and flow cytometry. This method enables the distinction of individual quiescent G0 cells from activated G1 cells based on their RNA content. Cells in early G1, called the G1a phase, have a greater RNA content than G0 cells but a lower RNA content than S phase cells. Cells in the G1b phase have higher total RNA contents than G1a cells. Moreover, G1b cells have RNA contents at the same level or above the RNA levels expressed by S phase cells with the lowest cellular RNA content. Interestingly, even though M phase cells have greater total RNA levels (ie, dsRNA + ssRNA) than G2 phase cells, M phase cells stain less strongly with PY than do G2 phase cells\textsuperscript{6}. This may be due to the lower levels of dsRNA that are present in M versus G2 phase cells. It is important to use PY staining with fixed cells since PY can label mitochondria in viable cells.

Please see the BrdU Flow Kit Staining Protocol (below) for the optional use of PY as an RNA stain. Examples of flow cytometric analyses of PY-stained cells can be found in Figures 7 and 8.

Determination of S Phase Activity using BromodeoxyUridine

The immunofluorescent staining of incorporated bromodeoxyuridine (BrdU) and flow cytometric analysis enable high resolution determinations of the frequency and nature of individual cells that have synthesized DNA in the course of a specific time interval. In this method, BrdU (an analog of the DNA precursor thymidine) is incorporated into newly synthesized DNA by cells entering and progressing through the S (DNA synthesis) phase of the cell cycle\textsuperscript{9,10}. The incorporated BrdU is stained with specific anti-BrdU fluorescent antibodies (that do not recognize thymidine). The levels of cell-associated BrdU are then measured by flow cytometry. Often, staining with a dye that binds to total DNA, such as 7-AAD, is coupled with immunofluorescent BrdU staining. With this combination, two-color flow cytometric analysis permits the enumeration and characterization of cells that have actively synthesized DNA (BrdU incorporation
for a defined time interval) in terms of their cell cycle position (ie, G0/1, S, or G2/M phases as defined by 7-AAD staining intensities).\textsuperscript{11, 12}

Prolonged exposure of cells to BrdU allows for the identification and analysis of actively-cycling, as opposed to non-cycling, cell fractions. Pulse labeling of cells with BrdU at various time points, permits a detailed examination of cell-cycle kinetics. BrdU incorporation studies have been used in a variety of experimental protocols. These include \textit{in vitro} and \textit{in vivo} labeling systems, and studies investigating T cell proliferative responses in mouse and human cells.\textsuperscript{21-26}

An important feature of BD Pharmingen\textsuperscript{TM} BrdU Flow Kits (Cat. No. 559619 and 557891, with FITC-anti-BrdU; and Cat. Nos. 552598 and 557892 with APC-anti-BrdU) is that they provide reagents for immunofluorescent BrdU staining with a protocol that is compatible with the use of additional fluorescent nucleic acid dyes (eg, 7-AAD and PY) and antibodies (eg, phycoerythrin- and/ or allophycocyanin-conjugated antibodies) specific for other cellular molecules. These latter molecules may include cell surface antigens or intracellular proteins (eg, cytokines or cyclins) whose expression or activity may be related to the cell’s activation, entry and progression through cell cycle or cell death. This is possible because the BrdU Flow Kit staining protocol avoids DNA-denaturing agents such as acid, ethanol, and high temperatures that can change cellular light-scattering characteristics and limit the recognition of antigens by fluorescent antibodies.\textsuperscript{13–15}

Fluorescent antibodies that are capable of recognizing cell surface antigens or proteins in cells (eg, cytokines) that have been fixed with paraformaldehyde and permeabilized with saponin can be used with the BrdU Flow Kit. With this combination of reagents, the expressed levels of various surface or intracellular proteins can be measured by flow cytometry relative to the cell’s DNA synthetic activity (BrdU incorporation level). The kits ensure consistent results by providing detailed instructions and all critical reagents necessary to implement the staining protocol.

Contents of the BD Pharmingen BrdU Flow Kits

- Fluorochrome-conjugated anti-BrdU antibody (FITC-, or APC-)
- BD Cytofix/Cytoperm\textsuperscript{TM} Fixation/Permeabilization Solution
- BD Perm/Wash\textsuperscript{TM} Buffer (10x)
- BD Cytoperm\textsuperscript{TM} Plus Permeabilization Buffer
- 7-AAD solution
- BrdU solution (10 mg/ml)
- DNase solution
- Instruction Manual

Please note that BD also offers a BD FastImmune\textsuperscript{TM} Anti-BrdU FITC reagent that combines an anti-BrdU antibody with DNase (Cat. No. 340649). This reagent has been optimized for use in the BD FastImmune procedure. Please see Chapter 4 for more details.

Unless otherwise specified, all products are for Research Use Only. Not for use in diagnostic or therapeutic procedures. Not for resale.
Protocol for BrdU Incorporation and Staining

1. Labeling of Cells with BrdU
   
   a. **In vitro**-labeling of cultured cells and cell lines with BrdU
      
      Cells can be incubated with BrdU (Mol. Wt. 301.9) at a final concentration of 10–20 μM in cell culture medium (ie 10 – 20 μl of 1 mM BrdU per ml of culture medium). Prolonged exposure of cells to BrdU allows for the identification of actively-cycling cell populations. Pulse labeling of cells by brief BrdU exposures at various time points permits the determination of cell-cycle kinetics (eg, the timepoint for initiation of DNA synthesis).
      
      To label cells **in vitro**, carefully add 10 μl of BrdU solution (1 mM BrdU in 1× DPBS) directly to each ml of tissue culture media. For this step, it is important to avoid disturbing the cells in any way (eg, by centrifugation steps or temperature changes) that may disrupt their normal cell cycling patterns. The cell culture density should not exceed 2 × 10⁶ cells/ml. The treated cells are then incubated for the desired length of time. For pulse-labeling experiments, the choice of time points and lengths of time for pulsing depend on the test cell population’s rate of cell cycle entry and progression. For example, an effective length of time for pulsing an actively proliferating cell line is 30 – 45 minutes. Cells from the same population that are not BrdU-labeled are the recommended negative staining control for this assay. This will allow determination of background staining levels for the anti-BrdU monoclonal antibody.
      
   b. Methods for **in vivo**-labeling of mouse cells with BrdU
      
      Two common methods for **in vivo** BrdU labeling of cells have been reported. In one method, a BrdU-containing solution can be injected into the peritoneum (i.p.) of each mouse. (A 10 mg/ml solution of BrdU in sterile 1× DPBS is provided in the BrdU Flow Kits for in vivo use. Inject mice i.p. with 100 μl [1 mg] of the BrdU solution.) Incorporation of BrdU can be readily detected in the thymus and bone marrow in as little as 1 hr post injection. In a second method, mice can be fed with BrdU by adding it to their drinking water. Dilute BrdU to 0.8 mg/ml in the drinking water. The BrdU mixture should be made up fresh and changed daily. Prolonged feeding of BrdU can have toxic effects for the animal. For long-term studies, some researchers have reported that feeding mice with BrdU for 9 consecutive days followed by a changeover to normal water has worked effectively. BrdU incorporation by cells from these animals has been detected past 70 days.
2. BrdU Flow Kit Staining Protocol (Cat. No. 559619, 557891, 552598 and 557892))

For a protocol adapted for staining in 96-well plates, please see the Application Note: Multicolor Flow Cytometric Analysis of S-phase (BrdU Incorporating) Cells Using the BD FACSArray Bioanalyzer.

a. Immunofluorescent staining of cell surface antigens.

1. Add BrdU-pulsed cells (10^6 cells in 50 μl of staining buffer) to flow cytometry tubes. Staining buffer comprises Dulbecco’s PBS with 3% Fetal Bovine Serum (heat inactivated) + 0.09% (w/v) sodium azide.
2. Add fluorescent antibodies specific for cell-surface markers in 50 μl of staining buffer per tube and mix well.
3. Incubate cells with antibodies for 15 minutes on ice.
4. Wash cells 1× by adding 1 ml of staining buffer per tube, centrifuge (5 min) at 200 – 300 × g, and discard supernatant.

b. Fix and permeabilize cells with BD Cytofix/Cytoperm™ Buffer.

1. Resuspend cells with 100 μl of BD Cytofix/Cytoperm Buffer per tube.
2. Incubate cells for 15–30 minutes at room temperature or on ice.
3. Wash cells 1× with 1 ml of BD Perm/Wash™ Buffer (as in Step 1d).

c. Incubate cells with Cytoperm Plus Buffer.

1. Resuspend cells with 100 μl of Cytoperm Plus Buffer per tube.
2. Incubate cells for 10 minutes on ice.
3. Wash cells 1× by adding 1 ml of BD Perm/Wash Buffer (as in Step 1d).

d. Re-Fixation of cells

1. Resuspend cells with 100 μl of BD Cytofix/Cytoperm Buffer per tube.
2. Incubate cells for 5 minutes at room temperature or on ice.
3. Wash cells 1× by adding 1 ml of BD Perm/Wash Buffer (as in Step 1d).

e. Treatment of cells with DNase to expose incorporated BrdU,17, 18

1. Resuspend cells with 100 μl of diluted DNase (diluted to 300 μg/ml in DPBS) per tube (ie, 30 μg of DNase to each tube).
2. Incubate cells for 1 hour at 37°C.
3. Wash cells 1× by adding 1 ml of BD Perm/Wash Buffer (as in Step 1d).

f. Stain BrdU and intracellular antigens with fluorescent antibodies.

1. Resuspend cells with 50 μl of BD Perm/Wash Buffer containing diluted fluorescent anti-BrdU and/or antibodies specific for intracellular antigens.
2. Incubate cells for 20 minutes at room temperature.
3. Wash cells 1× by adding 1 ml of BD Perm/Wash Buffer (as in Step 1d).
   
   **Note:** Proceed to Step i if the staining of total DNA and/or RNA levels is not desired.

g. *Optional* — Staining of Total DNA for Correlated Cell Cycle Analysis.

1. Resuspend cells with 20 μl of the 7-AAD solution.

   Representative data showing BrdU and 7-AAD staining and flow cytometric analysis of cells is shown in Figure 5.

h. *Optional* — Staining of RNA using Pyronin Y for Correlated Cell Cycle Analysis.

1. Add 20 μl of a 25 μg/ml solution of Pyronin Y (PY) (Sigma Cat. No. P-9172; stock solution is 1 mg/ml in distilled H₂O) after cells have been incubated with 7-AAD for at least 5 minutes. PY will stain DNA if it is not blocked by a DNA-specific stain. Incubate cells for 5 minutes and then analyze the cells by flow cytometry. PY fluorescence data is usually acquired with linear amplification.

i. Resuspension of cells for Flow Cytometric Analysis.

1. Add 1 ml of staining buffer to each tube to resuspend cells.

2. Analyze stained cells with a flow cytometer (run at a rate no greater than 400 events/sec).

   **Note:** Samples may be stored overnight at 4°C, protected from exposure to light, prior to analysis by flow cytometry.

Representative data showing PY, BrdU, and 7-AAD staining and flow cytometric analysis of cells is shown in *Figure 7*.

It is important to note that all of the different response phases, from cellular activation, to cell cycle entry and mitosis, and to cell death may be accompanied by the differential expression of intracellular and cell surface molecules (see *Chapters 1 and 3*, respectively). In addition to serving as cell cycle phase markers, analysis of the expression patterns (coexpressed levels) of functional molecules along with DNA and RNA levels permits high-resolution, multiparameter analysis (eg, by multi-color flow cytometric analysis) of the molecular mechanisms that underlie cell cycling (and differentiation) and apoptosis.

3. Flow Cytometric Analysis of Stained Cell Samples

The flow cytometric data presented in the following examples (*Figures 5 – 7*) were acquired using a BD FACS™ brand flow cytometer equipped with a 488 nm argon laser. This laser permits the excitation of the fluorescent dyes, fluorescein isothiocyanate (FITC) (FL1), phycoerythrin (PE) (FL2) and 7-AAD (FL3), as well as the generation of forward angle (FSC) and side-scattered (SSC) light signals from illuminated cells. Use of other fluorochromes (eg, allophycocyanin) that are excited by light wavelengths outside of the range generated by the argon laser, require flow cytometers such as the BD FACSCalibur™ that have an additional
laser light source (Figure 8). It should be noted that with the addition of each different fluorochrome used for multicolor staining, the more critical becomes the challenge of properly compensating overlaps in detection of emitted fluorescent signals. Fluorescent signals from the nucleic acid dyes are normally acquired in the linear signal amplification mode, whereas signals generated by fluorescent antibody staining are typically acquired in a logarithmic mode.

Additional cytometer setup guidelines and analysis tips can be found in the BD Pharmingen BrdU Flow Kits Instruction Manual.

Figure 5. BrdU and 7-AAD coexpression profile for an actively-proliferating cell population. D10.G4.1 T cells were pulsed with 10 µM BrdU for 30 minutes. The cells were then stained for BrdU and 7-AAD using the BrdU Flow Kit and analyzed by flow cytometry. As shown by the boxed region gates, significant proportions of cells are found to occupy distinct cell cycle phases including G0/G1, S, and G2/M. Region 6 identifies apoptotic cells as determined by their sub-G0/G1 levels of DNA (stained by 7-AAD), Region 3 shows cells within the G0/G1 phases (39%) of the cycle, whereas Region 4 includes BrdU+ or S phase cells (39%), with Region 5 showing cells that occupy the G2/M phases (14%).

Figure 6. Detection of in vivo-cycling cells. C57BL/6 mice were injected i.p. with 1 mg of BrdU in solution for various time intervals. Animals were sacrificed at 40 minutes, 2 hours, and 4 hours post injection. Thymus and bone marrow cell suspensions were then prepared and stained for incorporated BrdU (FITC-anti-BrdU) and total DNA (7-AAD) levels. The 40–minute timepoint shows
the characteristic “horseshoe” pattern that is seen for cell populations that are pulsed with BrdU for a short time. Cells from mice that were pulsed for 2 hours also show the horseshoe pattern. However, another cell population of G0/G1 cells that has incorporated BrdU and has returned to the G0/G1 phase is now detectable. These cells are positive for BrdU but have 1× DNA levels (ie, diploid chromosomal DNA levels) as determined by their cellular DNA content (7-AAD level). The 4–hour timepoint has an even larger population of BrdU+ G0/G1 phase cells.

In vivo pulsing with BrdU can be used as an important tool for determining the status of cycling cell populations within experimental animals. As shown in Figure 6, significant fractions of cell populations obtained from tissues such as bone marrow and thymus (primary lymphoid organs) incorporate in vivo-supplied BrdU readily, within 30 minutes of in vivo-pulsing.

**Figure 7. Multiparameter cell cycle analysis of BrdU-pulsed, antigen-stimulated human PBMCs.** Human PBMCs were cultured alone (Panels A and C) or with 0.5 µg /ml of tetanus toxoid for 6 days (Panels B and D). 2 cells were pulsed with BrdU (20 µM) for 2 hr prior to harvest. Cells were then stained for their levels of incorporated BrdU (FL1), PY (FL-2), and 7-AAD (FL-3). The results show that the unstimulated cells are primarily in G0 with baseline levels of DNA (7-AAD) and RNA (PY) (Panel A). In contrast, significant proportions of the activated cells either express higher RNA and the same DNA levels (G1 phase) or coexpress higher levels of both RNA and DNA (Panel B). Likewise, nonactivated cells show no significant incorporation of BrdU (and baseline RNA levels) (Panel C) whereas a large proportions of activated cells show higher levels of RNA with or without incorporated BrdU (Panel D) in keeping with the coexpression pattern seen in Panel B.

Multiparameter RNA and DNA analysis of proliferating cell populations. Utilizing the BrdU Flow Kit allows for the simultaneous staining and analysis of cells for their cell surface phenotype, total cellular DNA and RNA contents, and levels of actively-synthesized DNA detected by BrdU that was incorporated for a specific time interval (Figures 7 and 8).
Figure 8. Cell cycle analysis of antigen-activated CD4+ and CD8+ Human PBMCs. Human PBMCs from a tetanus-vaccinated individual were stimulated with 0.5 µg/ml of Tetanus Toxoid (List Biologicals) for 6 days. During the final 2 hours of culture, the cells were pulsed with 20 µM BrdU. Cells were harvested and then stained using the BrdU Flow Kit for incorporated BrdU (FITC-anti-BrdU) and total DNA (7-AAD). In addition, total RNA (PY), and cell surface CD4 and CD8 levels (ie, using a cocktail of APC-conjugated anti-CD4 and anti-CD8) were assessed with additional reagents. Panel A shows the 7-AAD/BrdU profile for the population. Approximately 12% of the cells are in S phase. Panel B shows the BrdU and PY coexpression profiles of cells. At least 3 major profiles are noted including BrdU^{low}\text{PY}^{low}, BrdU^{low}\text{PY}^{+}, and BrdU^{+}\text{PY}^{bright}. These patterns suggest that cells must express a certain threshold level of RNA before they can actively synthesize DNA (ie, enter the S phase). Panel C shows the total DNA/total RNA profile (7-AAD/PY). This profile can be used to separate cells within G0 (low RNA) and G1 (intermediate to high levels of RNA) phases of the cell cycle. The data suggests that the activated PBMC population has many cells in the G1 phase of the cell cycle. BrdU (Panel D), DNA (Panel E), and RNA (Panel F) fluorescence profiles for the CD4+ (intermediate APC fluorescence) and CD8+ (high APC fluorescence) cell subpopulations are shown. The data suggests that the CD4+ cells are primarily responding to antigen activation (ie, show cells with increased levels of cellular RNA and DNA).
Carboxy-fluoresceindiacetate Succinimidyl Ester for Tracking Cell Proliferation by Flow Cytometry

Carboxy-fluoresceindiacetate succinimidyl ester (CFDA SE) is a very effective reagent to study the division progress of proliferating cells. It passively crosses the cell membrane and covalently binds to free amine groups of intracellular macromolecules. Endogenous cytoplasmic esterases remove the carboxyl groups, converting non-fluorescent CFDA SE to fluorescent CFSE that remains cell associated. Upon cell division, CFSE is distributed uniformly between daughter cells. Each cell division reduces the CFSE fluorescent intensity of daughter cells by approximately half. Each successive generation can be counted by the number of discreet fluorescent frequency distributions (e.g., histogram “peaks” or dot plot “clusters”) that are revealed upon flow cytometric analysis. The multipeak histogram (Figure 9A) shows several successive divisions that human peripheral blood lymphocytes have undergone when cultured for 72 hr with phytohemaglutinin.

Figure 9. HPBMCs were loaded with 1 μM CFDA SE for 10 minutes at 37°C. Cells were washed twice in 1x PBS then stimulated with 1.5% PHA for 72 hrs. Cells were harvested and then stained with PE anti-human CD4 and allophycocyanin (APC)-anti-human CD8 then analyzed on a BD FACScalibur™. Panel A is the CFSE histogram for the viable cell population. Panel B is the two color dot plot generated by the flow cytometric analysis of cells stained with PE anti-human CD4 and CFSE. Panel C is the two color dot plot obtained for cells stained with allophycocyanin-anti-human CD8 and CFSE.
By using CFSE as a dye for following cell proliferation, one can select additional parameters (e.g., CD markers or intracellular cytokines) and perform further flow cytometric analysis to characterize the nature of cells within any cell generation. For example, as shown in Figure 9B and 9C, CFSE staining can be coupled with staining for cell surface CD4 and CD8 to identify the proliferative activities of individual cells within T cell subpopulations. CFSE labeling has also been used to determine the number of divisions required for cells to express new immunoglobulin isotypes or to express cytokines such as Interleukin-4. In addition to its use in experimental culture systems, CFSE-labeling is very useful for determining the proliferative and migratory behavior of cells transferred to adoptive recipient animals.

CFDA SE Labeling Protocol

Dilute CFDA SE in dimethylsulfoxide (5 mg/ml is equivalent to 8.8 mM) and store aliquots at −80°C. The working solution of CFDA SE is between 10 nM–5 mM. Researchers should determine the optimal loading concentration for their particular cell type. Normally, a solution of 1 µM CFDA SE in 1x PBS is used to load up to 5 × 10^7 cells. Cells are loaded at 37°C for approximately 10 minutes. Times can vary depending on how bright or dim you wish to load the cells. CFSE is not highly toxic, but may negatively affect cell function. To stop the reaction, wash the cells twice in 1× PBS. Cells are now ready to be activated or transferred to recipient experimental animals. It is recommended that you confirm the loading of your cells on a flow cytometer prior to proceeding with an experimental protocol.

Summary

A brief overview of reagents and methods for BrdU and nucleic acid staining of cells and the multiparameter flow cytometric analysis of their cell cycle positions has been presented. More detailed information for performing these types of flow cytometric cell cycle analyses is provided by the references listed at the end of this chapter. The reagents and/or methods referred to in this chapter were presented because they are useful for performing multiparameter flow cytometric analysis of cell populations that are of particular interest in immune function studies.

Cell Cycle Analysis using High Content Imaging

Bioimaging Certified Anti-BrdU: For Reproducibility and Flexibility

Fluorescence microscopy and automated imaging may be the measurement platform of choice for adherent cells, or when screening for cell responses to potential drug candidates. For assessment of cell cycle status by fluorescence microscopy or imaging, BD Biosciences offers Bioimaging Certified anti BrdU antibodies in three different direct conjugates: Alexa Fluor® 488, Alexa Fluor® 555, and Alexa Fluor® 647. Bioimaging Certified reagents, which need to meet several specific criteria, are optimal for the use in high-content applications where reproducibility is essential. The availability of direct conjugates to several fluorochromes broadens the flexibility when designing multi-color assays.
The validated staining procedure, which includes a gentle fixation and permeabilization (using BD Cytofix™ fixation buffer and BD™ Phosflow Perm Buffer III), as well as DNase treatment, is compatible with staining with a number of other antibodies and/or DNA dyes. For details of the staining procedure, please refer to the Technical Data Sheets of the individual products, available on our website.

Use of these reagents in the particular application of quantitative analysis of cell cycle phase is described in a detailed Application Note: Quantitative Analysis of the Cell Cycle in Imaging Applications.

BD™ Cell Cycle Kit

The new BD™ Cell Cycle Kit (Cat. No. 558662) allows simultaneous determination of the percentage of cells in M and S phase in a one-step staining reaction. Combining anti-BrdU to identify cells in S phase, with an antibody to phospho-Histone H3 (Histone H3 pS28) to identify cells in M phase, this kit can be used to examine the effects of various drug treatments, or to determine cell cycle kinetics. The two kit antibodies are directly conjugated with fluorochromes that fluoresce in the green (Alexa Fluor® 488) and red (Alexa Fluor® 647) channels. This allows for an additional reagent in the orange (for example, Alexa Fluor® 555) channel. For more details, please refer to the Technical Data Sheet on our website.

Figure 10. Confocal image (20x) of HeLa cells stained with the BD™ Cell Cycle Kit. HeLa cells were stained with three components of the BD™ Cell Cycle Kit (Cat. No. 558662): Alexa Fluor® 488 Mouse anti-BrdU (pseudo-colored green), Alexa Fluor® 647 Rat anti-Histone H3 (pS28) (pseudo-colored red) and Hoechst 33342 (pseudo-colored blue). Co-staining of Hoechst and Histone H3 (pS28) appears pink. Confocal image taken using a BD Pathway™ 435 Bioimaging system and a 20x (0.75 NA) objective. (Please refer to the Technical Data Sheet on our web for a full-color image.)

In-Situ Immunohistochemical Detection of Proliferating Cells with the BrdU In-Situ Detection Kit

The most common technical difficulty in obtaining effective BrdU staining by immunohistochemistry is caused by the inability of the antibody to penetrate the DNA strands. The Retrievagen A antigen retrieval solution featured in BD Pharmingen™ BrdU In-Situ Detection Kits does not include any harsh DNA denaturing agents, as do some other methods, making these kits superior in quality and compatible with multicolor immunostaining.
Highlights of the BD Pharmingen™ BrdU In-Situ Detection Kit:

- **Versatile**
  - Works on paraffin and frozen sections, cytospins and cultured cells
  - Works in multiple species (Mouse, Rat and Human tested) due to biotinylated anti-BrdU

- **Sensitive**
  - Specific staining and low background due to monoclonal antibody
  - Special antigen retrieval system does not compromise tissue morphology
  - Ultrasensitive Streptavidin-HRP detection system with DAB allows visual detection of proliferating cells with as little as 45 minutes of pulsing with BrdU

- **Multicolor Immunostaining**
  - Kit design enables multicolor immunostaining of other surface or cytoplasmic antigens in addition to BrdU

- **Convenient**
  - Reagents are easy-to-use or require only simple dilution prior to use
  - Instruction manual includes detailed protocols for staining different sample types
  - Positive control slides provide a reference control

*Figure 11. Double color immunostaining for BrdU and CD45/B220. BALB/c mice were injected with 1 mg of BrdU via the intra-peritoneal route and after 24 hrs the spleen, thymus and gastrointestinal tract were harvested, formalin-fixed, and processed for paraffin sections. Double color immunostaining was performed on the paraffin sections of the mouse spleen using the BrdU In-Situ Detection Kit with the DAB substrate to label the BrdU, and anti-CD45/B220 antibody with AEC substrate to label the B lymphocytes. Cells positive for BrdU alone can be identified by the brown staining of the nucleus (lowest arrow), B lymphocytes can be identified by the reddish color on the cell membranes (upper arrow) and B lymphocytes that have incorporated BrdU can be identified by the double color labeling of dark brown in their nucleus and red color on the cell surface (middle arrow). Shown in the insert is the isotype control. Magnification 1000x (Please refer to the Technical Data Sheet on our web for a full-color image.)*
References


References (continued)


Related BD Biosciences Literature


Application Notes

1. Multicolor Flow Cytometric Analysis of S-phase (BrdU Incorporating) Cells Using the BD FACSArray™ Bioanalyzer (using BD Pharmingen™ reagents) [Literature Part No. 04-790030-6A]

2. Simultaneous Detection of Proliferation and Cytokine Expression in Peripheral Blood Mononuclear Cells (using the BD FastImmune™ System) [Literature Part No. 23-3941-00]

3. Quantitative Analysis of the Cell Cycle in Imaging Applications [Literature Part No. XEUR7013-00].
User Manuals

1. BD Pharmingen™ BrdU Flow Kit Instruction Manual
2. BD Pharmingen™ BrdU In-Situ Detection Kit Instruction Manual
3. BD Pharmingen™ Cell Cycle Kit Technical Data Sheet

Available as downloads at: wwwbdbiosciences.com (search “BrdU”)

For more details about individual products, please refer to the Technical Data Sheet (available on our website).

Additional Technical Resources

Our Scientific Support Team is trained to help you with technical questions related to the use of our products. Just contact them at the numbers listed at the end of this manual, or e-mail: help.biosciences@europe.bd.com.
Chapter 9

BD™ ELISA Reagents for Quantitation of Soluble Biological Response Modifiers

Introduction

Due to the amplifying potential of enzyme labels, immunoassays that utilize enzyme-conjugated detection antibodies have become increasingly popular because of their high specificity and sensitivity. In 1971, Engvall and Perlmann coined the term “Enzyme-Linked ImmunoSorbent Assay” that is perhaps better known by the acronym, “ELISA”. An ELISA is an enzyme-based immunoassay method that is useful for measuring the concentrations of soluble antigens (analytes).

Sandwich ELISAs are sensitive enzyme immunoassays that can specifically detect and quantitate the concentrations of soluble analytes, such as cytokines, chemokines, inflammatory mediators, and their receptors that are present in biological fluids (eg, serum, plasma, urine, cell culture supernatants, or lysates). ELISAs are also very useful for measuring the levels of immunoglobulins, complete component soluble CD antigens, and adhesion molecules that are related to immune function. The basic Sandwich ELISA Method (see Figure 1) makes use of highly-purified, antigen-specific capture antibodies that are noncovalently adsorbed (“coated” – primarily as a result of hydrophobic interactions) onto the inner surfaces of plastic microwells. The most widely-used ELISA plate is the 96-microwell, plastic plate (polystyrene or polyvinyl chloride). Recently, plates with larger numbers of microwells (eg, 384- and 1,536-microwell ELISA plates) are being widely used for high throughput analyses. The immobilized antibodies serve to specifically capture soluble analytes present in samples that are applied to the plate. After washing away unbound materials, the captured analytes are detected by biotin-conjugated, antigen-specific detection antibodies followed by an enzyme-labeled avidin or streptavidin stage. Following the addition of a chromogenic substrate-containing solution, the level of colored product generated by the bound, enzyme-linked detection reagents can be conveniently measured spectrophotometrically using an ELISA-plate reader set to read absorbances at an appropriate light wavelength [expressed as optical density (OD)]. The level of colored product is proportional to the amount of analyte (and detection reagents) that is specifically bound in ELISA.
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<tr>
<th>Step</th>
<th>Description</th>
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<tr>
<td>1.</td>
<td><strong>CAPTURE ANTIBODY</strong>&lt;br&gt;Coat wells with analyte specific Capture Antibody</td>
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<tr>
<td>2.</td>
<td><strong>BLOCKING</strong>&lt;br&gt;Block unoccupied well sites with protein</td>
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<tr>
<td>3.</td>
<td><strong>STANDARD &amp; SAMPLES</strong>&lt;br&gt;Add ELISA standard or samples</td>
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<td>4.</td>
<td><strong>DETECTOR ANTIBODY</strong>&lt;br&gt;Add Biotinylated Detection Antibody</td>
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<tr>
<td>5.</td>
<td><strong>ENZYME-AVIDIN AND SUBSTRATE</strong>&lt;br&gt;a. Add Enzyme-avidin&lt;br&gt;b. Develop with substrate</td>
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**Figure 1.** Basic stages of the sandwich ELISA for measuring soluble cytokine and chemokine protein levels (see text for further description).

By including serial dilutions of a standard analyte solution of known concentration, the sandwich ELISA supports the development of standard curves as shown in *Figures 2 and 3*. Standard curves (aka “calibration curves”) are generally plotted as the standard analyte concentration versus the corresponding mean OD value of replicates. The concentrations of the putative analyte-containing samples can be interpolated from the standard curve. This process is facilitated by using a computer and software that can acquire, store and reanalyze ELISA data.\(^1\) Generally, it is useful to perform a dilution series of the unknown samples to be assured that their OD readings can be interpolated from the linear portion of the standard curve. Depending on the nature of the ELISA reagents used, researchers may choose to apply different curve fit analyses to their data, including either linear-log, log-log, or four-parameter transformations.\(^1,\(^4,\(^5\)
Figure 2. Standard curve from a sandwich ELISA that measures human IL-2 protein levels.
A standard curve was generated by a sandwich ELISA using the purified 5344.111 antibody (Cat. No. 555051) as the capture antibody, doubling dilutions of recombinant human IL-2 protein solution, and biotinylated-B33-2 (Cat. No. 555040) as the detection antibody. Avidin-HRP and the ABTS substrate (Sigma, Cat. No. A1888) were used for development. The standard curve is displayed as the concentration of recombinant human IL-2 versus the microwell absorbances [ie, OD measured with 405 nm incident light using a Microplate Reader (Molecular Devices, SpectraMAX 250)].

Although opinions differ, one convention for determining the ELISA sensitivity is to choose the lowest analyte concentration that gives a signal that is at least two or three standard deviations above the mean background signal value.\textsuperscript{6, 7} Because of the enzyme-mediated amplification of the detection antibody signal, the sandwich ELISA can specifically measure very low concentrations (ie, pg/ml levels) of analyte within complex biological fluids that may be physiologically relevant (eg, cytokines in sera from autoimmune mice). Although many different types of enzymes have been used, horseradish peroxidase and alkaline phosphatase are the enzymes that are often employed in ELISA methods.\textsuperscript{1, 8}

Application Notes

Sandwich ELISAs are exquisitely specific because antibodies directed against two (or more, see Figure 1) distinct epitopes are often used.\textsuperscript{9} Due to their high specificity, sandwich ELISAs can often be used to discriminate between different molecules that may have overlapping biological functions, and are not resolvable by bioassay methods (and thus, not quantifiable). Although sandwich ELISAs are very useful for analyte detection and measurement, several limitations for the interpretation of ELISA data must be mentioned.\textsuperscript{9} For example, because test samples often come from tissue culture supernatants or biological fluids that contain molecules produced by mixed cell populations, the ELISA data does not provide direct information on the identities and frequencies of individual cells that produce analytes and the amount of analyte produced per cell. Techniques such as the Immunofluorescent Staining of Intracellular Molecules for Flow Cytometric Analysis (Chapter 3), the BD FastImmune\textsuperscript{TM} Cytokine Flow Cytometry (Chapter 4), or BD\textsuperscript{TM} ELISPOT Assays for Cells That Secrete Biological Response Modifiers (Chapter 6) are required for acquiring this type of information.
Several key issues need to be considered when designing experiments that involve measurements of biological molecules using sandwich ELISA methods. For instance, it is well known that cytokine protein production by stimulated cell populations is transient and that the kinetics of expression of different cytokine genes may vary. For these reasons, it may be necessary to collect test samples at several time points to fully characterize cytokine-production by an experimental animal or by a cultured cell population. As an example, in the case of stimulated mouse CD4+ T cell populations, the levels of IL-2 produced are detected relatively early after stimulation whereas the accumulated levels of IL-5 protein rise later in culture. It should also be noted that cytokine production can be stimulus- and cell subset-dependent. For example in the case of T cells, it is well known that naive T cells have a limited cytokine production capability (ie, primarily can produce IL-2 shortly after activation); whereas, memory T cells can produce high levels and different types of cytokine proteins including IFN-γ and IL-4, as well as IL-2. Moreover, T cell subsets have been found to produce cytokines differentially in response to different stimuli. Another consideration is that cytokine protein concentrations, measured at any one time point, may reflect the concurrent processes of cytokine secretion, uptake by cytokine receptor-bearing cells, and cytokine protein degradation. Because of these processes, the measured level of cytokine protein (or by analogy, other biological molecules) may significantly underestimate the actual cytokine-producing potential of cells. In these cases, it may be necessary to use complementary techniques such as Immunofluorescent Staining of Intracellular Molecules for Flow Cytometric Analysis (Chapter 3), BD FastImmune Cytokine Flow Cytometry (Chapter 4) or BD™ ELISPOT Assays for Cells That Secrete Biological Response Modifiers (Chapter 6) to gauge the relative levels of cytokine expression by various test cell populations.

Figure 3. Standard curve from a sandwich ELISA that measures soluble human CD14 protein levels. A standard curve was generated by using the purified 55-3 (Cat. No. 551403) as the capture antibody, doubling dilutions of recombinant soluble human CD14 protein and biotinylated-3-C39 (Cat. No. 551405) as the detection antibody. Avidin-HRP and TMB substrate (Cat. No. 555214) were used to develop the ELISA.
The concentrations of immunoreactive analyte measured by ELISA may or may not correlate directly with the measured concentrations of bioactive analyte molecules.\textsuperscript{9, 14} For example, an ELISA may utilize anti-cytokine antibodies that cannot discriminate between the precursor (inactive) and mature (bioactive) forms of a cytokine protein such as TGF-β. Moreover, a sandwich ELISA may detect partially-degraded cytokine proteins that have retained their immunoreactive properties (ie, at least two recognizable epitopes) but may have lost their bioactivity. In conclusion, sandwich ELISAs are useful indicators of the presence and levels of analytes, but they do not actually provide information concerning the biological potency or bioactivity of the detected analytes.

In addition to measuring the concentrations of soluble ligands, sandwich ELISAs are also useful for detecting soluble forms of receptors. These soluble receptors may also be important in the regulation of ligand functions. For example, soluble cytokine receptors may act as antagonists or as carrier proteins for cytokines \textit{in vivo}.\textsuperscript{15} Depending on the specificities and affinities of the antibodies used, it may be that soluble receptors can interfere with the recognition of ligands (and vice versa). With these caveats in mind, from the types and amounts of different analytes present (ie, biologically-relevant molecules), one can infer the potential mechanisms by which the Immune System or particular cell populations perform their functions. The multiplex BD\textsuperscript{™} Cytometric Bead Array (CBA) (\textit{Chapter 2}) is another important platform that can perform simultaneous measurement of multiple analytes in a single small-volume sample.

Another consideration is that in some cases the level of cytokines in serum samples may not accumulate to levels that are detectable by a standard ELISA assay. For mouse cytokines, the \textit{In Vivo} Cytokine Capture Assay (IVC) (\textit{Chapter 10}) may offer a complementary approach for directly measuring levels of cytokines produced \textit{in vivo} (in mice).

\textbf{BD\textsuperscript{™} ELISA Reagents: Choose from Three Options}

Whether you wish to rely on pre-optimized, ready-to-use ELISA systems, or develop your own assay, BD Biosciences offers you maximum flexibility. Choose from a large menu of optimized BD OptEIA\textsuperscript{™} Kits and BD OptEIA Sets, or customize your ELISA system by combining individual matched capture and detection antibody pairs with corresponding recombinant protein standards.

BD Biosciences offers Sandwich ELISA reagents in three different formats:

- BD OptEIA ELISA Kits
- BD OptEIA ELISA Sets
- Matched Antibody Pairs and Protein Standards

\textbf{BD OptEIA ELISA Kits - For the Fastest, Easiest and Most Reliable Quantitation of Soluble Proteins in Serum, Plasma and Tissue Culture Supernatants}

BD OptEIA ELISA Kits contain pre-coated 96-well plates, breakable in 12 strips of 8 wells for convenience, and all necessary reagents provided ready-to-use, for
streamlined soluble protein quantitation. With a BD OptEIA™ Kit, you eliminate the time and resources required to develop and optimize an ELISA assay.

Developed to provide the highest accuracy in measuring analytes in a variety of samples, the BD OptEIA ELISA Kits show optimal spike recovery performances for measuring analytes within complex biological fluids such as serum and plasma (see the end of this chapter for spike recovery data). All our BD OptEIA Kits utilize specifically fragmented detection antibodies, to reduce interference caused by autoantibodies and plasma proteins such as complement components, acute phase proteins and fibronectin.

**BD OptEIA Kit Contents:**

- 2* pre-coated 96-well plates (breakable wells: 12 strips of 8 wells)
- Lyophilized Standard
- Detection Antibody
- Enzyme Concentrate (Streptavidin-horseradish peroxidase)
- Standard/Sample Diluent
- ELISA Diluent
- Wash Concentrate
- TMB Substrate Reagent
- Stop Solution (1M phosphoric acid)
- Plate sealers

**BD OptEIA ELISA Kit Features:**

- **Sensitivity**
  Detection as low as 1 pg/ml
- **Precision**
  Low inter- and intra-assay variation
- **Ease of Use**
  3-hour incubation time, at room temperature, no shaking required
- **Accuracy**
  Specifically formulated to minimize background

**Enhanced Kit II Format – for highest accuracy**

Several of our BD OptEIA ELISA Kits have been reformulated for enhanced sensitivity. Increased sensitivity is important, as many cytokines or other soluble proteins do not accumulate to high concentrations in the blood. These kits – referred to as Kit IIs – have been developed for highest accuracy with serum, plasma and other complex biological fluids. BD OptEIA ELISA Kit IIs demonstrate mean spike recovery improvements of 7% to 41% in serum and

*1 plate for human C3a, C4a and C5a BD OptEIA Kits*
plasma. Examples of BD OptEIA™ ELISA Kits with enhanced sensitivity include our BD OptEIA Human C5a ELISA Kit II (Cat. No. 557965) and our BD OptEIA Human IL-1β ELISA Kit II (Cat. No. 557966), which are capable of detection in the ranges of 0.078-5.0 ng/ml and 2.0-125.0 pg/ml, respectively.

Please refer to our on-line catalog for the most up-to-date list of BD OptEIA ELISA Kit IIIs available.

BD OptEIA ELISA Sets – An Economical Alternative to Test Serum, Plasma and Tissue Culture Supernatants

BD OptEIA ELISA Sets contain reagents sufficient for processing up to 20 96-well plates. They feature pre-validated antibody pairs, enzyme reagent and recombinant standard, packaged for convenience. All three critical ELISA reagents are pre-titered and Quality Control tested as a unit, allowing you to build your own ELISA while saving the time and the expense of tedious optimization experiments.

The Sets utilize specially-formulated F(ab’)_2 detection antibodies to reduce backgrounds caused by nonspecific binding. In addition, they have demonstrated quantitation of expected baseline analyte levels in various biological fluids including serum and plasma samples from healthy normal donors.

BD OptEIA Set Contents:

- Capture and Detection Antibodies (*both pre-titrated*)
- Enzyme Reagent (*Streptavidin-horseradish peroxidase, pre-titrated*)
- Recombinant Standard Protein

BD OptEIA ELISA Sets contain reagents sufficient for processing up to 20 96-well plates.
BD Biosciences Ancillary Reagents

recommended for use with BD OptEIA™ Sets

- BD OptEIA Reagent Set A, pH 6.5 buffer (Cat. No. 550536)
- BD OptEIA Reagent Set B, pH 9.5 buffer (Cat. No. 550534)
- BD OptEIA TMB Substrate Reagent Set (Cat. No. 555214)
- BD OptEIA Assay Diluent (Cat. No. 555213)
- BD Falcon™ 96-well clear, enhanced, ELISA Microplates (Cat. No. 353279)

BD OptEIA ELISA Set Features

- Matched, pre-optimized reagents in one package
- Designed to accurately measure cytokines, chemokines, and other BRMs in serum, plasma, cell lysates and cell culture supernatants
- F(ab’)2 detection antibodies used to reduce backgrounds caused by non-specific binding
- Lot-specific assay data provided
- Sufficient reagents for twenty 96-well ELISA plates
- Outstanding value

BD OptEIA ELISA Sets Data (typical standard curve)

Similar to our BD OptEIA Kits, several of our BD OptEIA ELISA Sets have been reformulated for enhanced sensitivity. Examples of BD OptEIA Sets with enhanced sensitivity include our BD OptEIA Mouse IFN-γ ELISA Set II (Cat. No. 551866) and our BD OptEIA Human IL-1β ELISA Set II (Cat. No. 557953), which are capable of detection in the ranges of 3.1-200.0 pg/ml and 4.0-250.0 pg/ml, respectively.

Please refer to our on-line catalog for the most up-to-date list of available BD OptEIA ELISA Sets.
Matched Antibody Pairs and Protein Standards – For Highest Flexibility and Cost Efficiency

We also offer a wide range of individual ELISA reagents, available à la carte. These include matched unlabeled capture antibodies and biotinylated detection antibodies, and recombinant protein standards. Preferably for use with tissue culture supernatants, this cost-effective approach to ELISA provides you the maximum flexibility to design your own ELISA assay.

Please refer to our on-line catalog for the most up-to-date list of available Matched Antibody Pairs for ELISA.

BD OptEIA ELISA Kit Assay Protocol

1. Add 50 µl of ELISA Diluent per well
2. Add 100 µl of standard or sample per well
3. Incubate 2 hr at RT, wash
4. Add 100 µl of the prepared Working Detector per well
5. Incubate 1 hr at RT, wash
6. Add 100 µl of TMB One-Step Substrate Reagent per well
7. Incubate 30 min at RT
8. Add 50 µl of Stop Solution per well, read absorbances at 450 nm

Please refer to the Kit Booklet or visit www.bdbiosciences.com/bd_opteia_elisa for a detailed specific protocol.

BD OptEIA ELISA Set Assay Protocol

1. Coat plates with 100 µl of diluted Capture Ab per well
2. Incubate overnight at 4°C, wash
3. Block plates with 200 µl Assay Diluent per well
4. Incubate 1 hr at room temperature (RT), wash
5. Add standard or sample at 100 µl per well
6. Incubate 2 hours at RT, wash
7. Add Working Detector at 100 µl per well
8. Incubate 1 hr RT, wash
9. Add Substrate Solution at 100 µl per well
10. Incubate 30 min at RT in the dark
11. Add 50 µl of Stop Solution per well, read absorbances of well samples at a 450 nm light wavelength

Please refer to the Set Insert or visit www.bdbiosciences.com/bd_opteia_elisa for a detailed specific protocol.
ELISA Protocol —
General Procedure using Matched Antibody Pairs
(For detailed explanations see Footnotes and References)

Capture antibody:
1. Dilute the purified capture antibody to 1 – 4 μg/ml in Coating Buffer. Add 50 – 100 μl of diluted antibody to the wells of an enhanced protein-binding ELISA plate (eg, BD Falcon™, 96-well clear, enhanced, ELISA microplates, Cat. No. 353279).
2. Seal plate to prevent evaporation. Incubate overnight at 4°C.

Blocking:
3. Bring the plate to room temperature, remove the capture antibody solution, and block non-specific binding by adding 200 μl of Blocking Buffer per well.
4. Seal plate and incubate at room temperature for 1 – 2 hours (best results may be obtained with longer incubation, eg, overnight at 4°C).
5. Wash ≥ 3 times with PBS/Tween e.

Standards and Samples:
6. Add standards and samples (diluted in Blocking Buffer/Tween e) at 100 μl per well.
7. Seal the plate and incubate for 1–4 hours at room temperature or overnight at 4°C.
8. Wash ≥ 4 times with PBS/Tween.

Detection antibody:
9. Dilute the biotinylated detection antibody to 0.5–2 μg/ml in Blocking Buffer. Add 100 μl of diluted antibody to each well.
10. Seal the plate and incubate for 1 hour at room temperature.
11. Wash ≥ 4 times with PBS/Tween e.

Avidin-Horseradish Peroxidase (HRP):
12. Dilute the avidin- or streptavidin- HRP conjugate or other enzyme conjugate to its pre-titered optimal concentration (eg, BD Biosciences, Cat. No. 554058) in Blocking Buffer. Add 100 μl per well.
13. Seal the plate and incubate at room temperature for 30 minutes.
14. Wash ≥ 5 times with PBS/Tween.
Substrate (TMB):

15a. Mix 5 ml of Reagent A with 5 ml of Reagent B (BD Biosciences, Cat. No. 555214), and immediately dispense 100 μl into each well. Incubate at room temperature (5–80 minutes) for color development. Add 50 μl of Stopping Solution\(^1\) to stop the color reaction.

16a. Read the optical density (OD) for each well with a microplate reader set to 450 – 570 nm.

Substrate (ABTS):

15b. Thaw ABTS Substrate Solution\(^1\) within 20 minutes of use. Add 100 μl of 3% H\(_2\)O\(_2\)\(^g\) per 11 ml of substrate and vortex. Immediately dispense 100 μl into each well. Incubate at room temperature (5 – 80 minutes) for color development. Add 50 μl of Stopping Solution\(^h\) to stop the color reaction.

16b. Read the optical density (OD) for each well with a microplate reader set to 405 nm.

Footnotes:

a. Coating Buffer: 0.1 M Na\(_2\)HPO\(_4\), adjust pH to 9.0 with 0.1 M NaH\(_2\)PO\(_4\). For measuring mouse IL-10, mMCP-1, mTNF, and rat GM-CSF the coating buffer must be adjusted to pH 6.0.

b. Phosphate Buffered Saline (PBS): 80.0 g NaCl, 11.6 g Na\(_2\)HPO\(_4\), 2.0 g KH\(_2\)PO\(_4\), 2.0 g KCl; mix with deionized (ddH\(_2\)O) water to make 10L of PBS ph to 7.0.

c. PBS/Tween: 0.5 ml of Tween-20 in 1 L PBS.

d. Blocking Buffer: Prepare 10% fetal bovine serum (FBS), 10% newborn calf serum (NBCS) or 1% bovine serum albumin (BSA; immunoassay grade) in PBS. The Blocking Buffer should be filtered to remove particulates before use.

e. Blocking Buffer/Tween: Add 0.5 ml Tween-20 to 1 L of Blocking Buffer.

f. ABTS Substrate Solution: Add 150 mg 2,2’-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt (eg, Sigma, Cat. No. A-1888) to 500 ml of 0.1 M anhydrous citric acid (eg, Fisher; Cat. No. A-940) in ddH\(_2\)O; pH to 4.35 with NaOH. Aliquot 11 ml per vial and store at -20°C. Add 100 μl 3% H\(_2\)O\(_2\) prior to use.

g. 3% H\(_2\)O\(_2\) Solution: Add 10 ml of 30% H\(_2\)O\(_2\) to 90 ml of H\(_2\)O. Protect from prolonged exposure to light.

h. ABTS Stopping Solution: (20% SDS/50% DMF): Add 50 ml of dimethylformamide (DMF) (Pierce, Cat. No. 20672) to 50 ml ddH\(_2\)O, then add 20.0 g sodium dodecyl sulfate (SDS) (CMS, Cat. No. 424-749).

i. TMB Stop Solution: Prepare 1.0 M Phosphoric Acid, Mix 115 ml of 85% Phosphoric Acid (Fisher Cat. No. A242) with deionized water to make 1 L of Solution.

Sandwich ELISA Helpful Hints

a. Determining Optimal Signal: To determine the optimal signal and lowest background for the ELISA, the capture antibody (1–4 μg/ml) and detection antibody (0.25 – 2 μg/ml) should be titrated against each other in a preliminary experiment. An appropriate range of serial dilutions for the ELISA standard should be included. A suggested range is generally provided in the Technical Data Sheets (TDS) for ELISA reagents.
Generally, use of the capture antibody at 2 μg/ml and the detection antibody at 1 μg/ml provides strong ELISA signals with low background.

b. **ELISA Standard Handling:** Please read the TDS for each ELISA Standard carefully. Handling instructions are lot-specific. For maximum recovery of an ELISA standard, the vial of standard should be quick-spun before opening. Lyophilized standards should be reconstituted as indicated on the lot-specific TDS. BD Biosciences recommends keeping the ELISA standard solution in a concentrated form (eg, ≥ 1 μg/ml) and in the presence of a protein carrier for long-term storage at −80ºC.

c. **Generating The Standard Curve:** The linear region of many sandwich ELISA standard curves is generally obtainable in a series of eight two-fold dilutions of the ELISA standard (eg, from 2000 pg/ml to 15 pg/ml works for many cytokines – however, use dilution ranges recommended in the ELISA antibody TDS from BD Biosciences). For best results, use recommended ELISA standards from related BD Biosciences Antibody Technical Data Sheets to generate appropriate standard curves. To increase sensitivity beyond that obtainable with the standard ELISA protocol, amplification kits, tertiary reagents, or alternate enzyme/substrate systems can be used. If the standard curve is not linear, check for pipetting errors, insufficient washing or improper preparation of standard stock solution.

d. **Background And Precision Issues:** High backgrounds in blank wells (ie, OD > 0.20) or poor consistency of replicates can be overcome by increasing the stringency of washes and optimizing the concentration of capture and detection antibodies. For example, during washes, the wells can be soaked for ~ 1 minute intervals; be sure all wash buffer is completely removed between washes. Lower concentrations of detection antibody or more washes after incubating the detection antibody can reduce background. Do not use chromogen that appears to have color prior to use. It may have been exposed to light. Evaporation of wells during the assay can also cause elevated background. For best results use a plate sealer for all incubation steps. When measuring analytes in complex fluids, such as serum, sample diluents that include irrelevant Ig are suggested.

e. **Optimal Sensitivity:** For optimal sensitivity, incubation of standards and samples for longer periods at room temperature or overnight at 4ºC is recommended.

f. **Weak or No Color Development:** If no signal is observed, then check the following:

i. Verify that the appropriate antibody clones were used.

ii. Check the activity of the enzyme/substrate system. For example, coat several ELISA wells with biotinylated detection antibody (1 μg/ml; several hours) in coating buffer. After blocking, wash the ELISA plate several times and then proceed with the sandwich ELISA protocol from Step 13. If the enzyme/substrate system is active, then a strong signal should be seen.

iii. Verify the activity of the ELISA standard or try a new sample of the
standard.

iv. If using peroxidase as the enzyme for color development, avoid sodium azide in wash buffers and diluents, as this is an inhibitor of peroxidase activity.

g. **Choosing Substrates:** The kinetics of color development of ABTS is slower than TMB substrate. When multiple plates are performed by one individual and tight control over the color development is needed, please use ABTS substrate for the best results.

**Performance Data using BD OptEIA™ ELISA Kits**

**BD OptEIA ELISA Kits Data (Human TNF Standard Curve)**

![Graph showing optical density vs. TNF concentration]

The Spike Recovery test assesses ELISA accuracy in various matrices by measuring the difference between the endogenous concentration of a sample and the concentration following addition of a purified sample of the analyte. The difference is expressed as a percentage of the amount of analyte added.

The Linearity test is performed by serially diluting positive samples of various matrices with Standard/Sample Diluent, and multiplying the result by the dilution factor.

**Spike and Recovery**

Three different amounts of TNF were spiked into various matrices. Results are compared with the same amounts of TNF spiked into Standard/Sample Diluent, as follows:

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Spike Concentration (pg/ml)</th>
<th>Average % Recovery</th>
<th>Average Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (n = 9)</td>
<td>250</td>
<td>89</td>
<td>81 – 95</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>86</td>
<td>75 – 93</td>
</tr>
<tr>
<td></td>
<td>62.5</td>
<td>93</td>
<td>78 – 109</td>
</tr>
<tr>
<td>Plasma (n = 5)</td>
<td>250</td>
<td>96</td>
<td>95 – 100</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>88</td>
<td>81 – 99</td>
</tr>
<tr>
<td></td>
<td>62.5</td>
<td>88</td>
<td>74 – 104</td>
</tr>
<tr>
<td>Cell Culture</td>
<td>250</td>
<td>98</td>
<td>88 – 106</td>
</tr>
<tr>
<td>Media (n = 3)</td>
<td>125</td>
<td>106</td>
<td>101 – 109</td>
</tr>
<tr>
<td></td>
<td>62.5</td>
<td>105</td>
<td>100 – 110</td>
</tr>
</tbody>
</table>
Linearity

Various samples were spiked with high concentrations of TNF, serially diluted with Standard/Sample Diluent, and run in the BD OptEIA ELISA Kit. ELISA testing results were as follows:

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Serum (n = 9)</th>
<th>Plasma (n = 5)</th>
<th>Cell Culture Media (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:2</td>
<td>Average % of Expected 104</td>
<td>106</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>Range 95 – 116</td>
<td>100 – 111</td>
<td>101 – 110</td>
</tr>
<tr>
<td>1:4</td>
<td>Average % of Expected 108</td>
<td>105</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>Range 90 – 124</td>
<td>97 – 114</td>
<td>95 – 108</td>
</tr>
<tr>
<td>1:8</td>
<td>Average % of Expected 116</td>
<td>104</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>Range 95 – 143</td>
<td>93 – 108</td>
<td>90 – 97</td>
</tr>
<tr>
<td>1:16</td>
<td>Average % of Expected 107</td>
<td>102</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>Range 85 – 140</td>
<td>89 – 133</td>
<td>78 – 98</td>
</tr>
</tbody>
</table>

References


**Relevant BD Biosciences Literature and Links**

**User Manuals**

Product-specific instructions for use are given in the Technical Data Sheet (TDS) for each product, available for download from our online catalog: [www.bdbiosciences.com/](http://www.bdbiosciences.com/)

Online Catalog » Research Reagents » ELISA Reagents »

**Video**


**Additional Technical Resources**

Our Scientific Support Team is trained to help you with technical questions related to the BD™ ELISA products. Just contact them at the numbers listed at the end of this manual, or e-mail: help.biosciences@europe.bd.com.
Chapter 10

In Vivo Capture Assays for Sensitive and Direct Measurement of Mouse Cytokine Production In Vivo

Introduction

Measurement of in vivo cytokine production is often difficult, owing to the short in vivo life-span of many cytokines. Rapidly utilized, catabolized and excreted, these often do not accumulate in serum to levels that are detectable by a standard ELISA assay. Cytokine production by cells re-stimulated in vitro is relatively easy to measure, using for instance ELISA or BD™ Cytometric Bead Array (CBA), however, cytokine production by cells in vitro may not always fully reflect in vivo cytokine secretion.

The In Vivo Cytokine Capture Assay (IVC), also known as the Cincinnati Cytokine Capture Assay, represents a complementary approach, for directly measuring levels of cytokines produced in vivo in mice.1,2

How the Assay Works

Mice are injected with a biotin-labeled monoclonal antibody that is specific for the cytokine targeted, and provided in a No Azide / Low Endotoxin (BD NA/LE™) format. This allows the targeted cytokine to accumulate in vivo for a defined period of time (2-72 hours) in the form of a long-lived, soluble cytokine:anti-cytokine antibody complex. The concentration of the complex – e.g. of the “in vivo captured” cytokine – is then

Figure 1. Overview of the In Vivo Capture Assay for Cytokines
measured in collected serum samples via a two-site sandwich ELISA, using microplate wells coated with a purified monoclonal antibody to a second epitope on the same targeted cytokine. The assay is developed with streptavidin-horseradish peroxidase (SAv-HRP) and a tetramethylbenzidine (TMB) chromogenic substrate solution. For quantitation, a standard curve is generated on each plate by adding serial dilutions of a known amount of recombinant mouse cytokine standard that is complexed with a known amount of biotinylated cytokine-binding antibody.

Advantages Compared to Conventional Cytokine ELISAs

Sensitivity
The *In Vivo* Capture Assay for Mouse Cytokines increases the sensitivity of detection of *in vivo* cytokine production 50–100-fold, over levels measurable using standard ELISA detection of serum cytokines.

Direct correlation
The amount of cytokine detected is proportional to the amount secreted *in vivo*, and this regardless of the site of cytokine production in the body. Also, the *In Vivo* Capture Assay does not affect ongoing cytokine-dependent immune responses: as only small quantities of antibody are injected, these “sample”, rather than neutralize, the secreted cytokine.

Ease of use
Both cytokine-binding antibodies as well as streptavidin-HRP are provided pre-titered. 10 μg of capture antibody is injected, serum collected, and samples simply analyzed using a slightly adapted ELISA procedure. Because only a small amount of serum is needed for analysis, the *In Vivo* Capture Assay does not require sacrificing animals.

Compatible with *in vivo* cytokine monitoring
Provided that sufficient time is allowed for loss of the initial bolus of injected antibody, the *In Vivo* Capture Assay can be used to measure *in vivo* cytokine production at different timepoints in the same mouse, allowing you to gain kinetic information on your studied immune process.

Applications
The *In Vivo* Cytokine Capture Assay has been applied in kinetic studies of *in vivo* immune responses, as well as in studies using cytokines as a biomarker to monitor pathogenic exposure, and in a mouse tolerance model.
Figure 2. Measurement of In Vivo IL-4 and IFN-γ production by IVC. BALB/c mice (5/group) were injected i.v. with 10 µg each of biotin-anti-IL-4 monoclonal antibody (mAb) (Cat. No. 557781) and biotin-anti-IFN-γ mAb (Cat. No 558491) ± 10µg of anti-CD3 mAb (Cat. No. 553057). Mice were bled 4 hours later and serum levels of cytokine-biotin-anti-cytokine mAb complexes were determined by ELISA.

Products

BD Biosciences offers In Vivo Capture Assay Sets for mouse IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF. Each assay set contains sufficient reagents for 10 96-well plates of assays.

Assay Set Components

- *In Vivo* Capture monoclonal antibody, biotinylated, and in BD NA/LE™ (No Azide/Low endotoxin) format
- Purified cytokine-specific monoclonal antibody for plate coating
- Recombinant cytokine standard
- Streptavidin-HRP

For more details about the assay procedure, please refer to the following general protocol, or to the corresponding technical data sheets provided on-line at www.bdbiosciences.com

Protocol

Additional Materials Required But Not Provided

- 96-well BD Falcon™ ELISA Plates (Cat. No. 353279) are recommended
- Plate Sealers with adhesive backing
- ELISA plate reader capable of measuring light absorbance at a 450 nm wavelength
- Single- and Multi-channel pipettes to deliver 50 µl, 100 µl, 200 µl or 300 µl volumes
- Graduated 1 ml, 5 ml, 10 ml, 25 ml pipettes for reagent preparation
- Deionized or distilled water
- Wash bottle or automated ELISA plate washer
- Log-log graph paper or computer and software for ELISA data analysis

Unless otherwise specified, all products are for Research Use Only. Not for use in diagnostic or therapeutic procedures. Not for resale.
• Tubes to prepare standard dilutions
• Absorbent paper
• Laboratory timer

Recommended Buffers and Solutions

Note: Do not use sodium azide in these preparations. Sodium azide inactivates the horseradish peroxidase enzyme.

1. Coating Buffer: 0.1 M Sodium Bicarbonate/Sodium Carbonate buffer, pH 9.5. Freshly prepare or use within 7 days of preparation, store at 2-8°C.

2. Assay Diluent / Blocking Buffer: Prepare 10% fetal bovine serum (FBS), 10% newborn calf serum (NBCS) or 1% bovine serum albumin (BSA; immunoassay grade) in Phosphate Buffered Saline (PBS) at pH 7.2–7.4. This buffer should be filtered to remove particulates before use. Freshly prepare or use within several days of preparation, store at 2–8°C.

Note: The BD OptEIA™ Assay Diluent (Cat. No. 555213) is recommended.

3. Wash Buffer: PBS with 0.05% Tween-20. Freshly prepare or use within 3 days of preparation store at 2–8°C.

4. Substrate Solution: Tetramethylbenzidine (TMB) and Hydrogen Peroxide.

Note: The BD OptEIA TMB Substrate Reagent Set (Cat. No. 555214) is recommended.

5. Stop Solution: 1 M H3PO4 (Phosphoric Acid) or 1 M H2SO4 (Sulfuric Acid)

Note: The BD OptEIA Reagent Set B (Cat. No. 550534) is recommended for the assay, as it contains 20-plate’s worth of the common ELISA reagents, including all of the buffers and solutions mentioned above.

Antibody Injection, Serum Collection and Handling

1. Inject mice intravenously or intraperitoneally with 10 µg of NA/LE™ Biotin-conjugated anti-mouse cytokine antibody (provided in the set) in 200 µl of sterile, endotoxin-free PBS. It is necessary to inject an antibody preparation that lacks preservatives, such as sodium azide, and that is low in endotoxin.

2. Collect blood samples from mice (e.g., in polypropylene microcentrifuge tubes) 2–72 hours post injection and prepare sera. 8

3. Allow blood samples to clot for 30–60 minutes (4°C).

4. Centrifuge the samples for 10 minutes (4°C) at 1000 x g.

5. Remove serum and assay immediately or store samples at ℉ ≤–70°C.

Note: Avoid repeated freeze-thaw cycles. Serum samples should be clear, non-hemolyzed, and non-lipemic.
ELISA Plate Coating

1. Dilute the purified, unconjugated anti-mouse cytokine antibody provided in the set to 2 μg/ml in Coating Buffer.

2. Add 50 μl of the diluted antibody to the wells of an enhanced protein-binding ELISA plate (e.g., BD Falcon™, Cat. No. 353279).

3. Seal the plate to prevent evaporation. Incubate overnight at 4°C.

Blocking

4. Bring the plate to room temperature, remove the coating antibody solution, and block non-specific binding by adding 200 μl of Blocking Buffer per well.

5. Seal the plate and incubate at room temperature for 0.5–1 hour.

6. Wash ≥ 3 times with Wash Buffer

Standard and Sample preparation and handling

7. Reconstitution of the Standard: After warming to room temperature, carefully open vial to avoid loss of material. Reconstitute 1 vial of lyophilized recombinant mouse cytokine protein standard with Assay Diluent as indicated on the Certificate of Analysis to generate stock standard solution. Allow the standard to equilibrate for 5-15 minutes before making dilutions. Vortex gently to mix well.

   Note: After reconstitution, immediately aliquot standard stock into polypropylene freezing vials and freeze at ≤ −70°C for up to 6 months. If necessary, store at 2–8°C for up to 8 hr prior to use.

   Do not leave reconstituted standard at room temperature.

8. In a tube labeled 1000 pg/ml, mix 1.0 ng of recombinant mouse cytokine (reconstituted from the stock standard solution) and 0.4 μg of NA/LE Biotin-anti-mouse cytokine antibody together in a final volume of 1 ml with Assay Diluent.

9. Add 300 μl of Assay Diluent to 5 tubes. Label as 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml, and 15.6 pg/ml.

10. Perform serial dilutions by first adding 300 μl of the 1000 pg/ml standard complex to the tube labeled 500 ng/ml. Repeat this action for the subsequent dilution tubes. Vortex dilution tubes gently between each transfer. The undiluted standard complex serves as the high standard (1000 pg/ml). The Assay Diluent alone serves as the zero standard (0 pg/ml).

11. Allow the BD NA/LE™ Biotin-anti-cytokine antibody and recombinant cytokine complex to incubate for 30 min at room temperature prior to adding to the ELISA plate. Add 50 μl to each well in duplicates.

12. Serum Samples: Investigators should determine the appropriate dilutions of their serum samples for their individual experimental model system. According to our data, we recommend serial dilutions starting from 1:5 for
negative control and experimental serum samples. Depending on the level of the particular cytokine typically produced (i.e. stimulus dependent), higher initial dilutions (e.g. 1:20 or 1:40) may be required for the experimental samples from treated mice. Add 50 μl of the diluted samples to each well in duplicates.

13. Seal the plate and incubate it for 2 hours at room temperature.

14. Wash the ELISA plate wells several times with Wash Buffer.

Horseradish Peroxidase–Streptavidin

15. Dilute the Horseradish Peroxidase-Streptavidin Conjugate at 1:1000 in Assay Diluent. Add 50 μl per well.

16. Seal the plate and incubate it at room temperature for 30 minutes.

17. Wash the ELISA plate wells several times with Wash Buffer.

Substrate (TMB)

18. Add 100 μl of Substrate Solution to each well. Incubate 10–30 minutes at room temperature for color development.

19. Add 50 μl of Stopping Solution to each well to stop the color reaction.

20. Read the absorbance at a 450 nm light wavelength within 15 minutes of stopping the reaction; values are expressed in optical density units, i.e., OD 450 nm. If wavelength correction is available, set it to 570 nm. This subtraction will correct for optical imperfections in the plate.

Calculation of Results

Calculate the mean absorbance for each set of duplicate standards and samples. Subtract the mean zero standard absorbance from each. Plot the standard curve on log-log graph paper, with cytokine concentration on the x-axis (abscissa) and absorbance on the y-axis (ordinate). Draw the best fit curve through the standard points. To determine the cytokine concentration of the unknowns, find the unknown’s mean absorbance value. At the point of intersection, draw a vertical line to the x-axis and read the cytokine concentration. If the samples were diluted, multiply the cytokine concentration by the dilution factor. Computer data reduction may also be employed, utilizing log-log linear regression analysis.

Standardization

This immunoassay is calibrated against purified insect cell-expressed recombinant mouse cytokine protein produced at BD Biosciences (Pharmingen™ brand).

Limitations of the Procedure

- Samples that generate absorbance values higher than the standard curve should be diluted with Assay Diluent and re-assayed.
- Interference by drug metabolites, soluble receptors, or other binding proteins in specimens has not been thoroughly investigated. The possibility of interference cannot be excluded.
• This In Vivo Capture Assay Set for Mouse Cytokine is intended for use as an integral unit. Do not mix reagents from different lots. Reagents from other manufacturers/other available clones should not be used in this Set.

Troubleshooting Tips

**Poor Precision:**

**Possible Source**
- Inadequate washing/aspiration of wells
- Inadequate mixing of reagents
- Imprecise/inaccurate pipetting
- Incomplete sealing of plate

**Corrective Action**
- Check function of washing system
- Ensure adequate mixing
- Check/calibrate pipettes
- Ensure complete seal on plate

**Poor Standard Complex Curve:**

**Possible Source**
- Improper standard/In Vivo Capture Assay Antibody handling or dilution
- Incomplete washing/aspiration of wells
- Imprecise/inaccurate pipetting
- Improper buffer/diluent used

**Corrective Action**
- Ensure correct preparation and storage of the standard and the In Vivo Capture Assay Antibody
- Check function of washing system
- Check/calibrate pipettes
- Check buffer/diluent preparation, pH

**Low Absorbances:**

**Possible Source**
- Inadequate reagent volumes added to wells
- Incorrect incubation times/temperature
- Incorrect antibody titration
- Improper buffer/diluent used
- Overly high wash/aspiration pressure from wash system

**Corrective Action**
- Check/calibrate pipettes
- Ensure sufficient incubation times/reagents warmed to RT
- Check Coating Antibody and Standard Complex preparation
- Check buffer/diluent preparation (e.g., for pH, presence of particulates)
- Utilize manual washing or an automated ELISA plate-washer with the proper pressure settings
References


Relevant BD Biosciences Literature and Links


Additional Technical Resources

Our Scientific Support Team is trained to help you with technical questions related to the In Vivo Cytokine Capture Assay. Just contact them at the numbers listed at the end of this manual, or e-mail: help.biosciences@europe.bd.com.
BD™ DimerX MHC:Ig Fusion Proteins for the Analysis of Antigen-specific T cells

Introduction

Applications and tools for studying the dynamics and frequency of antigen-specific T cells are critical in determining the underlying mechanism of viral infection, autoimmune diseases, and cancer. However, only recently have tools (immunological reagents) been available to directly enumerate and analyze those immunological processes. Antigen-specific T cells (CD8+) recognize antigenic peptides bound to the major histocompatibility complex (MHC) class I molecules expressed on target cells or antigen presenting cells (dual recognition). Monomeric peptide/MHC complexes fail to bind to the cognate T cells with high affinity and are not useful in tracking antigen-specific T cells by flow cytometry-based assays. Only in recent years, with the development of MHC multimers, has the ability to directly track antigen-specific T cells ex vivo using flow cytometry become possible. The MHC/peptide multimers, including dimers and tetramers, enable estimations of the frequency, distribution, phenotype, dynamics and functional state of antigen-specific T cells. Among the MHC multimers, dimer (BD™ DimerX) and tetramers are the only commercially available reagents. This chapter focuses on the BD DimerX MHC class I:Ig fusion protein reagents, which enable analysis of CD8+ cytotoxic T cells by flow cytometry.

The MHC molecules are polymorphic cell surface glycoproteins that play critical roles in the development of T cells in the thymus. In the periphery, they are expressed on the surface of antigen-presenting cells, such as B cells, macrophages, and dendritic cells. MHC molecules bind and present small antigenic protein fragments to the T cell receptor (TCR) expressed by antigen-specific T cells. MHC molecules (HLA in human, and H-2 in mouse) comprise two major classes. MHC class I molecules exist on the surface of almost all nucleated cells and consist of two separate polypeptide chains. The α chain is an MHC-encoded, transmembrane molecule containing three extracellular domains α1, α2, and α3, while the β chain is a non-MHC encoded small protein called β2 microglobulin (β2M) with a molecular weight of 12kDa. Peptide-binding is mainly carried out by the α1 and α2 domains of the heavy chain. The β2M is not a membrane protein and associates with the heavy chain in a non-covalent fashion. Although β2M is not directly involved in peptide-binding, it contributes to the integrity and conformation of the heavy chain and its function.

Class I molecules usually bind to antigenic peptides derived from intracellular antigens (eg, viral and some intracellular bacterial antigens) and then present to the CD8+ T cells. Those peptides are usually 8 – 9 amino acids in length because of the closed structure of the antigen-binding groove on Class I molecules. MHC class II molecules consist of two transmembrane polypeptide chains of almost equal length, which possess two extracellular domains. In this case, the α1 and β1 domain together constitute the peptide-binding region. The peptides bound to the MHC class II
molecules are usually derived from extracellular proteins and are usually longer and more varied in length than the class I-bound peptides. The class II/peptide complexes are generally recognized by CD4+ T cells. After the formation of TCR/MHC/peptide ternary complex, CD4 and CD8 molecules are joined through their interaction with the non-polymorphic regions of MHC, further strengthening the formation of the complex. The non-classical MHC class I molecules, such as CD1d, present glycolipid ligands to a special population of T cells, namely NK T cells, which express canonical TCR. The TCR primarily expressed by mouse NK cells is \( V_\alpha \) 14 J281 and by human NK cells, is \( V_\alpha \) 24.

BD™ DimerX reagents, from BD Biosciences, are MHC-immunoglobulin fusion proteins, developed to detect antigen-specific T cells. Three extracellular domains of MHC class I molecules are fused to the N terminal of the VH region of the mouse IgG1 through recombinant DNA technology (Figure 1). The expression vector containing the fusion protein is then co-transfected with genes containing human \( \beta_2\text{M} \) into a myeloma cell line J558L deficient in immunoglobulin heavy chain but retains the expression of immunoglobulin light chain (lambda). The secreted molecule is a three-chain complex consisting of a recombinant heavy chain of MHC-Ig fusion, an immunoglobulin light chain disulphide bonded to the heavy chain, and a non-covalently associated human \( \beta_2\text{M} \) molecule (Figure 1, and SDS-PAGE).

![Figure 1. Schematic representation of the MHC class I:Ig dimeric protein.](image)

The bivalent nature of peptide-binding sites increases the avidity of the BD DimerX molecule and results in stable binding to antigen-specific T cells. Furthermore, the hinge region in the immunoglobulin scaffold of BD DimerX provides a more flexible access for the T cell binding. Staining with the BD DimerX molecules is obtained by combining the purified BD DimerX with a second step reagent conjugated to a fluorescent molecule (such as PE) or by using directly conjugated BD DimerX molecules.
Key Advantages of BD™ DimerX Technology

Simplicity

In the past, the study of CD8+ antigen-specific T cell responses was restricted by a lack of quantitative assays. Traditional $^{51}$Cr-release assays required the use of radioisotopes, were time and labor consuming, limited to measuring responses in a population of cells, and were often only semi-quantitative. The limiting dilution assay (LDA) was the most quantitative tool available prior to the development of MHC multimers. However, LDAs required that the CTL precursors be expanded at least 10 replication cycles over a week or so for the detection of cytolytic activity and they lacked reproducibility and convenience.

In recent years, the intracellular cytokine (IC) staining and ELISPOT assays have been developed to provide additional powerful tools to evaluate antigen-specific immune responses at the single-cell level. These applications are primarily used for functional assays and their read-out systems do not depend directly upon the structural recognition of TCR. In combination with direct BD DimerX staining, these assays will provide a more complete picture of the nature of immune response (see Chapters 3, 4 and 6 for IC staining and ELISPOT details).

Versatility

With fluorescent staining of antigen-specific CD8+ T cells, we are able to monitor the frequency, distribution, and dynamics of these cells easily. In combination with other cell markers such as CD44, CD62L and CCR7, we can closely monitor the existence and function of memory T cells. Multicolor staining with activation markers, such as CD69 or CD38 and/or intracellular cytokine staining, allows researchers to analyze activation state and other functional properties of antigen-specific T cells simultaneously.

![Figure 2. Flow cytometric analysis of normal human lymphocytes from a cytomegalovirus (CMV) seropositive donor. BD DimerX HLA-A2:Ig was loaded with a CMV pp65-derived, HLA-A2-binding peptide (NLVPMVATV) at 640 molar excess. PBMC were stained with unloaded (left panel) or loaded (right panel) purified HLA-A2:Ig, then stained with PE-conjugated anti-mouse IgG1 (BD Biosciences, Cat. No. 550083), anti-CD14-APC and HLA-DR-APC (BD Biosciences Immunocytometry Systems, Cat. No. 340691), and anti-CD8-FITC (BD Biosciences, Cat. No. 551347). Antibody conjugates were chosen to be non-IgG, isotypes, so as not to interfere with detection of HLA-A2:Ig staining. Cells were collected with a lymphocyte gate and were subsequently gated to exclude APC-positive cells. Percentages shown are percent of CD8+ cells stained for HLA-A2:Ig.](image-url)
Flexibility

Since the BD DimerX proteins are produced in mammalian cells, the conformational integrity of the molecule is largely intact. During the intracellular transport process, the MHC binding grooves of the BD DimerX molecules are believed to be filled with a variety of endogeneous peptides. Loading of specific peptide into the binding groove of the BD DimerX construct is facilitated by passive exchange in the presence of excess peptide of interest under natural or mild denaturing conditions. The BD DimerX molecule can be loaded with your peptide of choice (relevant, irrelevant) to be used in a variety of different experimental systems and thereby, providing a convenient tool for T cell epitope mapping experiments.

Stability

The BD DimerX construct is not produced in bacteria and does not go through a denaturation-renaturation process, hence the protein molecules are highly stable.

Staining Protocol for Optimal Signal

The majority of our BD DimerX reagents are available in an unconjugated form, thus requiring a two-part staining procedure. We have developed an improved protocol to make your BD DimerX stainings as easy as possible, while ensuring the highest quality signal.

The optimized BD DimerX staining procedure consists of two parts.

Preparation of the ligand-loaded DimerX:Ig staining reagent:

- Load BD DimerX reagent with peptide (or lipid) of choice
- Pre-incubate peptide- (lipid-) loaded dimer with fluorochrome-conjugated detection antibody
- Add mouse IgG1 isotype control to block excess binding sites of fluorochrome-conjugated detection antibody.

The resultant mixture is ready to use for staining antigen-specific T cells in combination with other antibodies.

The main advantages of pre-incubating the peptide-dimer complex with the detection reagent are:

- Decreases potential background staining of the dimer on non-CD8+ cells (notably cells expressing Fc receptors)
- Reduces the number of incubations with the cell sample to a single staining step
- Increases staining intensity in most cases.

This optimized procedure allows for antigen-specific T cell staining in multi-color flow cytometric settings, with minimal background staining on non-CD8+ cells, and optimal signal-to-noise ratios (see Figure 3).
Ligand Loading of DimerX:Ig Dimeric Protein

For most BD DimerX interactions the ligands are peptides, however, for CD1d:Ig the ligands are glycolipids. Below we describe the peptide-loading. Several peptide-loading protocols have been described. The method used at BD Biosciences involves passive loading of excess peptide in solution with the MHC:Ig protein. We have found that passive loading works particularly well in the case of high affinity peptides. For lower-affinity peptides, an increase in the molar ratio of peptide to MHC:Ig may improve loading, as determined by flow cytometric analysis. It is suggested that for each peptide, parameters such as the dose of MHC:Ig per million cells, molar ratio of peptide to MHC:Ig, and peptide loading time be determined empirically by the investigator. Parameters and minimal requirements for peptide binding to MHC have been reported in the literature. While the DimerX products contain β2 microglobulin (an added excess, or, for CD1d:Ig, co-expressed), for investigators requiring excess recombinant human β2 microglobulin, we recommend Cat. No. 551089.

Peptide preparation and passive loading:

1. The molecular weight (MW) of a peptide of interest will need to be determined. A peptide’s MW can be estimated by multiplying its number (n) of amino acids (AA) by 130 daltons (d) per amino acid:

   \[ \text{MW of peptide (d)} = n \text{ (AA)} \times 130 \text{ (d/AA)} \]

2. A stock of peptide may be prepared at 20 mg/ml in DMSO. Dilute the peptide solution to 2 mg/ml in sterile DPBS, pH 7.2 for use in the MHC:Ig loading protocol.

   Note: Peptides should be 8 – 9 amino acids in length with purity over 95% (HPLC purified). To prepare a stock solution, dissolve the peptide in DMSO at 20 mg/ml and further dilute in sterile PBS to 2 mg/ml. The stock solution may then be aliquoted and frozen at –20°C until use.

3. Mix MHC:Ig protein with specific or control peptide at 40, 160, or 640 molar (M) excess.

   The following calculation, using an 8-amino-acid peptide as an example, may be used:

   \[ D_p = \text{Molecular weight of peptide} \]

   \[ D_{dimer} = 250,000 \text{ daltons} \]

   \[ R = \text{desired excess of molar ratio: eg, 160} \]

   \[ M_p = \text{microgram peptide of interest} \]

   \[ M_{dimer} = \text{mg of BD DimerX in the reaction. A typical amount of loaded BD DimerX for flow cytometry is 0.25 to 4 μg/million cells.} \]

   \[ M_p = \frac{M_{dimer} \times R \times D_p}{D_{dimer}} \]
In this example, one would add 2.66 μg of peptide and 4 μg of MHC:Ig in solution for the optimal peptide loading of the MHC:Ig.

**Note:** In general, it is advantageous to perform the peptide loading into the DimerX product in as small a volume as possible. The higher concentration will promote good interaction between the small peptide and the DimerX MHC peptide binding pocket.

4. Mix peptide and MHC:Ig together in PBS, pH 7.2, incubate at 37°C overnight. The peptide-loaded MHC:Ig can be stored at 4°C for up to 1 week.

**Note:** Usually incubation overnight at 37°C provides good staining results (Figure 2). It has been published that raising the temperature to 42.5°C may increase the peptide exchange.\(^1\) Loading at 4°C for a greater length of time is also common (see Figure 4). After loading, the complex can be stored at 4°C for up to one week before testing. Longer storage of the BD DimerX/peptide mixture is not recommended. Removal of excess peptide is not necessary before use.

**Alternative to passive loading: Active peptide loading by alkaline or acid stripping:**

In some cases, alkaline or acidic stripping of the endogenous peptides (bound during the bio-production of the DimerX reagents) may help to improve loading efficiency.\(^2\)

**Alkaline Stripping**

a. Incubate BD DimerX reagent with 5 volume equivalents of high pH peptide-stripping buffer (150 mM NaCl, 15 mM Na\(_2\)CO\(_3\), pH 11.5) for 20 minutes at room temperature.

b. Add different molar excesses of peptide of interest to the mixture and adjust the pH to 7.2 with neutralizing buffer (250 mM Tris/HCl, pH 6.8).

c. The mixture may be stored for 24 to 48 hours at 4°C.\(^2\)

**Acid Stripping**

a. Incubate BD DimerX reagent with 5 volume equivalents of citrate-phosphate buffer (131 mM citrate 124 mM Na\(_2\)PO\(_4\), pH 6.4).

b. Incubate the mixture at 37°C for 1.5 to 2 hours in the presence of a high molar excess of peptide of interest.

c. Adjust the pH to 7.2 and incubate at least 24 hours at 4°C to allow the protein to refold.\(^2\)

**Immunofluorescent Staining Protocol**

1. Prepare peptide-loaded MHC:Ig protein staining cocktail by mixing 1 - 2 μg of peptide-loaded MHC:Ig protein/test with 1 - 2 μg of PE-conjugated A85-1 mAb (anti-mouse IgG\(_1\), Cat. No. 550083)/test at a ratio of 1:1 or 1:2 of dimer:A85-1 mAb. Incubate the mixture for 60 minutes at RT, protect from exposure to light.
2. Add 1 - 2 μg of purified mouse IgG1 isotype control mAb A111-3 (Cat. No. 553485)/test to the staining cocktail (from Step 1 above). Incubate the staining cocktail for 30 minutes at RT, protect from exposure to light.

3. Resuspend target cells in FACS staining buffer* [eg, BD Pharmingen™ Stain Buffer with BSA, Cat. No. 554657, or DPBS, 1% FCS, 0.09% NaN3], at a concentration of approximately 10^6 cells per 50 μl.

   *Note:* the staining buffer can already contain Fc receptor blocking reagent, as described in the next step, or it should be added in the next step.

4. Add the appropriate reagent to the cell suspension to block non-specific binding of BD DimerX or antibody reagents to surface Fc receptors

   • For mouse cells, the FACS staining buffer in the previous step should contain the appropriate amount of Mouse BD Fc Block™ (purified anti-mouse CD16/CD32 mAb 2.4G2, Cat. No. 553141/553142)

   • For human cells, 10 μl (2 μg) of a polyclonal human IgG stock (0.2 mg/ml in PBS, pH 7.2) should be added per 50 μl of cell suspension.

5. Incubate 10 minutes at RT.

6. Add ~1x 10^6 cells per staining tube (eg, 12 x 75 mm tube, BD Falcon™ Cat. No. 352008).

7. Add 50 μl FACS buffer containing the optimal per test amount of the staining cocktail, plus any other cell-surface marker specific antibodies to be used to each sample.

   *Note:* BD DimerX reagents are fusion proteins containing mouse IgG ww heavy chain regions. For multicolor staining, it is important to choose antibodies that are not mouse IgG1 isotypes, in order to avoid possible staining by the secondary reagent.

8. Wash cells 1x with 2 ml FACS buffer, centrifuge for 5 minutes at 250 x g, and aspirate supernatant. Resuspend in FACS buffer and analyze by flow cytometry.

   *Note:* Since the frequency of cells which are capable of recognizing specific peptide-MHC complexes is typically very low, e.g., <1%, we recommend acquisition of at least 100,00 lymphocytes for flow cytometric analysis in order to optimally detect this subpopulation.

For a detailed protocol for each specific BD DimerX reagent, please review the individual BD DimerX Technical Data Sheets packaged with the reagent or available online at bdbiosciences.com

**Alternative Immunofluorescent Staining Protocol**

(without pre-incubation of loaded BD DimerX reagent with detection antibody)

1. Resuspend PBMCs or target cells in FACS staining buffer [e.g., BD Pharmingen™ Stain Buffer with BSA, Cat. No. 554657], at a concentration of approximately 10^6 cells per 50 μl.
*Note: the staining buffer can already contain Fc receptor blocking reagent, as described in the next step, or it should be added in the next step.

2. Add the appropriate reagent to the cell suspension to block non-specific binding of BD DimerX or antibody reagents to surface Fc receptors

- For mouse cells, the FACS staining buffer in the previous step should contain the appropriate amount of Mouse BD Fc Block™ (purified anti-mouse CD16/CD32 mAb 2.4G2, Cat. No. 553141/553142)
- For human cells, 10 μl (2 μg) of a polyclonal human IgG stock (0.2 mg/ml in PBS, pH 7.2) should be added per 50 μl of cell suspension.

3. Incubate 10 minutes at RT.
4. Add ~1x 10⁶ cells per staining tube (eg, 12 x 75 mm tube, BD Falcon™ Cat. No. 352008).
5. Add 1 to 2 μg of peptide-loaded BD DimerX:Ig protein to each sample. Incubate 60 minutes at 4°C.
6. Wash cells 1x with 2 ml FACS buffer, centrifuge for 5 minutes at 250 x g, and aspirate supernatant.
7. Add 100 μl FACS buffer containing appropriately diluted fluorescent secondary reagent. We typically use PE-conjugated A85-1 mAb (anti-mouse IgG1, Cat. No. 550083). Incubate 30 minutes at RT.

Note: Additional antibodies specific for markers such as CD4, CD8, or MHC may be included at this step by adding appropriately diluted fluorescently-conjugated antibodies. BD DimerX reagents are fusion proteins containing mouse IgG1 heavy chain regions. For multicolor staining, it is important to choose antibodies that are not mouse IgG₁ isotypes, in order to avoid possible staining by the secondary reagent.

8. Wash cells 1x with 2 ml FACS buffer, centrifuge for 5 minutes at 250 x g, and aspirate supernatant. Resuspend in FACS buffer and analyze by flow cytometry.

Note: Since the frequency of cells which are capable of recognizing specific peptide-MHC complexes is typically very low, e.g. <1%, we recommend acquisition of at least 100,00 lymphocytes for flow cytometric analysis in order to optimally detect this subpopulation.
Troubleshooting

Background on non-T cells

Background staining on non-T cells is a common occurrence with the use of both tetramer and BD DimerX reagents. Background can be reduced by preincubating the mouse cells with Mouse BD Fc Block™ CD16/CD32 (FcγIII/II Receptor (Cat. No. 553141 or 553142) or human PBL with purified human immunoglobulin. Typically, it is recommended that non-T cells be excluded from data analysis of staining using MHC multimers. This can be accomplished in a multicolor staining experiment by gating only on CD3⁺ or CD8⁺ cells, or by excluding non-T cells by using antibodies to B cells and macrophages, such as B220 and Mac-1 in the mouse and CD14 in human.

In experiments using purified BD DimerX molecules, a second step reagent is necessary for detection. For multi-color staining, other antibodies used should be non-mouse IgG₁ isotype.

No staining or weak staining

A known positive T cell population or T cell line should be used for every experiment. All peptides must be pure, as contaminants can compete and inhibit the binding process. The loading procedure should be performed in a sterile setting to avoid contamination of either peptide or BD DimerX.

Increase the loading temperature to 37°C or 42.5°C to facilitate the peptide exchange and improve staining.
Negative control

For mouse experiments, an “irrelevant peptide” that binds to the relevant BD DimerX should be used. For human PBL experiments, use unloaded BD DimerX or an irrelevant peptide as negative controls.

Figure 4. Antigen-specific T cell identification of OT-1 TCR transgenic mouse splenocytes.

Splenocytes from OT-1 TCR transgenic mice were incubated with Mouse FC Block™ antibody (anti-mouse CD16/32, Cat. No. 553141/553142, Both Panels) and immunofluorescently stained with FITC-conjugated anti-mouse CD8 (clone 53-6.7. Cat. No. 553030/553031, Both panels) and PE-conjugated Mouse BD DimerX H-2kb:Ig fusion protein (BD Biosciences, Cat. No. 552944, both Panels) loaded overnight at 4˚C with OVA peptide (Panel A) or SIY peptide (Panel B). Data shown is for viable cells only (10% probability plot). Cells were gated based on propidium iodide staining as well as light scatter characteristics. Data was acquired on a BD FACSCalibur™ (BD Biosciences Immunocytometry Systems, San Jose CA) flow cytometer.
References


Chapter 12

Cell-based Assays for Biological Response Modifiers

Cytokine Biological Assays

Introduction

The identity of cytokines is determined by their physiochemical as well as biological properties. Prior to informatics, cytokines were initially identified based upon their biological activity within in vitro assays termed bioassays. Bioassays were used to characterize the biochemical properties of the novel cytokines and were therefore designed to be quantitative, sensitive, and relatively rapid. The bioassays were based upon the ability of cytokines to stimulate proliferative, cytolytic, antiviral, inhibitory, chemotactic, or colony-forming cellular responses. The responding indicator cell population was either primary hematopoietic cells or established cell lines. The indicator cells responded to cytokine in a dose-dependent manner, generally over a 10 – 100 fold range, with a signal to noise ratio of ≥ 5. This type of biological response enabled quantitation that was measured as Units of biological activity per milliliter.

Once purified cytokine became available, the biological activity of the cytokine could be described in terms of a Specific Activity or Units of biological activity per unit mass (U/mg). Alternatively, the biological potency of the cytokine preparation was described in terms of an ED$_{50}$ or concentration where 50% of the saturating maximal response is observed (see Bioassays section, for further details).

The availability of high concentrations of purified cytokine allowed researchers to establish that cytokines could act in a pleiotropic fashion. The pleiotropic nature of cytokines along with the generation of new bioassay indicator cell lines, led to the existence of multiple bioassays for each cytokine. The biological activity of each cytokine was not defined by a unique bioassay. Instead, because numerous bioassay methods and indicators for each cytokine were reported, a range of biological activities could be associated with each cytokine.

The potency of cytokine preparations is therefore best compared with in-house standards. Alternatively, potency can be calibrated with respect to vendor or international standards. The NIBSC and NIH BRMP Programs have prepared International/National Calibration Standards that are intended for researchers to calibrate, by comparative assay, cytokine preparations. These international standards are not intended to be examples of the purest material and consequently cannot be used to establish definitive ED$_{50}$, or specific activities, for cytokine preparations. The calibration standards do, however, permit comparison of experimental results between laboratories, and can facilitate transition into the use of new cytokine preparations.
Not only do many cytokines possess pleiotropic biological activity, but most bioassays themselves can also detect more than a single cytokine. Nevertheless, bioassays are an essential component in cytokine characterization. The detection sensitivity of bioassays is often greater than immunoassays. In addition, bioassays allow an assessment of intact, biologically-active cytokines. In contrast, immunoassays may measure degradation products, inactive pro-cytokines, or inactive isomers.

BD Biosciences has put in place, with minor modifications, quantitative cytokine bioassays that have been previously reported in the literature. Table 1 (at the end of this chapter) provides a brief description of the cytokine bioassays currently used to evaluate cytokine biological activity at BD Biosciences. These bioassays are used to quantify the biological activity of our cytokine preparations and the neutralizing activity of our anti-cytokine/cytokine receptor antibody products. The cytokine products are ≥ 95% pure as assessed by SDS-PAGE analysis and quantified with an absorbance assay based on Beer Lambert Law. In addition, the endotoxin levels are ≤ 0.1 ng per μg cytokine protein as determined by a kinetic quantitative chromogenic LAL assay using E. coli 055:B5 as the endotoxin standard. The physiochemical and biological potency of all cytokine preparations are compared to previous in-house standards. The biological activity of released cytokine preparations is comparable, being 80 – 200% of previous control lots.

Cytokine Indicator Cells

Established cell lines are the preferred indicator cells because of their consistency and ease of use. Because they respond in a consistent fashion and provide the best signal-to-noise ratios and dose-response curves, cytokine-dependent cell lines, whose growth and survival require exogenously added cytokines, are the best indicator cells. Cytokine-dependent cells are ideal for short-term bioassays because they rapidly die in the absence of exogenous cytokines. The specific indicator cells used at BD Biosciences were chosen based upon their availability and freedom from mycoplasma contamination. When the indicator cells are primary human peripheral blood cells, cells from multiple donors are generally tested because of potential donor variability.

Bioassay Detection Methods

In culture, cytokines stimulate a variety of cellular metabolic responses. Cytokines can stimulate indicator cells to produce new cytokines that in turn can be detected in a secondary bioassay or an ELISA.

Cell growth, cytostasis, or cytolysis can be measured indirectly by measuring the conversion of a redox sensitive tetrazolium salt (MTT). MTT is converted to dark blue formazan crystals when exposed to dehydrogenase activity in actively metabolizing cells. MTT conversion can be used as an indirect, nonradioactive method to measure cell growth with cytokine-dependent cells. In the absence of exogenously added cytokines, the indicator cells die.
Tritiated thymidine ([3H]-TdR) incorporation into DNA directly measures the DNA synthetic rate, and therefore correlates with cell proliferation, cytostasis, or cytolysis.¹

Bioassay detection methods are not limited to those listed above. There are alternate detection methods based upon modified cell surface protein expression, direct cell enumeration, radioactive chromium release by lysed cells, and calcium flux.⁷, ⁸

Bioassays

A schematic bioassay procedure for most bioassays is shown in Figure 1. Indicator cells are incubated with serial 2 – 3 fold dilutions of cytokine in 96-well flat-bottomed microculture plates in final volumes of 100 μl to 200 μl. Three or four replicates are assayed for each sample. In co-stimulatory assays, a fixed suboptimal concentration of co-stimulatory cytokine is added to the culture mixture. The test cytokine and co-stimulant act synergistically to stimulate indicator cell responses. In anti-viral (interferon) assays, the indicator cells are treated with serial dilutions of cytokine with a fixed concentration of cytolytic virus (Plaque Forming Units). In neutralization assays, once the neutralizing activity of the antibody is established, cells are cultured with serial dilutions of antibody and with fixed, suboptimal concentrations of the relevant cytokine.

In all bioassays, a positive control appropriate for the test cytokine is included to establish potency of the test sample. In addition, where possible, a second positive cell, or assay control is run to validate indicator cell health and assay conditions.

Indicator cells are cultured with the appropriate reagents for 1 to 72 hours at 37°C in a humidified CO₂ incubator. After the primary incubation period, the bioassay detection phase begins. Cultured supernatants may be collected and tested for the presence of cytokine. MTT dye, or [3H]-TdR can be added to the cell cultures for an additional 4 – 8 hours to measure cell viability or growth. The primary and secondary incubation takes place under identical temperature and CO₂ conditions.

Secondary cytokines present in culture supernatants are measured in either a second bioassay or an ELISA. The conversion of MTT to blue formazan crystals by actively metabolizing cells is measured spectrophotometrically in a plate reader after the formazan crystals have been solubilized in an acidified SDS-dimethyl formamide solution. Incorporation of [3H]-TdR into DNA is measured by harvesting the labeled indicator cells onto glass fiber filters followed by extensive washing to remove free [3H]-TdR. The filters are dried and the incorporated [3H]-TdR is measured by liquid scintillation counting. The results obtained using the above detection methods are then quantitated in the manner described in Figure 3.
Bioassay Results and Quantitation

The bioassay data are tabulated, the appropriate control values are subtracted, and the resulting data are graphed by plotting the cellular response values (y-axis) versus the reciprocal dilution of the test sample (x-axis). Proliferation and anti-viral type assay results will have descending dose-response curves similar to Figure 2. Cytolytic and cytostatic responses are expected to display curves with an ascending dose-response curve. The graphs of the raw data are used to determine the saturating maximal response value and the linear portion of the dose-response curve. The values obtained using this quantitation method are considered valid if the sample slopes are parallel with the cytokine standard. A lack of parallelism may be an indication of poor assay performance (technique), non-identity or presence of inhibitory/stimulatory substances. An example of cytokine quantitation is shown in Figure 3. The following terminology is used to describe cytokine biological activity or potency:

**Unit:** The amount (mass or volume) of cytokine required to stimulate a half-maximal response at cytokine saturation.

**Specific Activity:** Units of biological activity per unit mass of cytokine (U/mg).

**ED$_{50}$:** Cytokine concentration or dilution at which a half-maximal response is observed.
Dose-Response Range: Concentration or dilution range where a dose-related biological response is observed.

Figure 2. Idealized cytokine bioassay results. At high concentrations, the cytokine induces a saturating maximal response. As the cytokine is serially diluted, a dose-response relationship is observed. We have generally found the range where we observe a biological effect to be 10 – 100 fold.* The dilutions of sample required to stimulate a half-maximal response normally fall within the linear portion of the dose-response curve. This part of the curve is therefore used, like enzymatic assays, to define a unit of activity.

* The exception is Interferon (IFN) assays where the slope of the dose-response curve is very steep, often dropping precipitously over a 2 – 4 fold range of IFN.
Figure 3. Proliferative response of the CTLL-2 indicator line to recombinant mouse IL-4 as measured by $[^{3}H]$-Tdr incorporation. In the upper half of the figure, the raw data minus background incorporation is plotted versus the reciprocal IL-4 sample dilution. The curve is used to establish the maximum of 12,200 cpm at saturating IL-4 concentrations. It reveals the concentration range where a linear dose-response relationship is observed. The linear region is subjected to linear regression analysis (lower half of figure) using a logarithmic linear least-squares method to determine “best fit” for the curve. The x-intercept where a 50% maximal response occurs is used to calculate a Unit of activity, the Specific Activity, and the ED$_{50}$ of the sample.

Characterization of Cytokine-Neutralizing Antibodies

Neutralizing antibodies have proven to be valuable tools for discerning the role of cytokines in many in vitro and in vivo biological responses. BD Biosciences neutralizing antibodies contain no sodium azide and possess $\leq 0.01$ ng of endotoxin per μg neutralizing antibody (denoted by the No azide/Low endotoxin, or NA/LE™ format). The neutralizing reagents are initially characterized by cross-matrix analysis of titrated cytokines and antibody diagrammatically shown in Figure 4, with the exception that 3 additional serially-diluted cytokine concentrations are routinely tested. The cytokine is tested at suboptimal concentrations. The cross-matrix analysis allows identification of conditions where we observe both 50% neutralization for potency and 90% neutralization for experimental use. Once we have established a cytokine concentration where both 50% and 90% inhibition are observed, future antibody preparations are tested using this single, fixed concentration of cytokine. The 50% neutralization activity of new test antibody preparations is 80% – 120% of previous control antibody preparations.
Chemokine Biological Assays

Introduction

Chemokines are a group of small (8 to 14 kDa), structurally-related, mostly basic and heparin-binding cytokines. Over 45 chemokines have been identified in humans. Based on the arrangement of the first two amino-terminal cysteine residues, chemokines can be subdivided into four families: CC (CCL1–CCL28), CXC (CXCL1–CXCL16), C (XCL1) and CX3C (CX3CL1). All chemokines exert their biological function via a group of seven-transmembrane, G protein-coupled receptors (GPCRs). Like chemokines, their receptors can also be divided into 4 families based on the ligands they bind to: CC chemokine receptors (CCR1 – CCR11), CXC chemokine receptors (CXCR1 – CXCR6), C chemokine receptor (XCR1) and CX3C chemokine receptor (CX3CR1).

Chemokines were originally known to attract mainly granulocytes and monocytes and to be involved in acute and chronic inflammatory responses. Recently, newly discovered chemokines were shown to be involved in controlling leukocyte trafficking. These new chemokines are functionally and genetically distinct from the classical “inflammatory chemokines” and may be classified as the “lymphoid chemokines” or “homeostatic chemokines”.

Biological Assays for Chemokines and Chemokine Receptors

The chemotaxis assay and the calcium mobilization assay are widely used to characterize the biological activity of chemokines and their receptors.

1. Chemotaxis Assay

The chemotactic assay is based on the directional migration of target cells in response to chemokine gradients. The apparatus used for most assays is the Boyden chamber developed in the 1960’s. Modern chemotaxis assays have adapted several modifications such as precoating the membranes with extracellular matrix proteins (collagen, fibronectin, etc.) or endothelial cell monolayers to mimic in vivo environments.

The chemotaxis assay we employ at BD Biosciences utilizes a 48-well chemotaxis chamber (Neuro Probe, Cabin John, MD). Figure 5 illustrates the chemotaxis assay scheme used. Briefly, different concentrations of

![Chemokine Biological Assays](image)

**Figure 4.** BD Biosciences Cross-Matrix Neutralization Format
samples are added into the lower chamber and the target cells are added into the upper chamber. A polycarbonate filter (Osmonics, Livermore, CA) separates the upper and lower chambers. Different sizes for different cell types are used, e.g., 3 μm pore-sized filters for neutrophils and 5 μm pore-sized filters for monocytes and eosinophils. To measure lymphocyte chemotaxis, 5 μm pore-sized filters precoated with mouse type IV collagen are used. After incubation, the filter is stained and the cells that have migrated into the filter are counted using a microscope.

Another modified chemotaxis assay that enumerates the migrated cells by the measurement of lactate dehydrogenase (LDH) upon cell lysis is also used to determine the biological activity of chemokines. Briefly, the transwell inserts with 3 or 5 μm pore-size membranes are suspended in the wells of a 24-well plate containing controls and chemokines. The target cells are added into the transwell inserts. After incubation, the number of migrated cells is determined by LDH assay.16 The amount of released LDH is proportional to the enzymatic conversion of a tetrazolium salt (INT) into a red formazan that can be easily measured at a 490 nm wavelength of light using an ELISA plate reader.

Figure 5. Scheme of chemotaxis assay using a 48-well chemotaxis chamber. (Based on Figure 6.12.1 in Current Protocols in Immunology, 1995, Unit 6.12.) (A) Samples (25 – 30 μl) are added to the wells in the lower chamber. A polycarbonate filter is placed over the wells. After chamber assembly, the target cell suspension (55 – 60 μl) is added to the wells in the upper chamber. (B) After incubation, the filter is removed from the chamber. The non-migrating cells are removed by scraping the filter against the wiper. (C) The Diff-Quik-stained filter is cut in half and placed on a microscopic slide. The cells that have migrated into the filter are counted using a microscope.
Figure 6. Dose-response curves for chemotaxis of human neutrophils in response to recombinant human IL-8. Each point represents the averaged cell numbers per field (1000×). A 3 µm pore-size polycarbonate filter separated the upper and lower chambers.

An example of a dose-response curve for the chemotactic response to recombinant human IL-8 is shown in Figure 6. The bell-shaped dose-response curve obtained in the experiment is typical for chemokines as a result of receptor desensitization. The results reported in the literature, as well as our own results, indicate that leukocytes obtained from different individuals might respond differently to the same chemokines. Therefore, donor-dependent variation in results of chemotaxis assays should be anticipated.

Figure 7. Inhibition of human lymphocyte chemotaxis by monoclonal anti-CXCR4 antibody (Clone 12G5). Chemotaxis was measured by using a 48-well Boyden chamber and a 5 µm pore-size filter precoated with mouse type IV collagen. The concentration of human SDF-1α added in the lower chamber was 200 ng/ml.
With the same protocols, antibodies directed against chemokines or their receptors can also be screened for their neutralization activities. Figure 7 represents an example of a dose-response curve for a neutralizing antibody directed against human CXCR4. The activity of a neutralizing antibody is expressed as an ND\textsubscript{50} that is defined as the antibody concentration yielding 50% neutralization of the response.

2. Calcium Mobilization Assay

Transient increases in cytosolic free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) can provide an indication of cellular activation for many ligand-transmembrane receptor systems that are involved in cell signaling. For this reason, assays that measure the levels of transient [Ca\textsuperscript{2+}]\textsubscript{i} flux caused by ligands, or that are prevented by antibodies directed against activating ligands or their receptors, can be used to determine the levels and specific activities of these biologically-active molecules.

Calcium ions play a unique role in intracellular signaling and are considered as an important second messenger for cellular signaling pathways. Chemokines, anaphylatoxins, and other inflammatory mediators may trigger calcium mobilization responses upon binding to their cellular receptors. In these cases, receptor-ligand interactions activate the guanine nucleotide binding proteins located on the inside of the membrane. Consequently, heterotrimeric G proteins activate phospholipase C to cleave phosphatidyl inositol 4,5-bisphosphate, releasing diacylglycerol and inositol triphosphate. Inositol triphosphate causes the release of Ca\textsuperscript{2+} from intracellular stores, while diacylglycerol and the increased cytosolic Ca\textsuperscript{2+} levels have been implicated in the activation of protein kinase C inside the cell. Increased phosphorylation events have in turn been related to oxidant production and secretory function by these activated cells.

There are a wide variety of available fluorescent indicator dyes, such as Indo-1 and Fura-2 that change their fluorescent properties after complexing with Ca\textsuperscript{2+}. For example, when using a spectrofluorometer with an excitation light wavelength set at ~358 nm, the fluorescence emission maximum of Indo-1 shifts from ~485 nm in Ca\textsuperscript{2+}-free medium to ~405 nm when the dye is saturated with Ca\textsuperscript{2+}. The ratio of fluorescence of the Ca\textsuperscript{2+}-bound dye and the Ca\textsuperscript{2+}-free dye can be used to determine [Ca\textsuperscript{2+}]. The cell-permeable acetoxyethyl (AM) esters of these dyes can be passively loaded into cells, where they are cleaved to cell-impermeable products by intracellular esterases.

To perform a calcium mobilization assay, the target cells are loaded with Indo-1, placed in a temperature-controlled (37°C) stirred cuvette inside the spectrofluorometer, and are excited at a 358 nm wavelength of light. After determining the baseline emission at 405 and 485 nm, the stimulant (chemokine, anaphylatoxin or other inflammatory mediator) is rapidly injected into the cell suspension. Emitted fluorescent light signals are continuously monitored and recorded for the next 120 – 300 seconds. The level of cytosolic free Ca\textsuperscript{2+}, as reflected by the ratio of emissions
(E405/E485), increases rapidly if the ligand is stimulatory, (ie, causes its receptor to transduce signals inside the cell that result in the mobilization of Ca\textsuperscript{2+} from intracellular stores into the cytosol). This response is followed by a decrease of the [Ca\textsuperscript{2+}] back to baseline levels. The amplitude of the transient increase of cytosolic free Ca\textsuperscript{2+} is dependent on the stimulatory ligand concentration used to activate the target cells allowing for the determination of an ED\textsubscript{50} (see Figure 8). Alternatively, the ND\textsubscript{50} of a neutralizing antibody directed against the ligand or the receptor can be determined using the same protocol (see Figure 9).

For more detailed protocols for calcium mobilization assays, researchers should refer to other published protocols (see References 17 and 18).

![Figure 8. Transient increase in cytoplasmic free calcium induced by various concentrations of recombinant rabbit IL-8. A dose-response titration was performed using human neutrophils.](image)
Figure 9. Monoclonal anti-rabbit IL-8 antibody (Clone 2g3) inhibits rabbit IL-8-induced calcium flux in human neutrophils. A dose-response titration of the antibody is shown using recombinant rabbit IL-8 at 125 ng/ml as the stimulant.

3. Other Methods:

In addition to the methods described above, several methods have been used to determine the biological activities of certain chemokines. These include CD11b/CD18 up-regulation assays for both CC and CXC chemokines; neutrophil elastase or β-glucuronidase release assays and neutrophil oxidative burst assays for CXC chemokines; hematopoietic colony formation assays for MIP-1α and MIP-1β; and histamine release assays for CC chemokines. For more information on these assays, please refer to Reference 17.
References

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</table>
### Cell-based Assays Table 1

<table>
<thead>
<tr>
<th>Cytokine*</th>
<th>Assay Type</th>
<th>Indicator Cell</th>
<th>Final Cell Density/ml</th>
<th>Incubation Time (hr)</th>
<th>Detection Method and Length</th>
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<tr>
<td><strong>Chemokines</strong></td>
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<tr>
<td>Eotaxin</td>
<td>Calcium flux</td>
<td>hCCR3 transfectant cells</td>
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<td><strong>Growth Regulated Oncogene</strong></td>
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<td>GROα, h</td>
<td>Calcium flux</td>
<td>Human peripheral blood leukocytes</td>
<td>$1 \times 10^6$ seconds</td>
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<tr>
<td><strong>Interleukin-8</strong></td>
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<tr>
<td>IL-8, h</td>
<td>Calcium flux</td>
<td>Human peripheral blood leukocytes</td>
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<td><strong>Gamma interferon inducible protein-10</strong></td>
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<tr>
<td>IP-10, h,m</td>
<td>Calcium flux</td>
<td>hCXCR3 transfectant cells</td>
<td>$1 \times 10^6$ seconds</td>
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<tr>
<td><strong>Monocyte Chemoattractant Proteins</strong></td>
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<tr>
<td>MCP-1,2,3</td>
<td>Calcium flux</td>
<td>THP-1 (ATCC TIB-202)</td>
<td>$1 \times 10^6$ seconds</td>
<td>Calcium flux</td>
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<tr>
<td><strong>Monokine Induced by gamma interferon</strong></td>
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<tr>
<td>MIG, h,m</td>
<td>Calcium flux</td>
<td>hCXCR3 transfectant cells</td>
<td>$1 \times 10^6$ seconds</td>
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<tr>
<td>RANTES, h</td>
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<td>THP-1 (ATCC TIB-202)</td>
<td>$2.5 \times 10^5$</td>
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<tr>
<td>SDF-1, h</td>
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<td>Human PBMC</td>
<td>$1.5 \times 10^5$</td>
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<td>Enumerate cells</td>
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</table>

* h = human, m = mouse, r = rat, p = porcine

** 0.5 µCi of tritiated thymidine per well

*** Virus (24 hr) → IFNγ (48 hr)
Chapter 13

Tools to Study the Complement System

Introduction

The Complement System is an integral part of the Innate and Adaptive Immune System.\textsuperscript{1,2} Complement plays an important role in microbial killing, and is essential for the transport and clearance of immune complexes. Many of the activation products of the Complement System are also associated with immunoregulatory or proinflammatory functions.

The Complement System consists of a group of soluble plasma proteins, that interact with one another in three distinct enzymatic-activation cascades. The Classical, Alternative, and recently-described Lectin Pathways can lead to the formation of the Terminal Complement Complex (TCC) and an array of biologically-active molecules (see \textit{Figure 1}). This process is carried out through enzymatic amplification steps, and controlled by an array of soluble regulatory proteins. In addition to the soluble components, membrane-associated molecules act as receptors/regulators for fragments of the activated complement components. Complement activation is initiated either by specific antibodies, recognizing and binding to a variety of pathogens and foreign molecules, or by direct interaction of complement proteins with foreign substances.\textsuperscript{3} Complement-mediated cellular lysis via the TCC is mediated by insertion of pore-forming protein complexes into targeted cell membranes. Complement-mediated cell activation, including chemotaxis, lysosomal enzyme release, enhanced phagocytosis or immune-complex clearance is achieved by binding of complement proteins or protein fragments to specific membrane receptors.\textsuperscript{1,2}

\textit{Figure 1.} Overview of complement cascades.
Analysis of Complement Function and Protein Levels

Functional hemolytic assays (Classical Pathway CH$_{50}$ and Alternative Pathway APC$_{50}$ measurements) provide information on complement function as a whole. This type of assay uses antibody-sensitized or unsensitized sheep erythrocytes. The values are expressed as 50% hemolytic complement units per ml (CH$_{50}$, APC$_{50}$). One CH$_{50}$ and/or APC$_{50}$ unit is defined as the quantity or dilution of serum required to lyse 50% of the red cells in the test. Low CH$_{50}$ and/or APC$_{50}$ values are indicators of complement protein deficiencies or in vivo complement activation. These assays can also be used in in vitro experimental systems to identify complement activating-substances or to evaluate potential inhibitors for therapeutics.

Limiting-dilution hemolytic assays serve as functional tests for individual complement components. These assays use antibody-sensitized sheep erythrocytes and a serum source, that has an excess of all complement components, but is deficient for the one being measured in the sample. The extent of hemolysis is therefore dependent on the availability of the measured component in the test sample, which provides the basis for quantitation.

Several immunochemical methods are applicable to measure individual complement component concentrations, such as radial immunodiffusion, nephelometric assays as well as sandwich ELISAs.$^{4,5}$

**Determination of Complement Fragment Levels**

A number of proteolytic cleavage products, and activation-specific complexes are generated during complement activation, such as C3a, C4a, C5a, Bb, and sC5b-9. These products reflect complement activation, independent of fluctuations in individual complement component levels.

Anaphylatoxins C3a, C4a and C5a are bioactive cleavage products released from plasma components C3, C4 and C5 during complement activation.$^{6,7}$ They are involved in the mediation of a variety of cellular immune responses, as well as being potent proinflammatory agents.$^{3}$ The intact anaphylatoxins in serum or plasma are quickly converted into the more stable, less active C3a-desArg, C4a-desArg or C5a-desArg forms, by Carboxypeptidase N. Radioimmunoassay or ELISA determinations of anaphylatoxin levels in serum or plasma samples frequently require extra sample preparation steps, such as removal of the precursor molecules by precipitation methods.$^{8}$ These preparation steps are time consuming and can lead to under- or over-estimation of the analyte levels. The ELISA-based quantitation of C3a-desArg, C4a-desArg and C5a-desArg in plasma or experimental samples using neoepitope-specific monoclonal antibodies avoids these technical difficulties. It provides a more reliable, faster measurement of the level of complement activation.

BD Biosciences offers BD OptEIA™ ELISA kits for human C3a-desArg, C4a-desArg and C5a-desArg (Cat. Nos. 550499, 550947 and 557965), a BD OptEIA ELISA Set for C5b-9 (the TCC, or membrane attack complex; Cat. No. 558315), and the BD™ Cytometric Bead Array (CBA) Human Anaphylatoxin Kit (Cat. No. 552363). These kits employ neoepitope-specific monoclonal antibodies for the easy, accurate
detection of complement activation products in plasma and other biological fluids. The Anaphylatoxin BD OptEIA ELISA Kits are useful for specifically determining the levels of individual anaphylatoxins, and offer a choice in assay development. The BD CBA Human Anaphylatoxin Kit provides complete and ready-to-use reagents to perform simultaneous, highly-sensitive measurements of human anaphylatoxins C3a-desArg, C4a-desArg and C5a-desArg from one small volume sample. For analysis of mouse complement proteins, BD Biosciences now offers Matched Antibody Pairs and protein standards for mouse C3a (Cat. No. 558250, 558251 and 558618) and C5a (Cat. No. 558027, 558028, and 622597). For a general description of the OptEIA ELISA Kit and Set protocols, please refer to Chapter 9 of this handbook. For detailed descriptions of sample preparation, assay and data analysis using the BD OptEIA Human Anaphylatoxin ELISA Kits, please refer to the kit manual. For a general description of the CBA Kit protocol, please refer to Chapter 2 of this handbook.

For a detailed description of sample preparation, assay and data analysis using the Human Anaphylatoxin CBA Kit, please refer to the kit manual.

The ability to accurately measure complement activation products from in vivo samples has been problematic because the in vitro processing of the sample results in the continuation of the Complement Cascade. This issue for the anaphylatoxins has been resolved by the use of an inhibitor, FUT-175. Past research has shown that addition of FUT-175 (Futhan), a broad range serine-protease inhibitor, to plasma samples at the time of sample collection provides additional protection from ex vivo complement activation, and therefore ensures more accurate measurements. Samples stabilized with FUT-175 reflect the circulating levels of complement activation products at the time of the sample collection. BD Biosciences now offers FUT-175 (Cat. No. 552035) as an additive to stabilize plasma samples for complement measurements. See protocol (below) for using FUT-175 to prevent in vitro complement activation.

Analysis of Cells That Express Complement Receptors and Membrane Proteins

Multiparameter flow cytometry and immunohistochemical methods can be used to examine the distributions and nature of cells that coexpress complement receptors and the membrane bound complement regulatory proteins. BD Biosciences offers a wide selection of reagents to detect human, mouse and rat cell surface molecules that are part of the Complement System and function as Complement Receptors or Complement Regulators. These include antibodies that recognize human C5aR (CD88), C3aR, C1qRp (CD93), CR1 (CD35), CR2 (CD21), CR3 (CD11b/CD18), CR4 (CD11c/CD18), MCP (CD46), DAF (CD55) and CD59, as well as many of the mouse and rat counterparts of these molecules. These reagents provide the researcher with the ability to perform multiparameter analysis of the cells. For complete listing of these reagents, go to our web at bdbiosciences.com and search for "complement". Some of these reagents are also capable of blocking ligand-receptor interactions and/or receptor-mediated cellular activation. For a general description of immunofluorescent surface staining and flow cytometric analysis protocols refer to Chapter 1 of this handbook.
Use of FUT-175 to Stabilize Plasma for Anaphylatoxin Measurements

Background

FUT-175 [Futhan, or Nafamstat Mesilate (6-amidino-2-naphthyl p-guanidinobenzoate dimethanesulfonate)] is a synthetic, broad specificity, low molecular weight (MW = 539.58) protease inhibitor and an inhibitor of the Classical and Alternate Pathways of Complement. 9,10

Although chelation of bivalent cations by EDTA inhibits the activity of a number of plasma proteases, including those involved in the coagulation and complement pathways, it has been reported that cleavage of certain complement components still occurs in EDTA plasma. This makes measurements of the in vivo-generated complement cleavage products less accurate. Addition of FUT-175 to plasma samples at the time of sample collection provides additional protection from ex vivo activation, and therefore ensures more accurate measurements, that reflect the circulating levels of activation products. This benefit applies particularly to measurements of the anaphylatoxins C3a-desArg and C4a-desArg. 11,12

Helpful tips for using FUT-175 as a sample stabilizer:

Adding FUT-175 to blood or plasma samples that are collected for anaphylatoxin measurements prevents ex vivo complement activation due to handling and processing at room temperature and freeze-thawing. Prior to use, reconstitute FUT-175 (Futhan) with 1 ml dH2O to get a 5 mg/ml = 100 × stock solution. After reconstitution, FUT-175 is stable for 4 weeks if stored at 4ºC and several months if stored at –80ºC.

a. Add FUT-175 to freshly-drawn EDTA-treated blood;
   1. Draw blood into EDTA-containing BD Vacutainer™ tube (purple top).
   2. Carefully remove stopper from BD Vacutainer tube, add 10 μl FUT-175 100× stock solution per each ml of blood.
   3. Mix well, centrifuge in pre-cooled centrifuge (400 × g, 10 min.).
   4. Collect plasma in separate tube.
   5. Aliquot plasma and store frozen until Anaphylatoxin determination.

b. Add FUT-175 to freshly collected EDTA plasma;
   1. Draw blood into an EDTA-containing BD Vacutainer (purple top).
   2. Keep tube on ice until centrifugation.
   3. Centrifuge in pre-cooled centrifuge (400 × g, 10 min.) within 30 min of blood collection.
   4. Remove stopper from Vacutainer tube, collect plasma in a separate tube.
   5. Add 10 μl FUT-175 stock solution per each ml of plasma.
   6. Mix well, aliquot and store frozen until Anaphylatoxin determination.
Experiment to Demonstrate Benefit of Using FUT-175

a. Reagents

1. Lyophilized samples of FUT-175 (Cat. No. 552035). Each vial contains 5 mg FUT-175 (Futhan). Store lyophilized vials at 4°C, protected from light and moisture. Prior to use, reconstitute FUT-175 (Futhan) with 1 ml dH$_2$O to get a 100× stock solution. After reconstitution with dH$_2$O, FUT-175 is stable for 4 weeks if stored at 4°C and several months if it is stored at –80°C.

2. EDTA-treated plasma can be used along with FUT-175 for optimal stabilization of plasma samples in preparation for analysis by immunoassays. Specimens should be clear, non-hemolyzed, and without particulate matter. For plasma collection, EDTA-blood samples should be kept on ice and spun (400 × g, 10 min) within 30 min of blood collection in a refrigerated centrifuge. Plasma specimens can be collected ahead of time and stored in aliquots at ≤ –80°C. If using frozen plasma specimens, then thaw them at RT and keep them on ice until use. If freshly collected plasma is used, then keep it on ice until use.

Caution: Human Plasma is a potential source for HIV-1, hepatitis, and other infectious agents. Therefore, all specimens should be handled as if capable of transmitting disease. Gloves should be worn at all times during the procedure.

3. To measure C4a anaphylatoxin levels use the BD OptEIA Human C4a-desArg ELISA Kit, Cat. No. 550947, or the Human Anaphylatoxin BD CBA Kit, Cat. No. 552363 (see alternative protocol for CBA measurements).

b. Experimental setup

Results from the following experiment are shown to illustrate the benefit of using FUT-175 to stabilize plasma samples and to avoid the post-collection generation of complement fragments.

Previous studies have shown that in EDTA plasma samples, incubated at RT or 37°C for a period of time, significant amounts of C4a are generated. The addition of FUT-175 to EDTA plasma samples prevents in vitro C4a generation.

1. Into each of four Eppendorf tubes, labeled A–D, aliquot 200 µl of EDTA plasma.

2. Add 2 µl FUT-175 100× stock into samples C and D to get a final concentration of 50 µg/ml FUT-175 (see Table 1 below); add none to samples A and B.
Table 1. Experimental setup to test the protective effect of FUT-175 in EDTA-plasma

<table>
<thead>
<tr>
<th>Tube</th>
<th>Test Conditions</th>
<th>Typical C4a-desArg Values* (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>No Futhan, 1 hour @ 0°C</td>
<td>150–450</td>
</tr>
<tr>
<td>B</td>
<td>No Futhan, 1 hour @ 37°C</td>
<td>8,000–12,000</td>
</tr>
<tr>
<td>C</td>
<td>50 µg/ml Futhan, 1 hour @ 0°C</td>
<td>150–450</td>
</tr>
<tr>
<td>D</td>
<td>50 µg/ml Futhan, 1 hour @ 37°C</td>
<td>150–450</td>
</tr>
</tbody>
</table>

*Based on donor to donor variation, and plasma collection/handling

3. Mix well and incubate samples A and C for 1 hour @ 0°C, incubate samples B and D for 1 hour @ 37°C.

4. Cool tubes by placing on ice, and test samples immediately for C4a levels using BD OptEIA Human C4a ELISA Kit, or the Human Anaphylatoxin BD CBA Kit. Alternatively, samples can be frozen and kept at –80°C until ready to be tested.

5. To determine C4a-desArg levels use the BD OptEIA ELISA Human C4a Kit, (Cat. No. 550947). Please refer to the kit manual for a detailed description on performing the assay. Also refer to Chapter 9 of this handbook.

6. Samples need to be diluted to the detection range of the BD OptEIA Human C4a ELISA Kit (for A, C and D, start sample dilution at 1:200; for sample B, due to the anticipated, elevated levels of the analyte, start the dilution at 1:8,000). Follow standard assay procedures as described in the kit manual.

7. Plot the Standard Curve on log-log graph paper with the C4a concentration on the x-axis and OD values on the y-axis. Draw the best-fit straight line through the standard points. Alternately, since most ELISA readers are connected to a computer, it is desirable to collect and store data in the computer and to analyze it with a software program that is specialized for analyzing ELISA data. The program enables the use of statistics and linear regression, to give the best fitting curve, and the final report, with calculated values of C4a for the unknown samples. Please refer to Chapter 9 for additional information on ELISA methods.

8. Interpretation of results: For evaluating FUT-175 effects, calculate the % of C4a generation in the FUT-175-containing sample, relative to the non-treated sample: divide the net change of C4a levels in FUT-175 containing samples (ie, tube D minus tube C) by the net change of C4a levels without FUT-175 (ie, tube B minus tube A) and multiply by 100 to get percentage.

\[
\frac{(D) \ C4a_{\text{with FUT-175 @ 37°C}} - (C) \ C4a_{\text{with FUT-175 @ 0°C}}}{(B) \ C4a_{\text{without FUT-175 @ 37°C}} - (A) \ C4a_{\text{without FUT-175 @ 0°C}}} \times 100\%
\]
The C4a levels in the presence of FUT-175 should be less than 5% of the untreated samples.

Alternative protocol: Assay protocol to determine plasma C3a-, C4a- and C5a-desArg levels using the Human Anaphylatoxin CBA Kits

a. Reagents

1. Use samples generated as described in the experimental setup, and Table 1 above.

2. Use the Human Anaphylatoxin CBA Kit, (Cat. No. 552363). Please refer to the kit manual for a detailed description on performing the assay. Also, please refer to Chapter 8 of this handbook.

b. Experimental setup

1. Prepare standard dilution, as described in the kit manual.

2. Prepare sample dilutions: Samples need to be diluted to the detection range of the Human Anaphylatoxin CBA Kit. (for samples A, C and D, dilutions of 1:400 1:800 and 1:1600 are recommended; for sample B, due to the anticipated, elevated levels of the analytes use dilutions 1:1600, 1:3200 and 1:6400)

3. Continue by adding capture beads and PE detector antibodies. Incubations and wash steps as described in the kit manual.

4. Set up Flow Cytometer and analyze beads.

5. Use the BD CBA Software to calculate anaphylatoxin concentrations in the samples incubated under different conditions.

6. Calculate the effectiveness of FUT-175 to block in vitro generation of anaphylatoxins using the formula above.
Figure 2. **Anaphylatoxin levels measured by CBA analysis.** Anaphylatoxin levels were measured using the BD CBA Human Anaphylatoxin Kit from samples B (left panel) and D (right panel) from the experimental setup described above (Table 1), using 1:1600 sample dilution. Bivariate dot plots (FL2 versus FL3) from an actual experiment are shown. There is a significant elevation of C4a levels in sample B (@ 37°C for 60 min without FUT-175) as revealed by the increase in FL2 intensity compared to sample D (@ 37°C for 60 min with FUT-175). Anaphylatoxin concentrations from these samples were calculated using the BD CBA Software as follows:

<table>
<thead>
<tr>
<th>Sample</th>
<th>C3a (ng/ml)</th>
<th>C4a (ng/ml)</th>
<th>C5a (ng/ml)</th>
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<tr>
<td>EDTA plasma @ 37°C for 60 min without FUT-175</td>
<td>448.6</td>
<td>&gt;8,000.0</td>
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<tr>
<td>EDTA plasma @ 37°C for 60 min with FUT-175</td>
<td>339.5</td>
<td>1,446.0</td>
<td>13.3</td>
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Average anaphylatoxin levels determined by the Human Anaphylatoxin CBA Kit from freshly drawn EDTA-plasma (n=10) are 111, 467, and 8.8 ng/ml for C3a, C4a, and C5a respectively.
References/Recommended Reading:

Complement Reviews and Protocols:


FUT-175 (Futhan)


BD Biosciences Literature


For additional information and updated lists of related products, please visit www.bdbiosciences.com/immune_function

Do you know about our protocols on the web?
www.bdbiosciences.com/pharmingen/protocols

BD Biosciences Scientific Support
Our team of experts is trained to help you with your technical questions about our products and their use.

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