Techniques for Immune Function Analysis

Application Handbook 1st Edition

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About the Cover

A set of graphics was selected that represent the various technologies, applications, and reagents offered by BD Biosciences, which are useful for studying Immune Function. The graphics were mapped together onto the surface of a sphere, which symbolizes a cell, the fundamental biological unit involved in the generation and mediation of immunological and inflammatory responses. The composite graphic represents the integrated set of tools and solutions that are available for multiparameter, high-resolution analyses of the molecular and cellular mechanisms that underlie immune function.

Preface

The study of the immune system attracts large numbers of researchers from diverse scientific disciplines because of its central importance in providing immunological host defense and its intercommunication with other systems that maintain bodily homeostasis. The immune system is often studied in its intact form but it can also be readily disassembled into its cellular and molecular components (eg, lymphoid cell populations and effector molecules), recombined and modified in various ways, and analyzed in an *in vitro* or an *in vivo* setting. Due to the creative development and application of a wide variety of experimental protocols, often using new technological platforms and reagents, vast amounts of new information concerning immune function become available on a daily basis. Researchers busily scrutinize this information hoping to better define and understand the networks of cellular and molecular mechanisms that underlie immunity and inflammation in health and disease.

BD Biosciences is pleased to introduce the new ***Techniques for Immune Function Analysis, Application Handbook 1st Edition***. This handbook grew out of the original Cytokine/ Chemokine Application Manual that was first published in 1997. The original manual was based on BD Biosciences technical publications and presentations and with a tremendous amount of input from customers dealing with immune function studies from a "Genes to Proteins to Cells" perspective. The new title for this publication reflects the enlarged scope of the book that served as a guide for applications and reagents designed to study the roles played by cells and the regulatory and effector molecules (ie, biological response modifiers including cytokines, chemokines, inflammatory mediators and their receptors) that mediate inflammation and natural and acquired immunity. New chapters dealing with the BD™ Cytometric Bead Array, the BD FastImmune™ System, the BD™ ELISPOT Assay, BD™ DimerX MHC:Ig Molecules, Immunofluorescent Staining of Cell Surfaces for Flow Cytometric Analysis, and Inflammatory Mediators have been added to this handbook. Previous chapters pertaining to the BD RiboQuant™ Multi-Probe RNase Protection Assay System, ELISA, BD OptEIA™ ELISA Sets and Kits, Immunofluorescent Staining of Intracellular Molecules for Flow Cytometric Analysis, and Bioassays have been revised as well with a presentation of new reagents and methods discussed therein. For additional information please access the new Immune Function Homepage at **www.bdbiosciences.com/immune_function**.

Abbreviations

7-AAD	7-aminoactinomycin
ABTS	2, 2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
AEC	3-amino-9-ethyl-carbazole
aka	also known as
APC	allophycocyanin or antigen-presenting cell
BrdU	bromodeoxyuridine
BRM	biological response modifier
BSA	bovine serum albumin
CO_2	carbon dioxide
cpm	counts per minute
DAPI	4′,6-diamidino-2-phenylindole*2HCl
ddH_2O	distilled deionized water
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
ED_{50}	50% effective dose
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assay
FACS	fluorescent activated cell sorting
FcR	immunoglobulin Fc receptors
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte-macrophage colony-stimulating factor
H_2O_2	hydrogen peroxide
hr	hour
HRP	horseradish peroxidase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
kDa	kilodalton
L	liter
LAL	limulus amebocyte lysate
LPS	lipopolysaccharide
MCP	monocyte chemoattractant protein
min	minute
MIP	macrophage inhibitory protein
mRNA	messenger RNA
NA/LE	no azide/low endotoxin
ND_{50}	50% neutralizing dose
OD	optical density
NBCS	newborn calf serum
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS-Tween	PBS containing 0.05% Tween-20
PE	phycoerythrin
PerCP	Peridinin chlorophyll protein
pfu	plaque forming units
PBMC	peripheral blood mononuclear cells
PI	propidium iodide

Abbreviations *(continued)*

PMA	phorbol myristate acetate
PMT	photomultiplier tube
PY	pyronin Y
RANTES	Regulated upon Activation, Normal T Expressed and presumably Secreted
rhIL	recombinant human interleukin
RNA	ribonucleic acid
RPA	ribonuclease protection assay
RT	room temperature
SDS	sodium dodecyl sulfate
[^{3}H]-TdR	tritiated thymidine
TBE	Tris borate EDTA
TCC	terminal complement complex
TCR	T cell receptor
TDS	Technical Data Sheets
TE	Tris EDTA
TMB	tetramethylbenzidine
TNF	tumor necrosis factor
U	unit

1. BD Cy-Chrome™ is now listed as PE-Cy5.
2. TNF-α is now listed as TNF.

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_c (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 β_c 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.[4] Cytokine receptors transduce external biological signals into intracellular events by various signal transducing proteins including Protein Tyrosine Kinases.[1–3, 5–7]

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 TNFRs, IL-2Rs, IL-4Rs, and IL-6Rs).[8–10] Soluble cytokine receptors may regulate cytokine actions by specifically binding their cognate cytokine and thus inhibiting its interaction with receptors expressed on target cells.[2] Alternatively, soluble receptors may potentiate the effects of their bound cytokine by extending its half-life in the circulation.[2] 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, CX_3C receptor, and orphan receptors.[1,2]

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.[1] 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).[11-13] 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 (radioreceptor assay).[13] Although the radioreceptor 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.[14] 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).[15]

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.

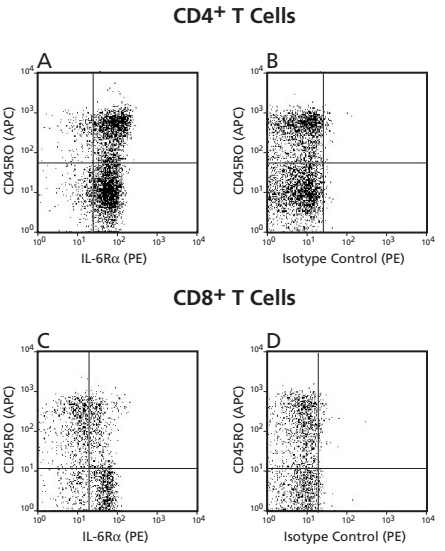

Figure 1. Differential expression of human IL-6Rα (CD126) chain on CD4⁺ and CD8⁺ T cells. Human PBMCs were isolated by density gradient centrifugation (Ficoll-Paque™) and were stained with FITC-anti-human CD4 (Cat. No. 555346, Panels A and B), PerCP-anti-human CD8 (custom made by the Custom Technology Team, BD Biosciences Pharmingen, 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 IgG_1,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.

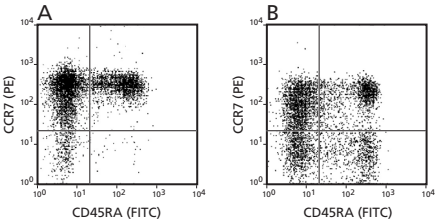

Figure 2. Detection of human CCR7 expression on CD4⁺ and CD8⁺ human lymphocytes by PE-conjugated anti-human CCR7 antibody. Human PBMCs were stained with PE-conjugated anti-human CCR7 (clone 3D12, Cat. No. 552176, Panels A and B) and FITC-conjugated anti-human CD45RA (Cat. No. 555488, Panels A and B). 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.

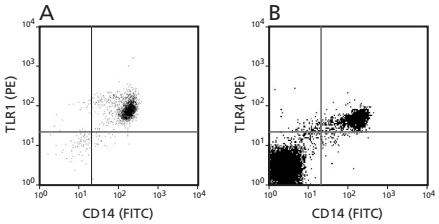

Figure 3. Detection of TLR1 and TLR4 expression on human peripheral blood monocytes. Human peripheral blood mononuclear cells were either treated with BD Pharm Lyse™ (Cat. No. 555899) to lyse red blood cells (Panel A) or were purified by density gradient centrifugation (Ficoll-Paque™) to isolate PBMCs (B). The cells were subsequently stained with either purified anti-human TLR1 (clone GD2, Cat. No. 552033, Panel A), or purified anti-human TLR4 (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 IgG_{2a} (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.

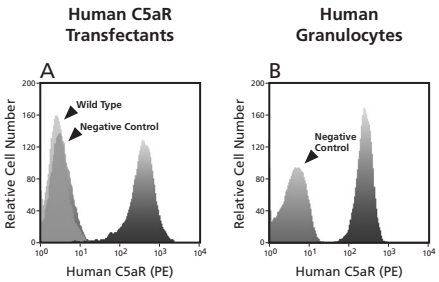

Figure 4. Expression of human C5aR on C5aR transfectants and granulocytes. Human C5aR transfected and wild type mouse L cells were treated with Mouse BD FcBlock™, CD16/CD32 (FcγII/II Receptor, Cat. No. 553141 and 553142) to block Ig Fc- receptors and were stained with PE-conjugated anti-human-C5aR antibody, (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.

Other Assays Used to Study BRM Receptor Biology

Multiprobe Ribonuclease Protection Assay (RPA): The multiprobe RPA can be used to measure the mRNA levels for multiple BRM ligands and receptors within cell or tissue lysate samples (described in ***Chapter 11***). The RPA does not provide information concerning the types and levels of transcripts at the level of individual cells. Due to potential post-transcriptional and post-translational modifications, it is desirable to follow up RPA analyses with analyses conducted at the protein level (eg, by immunofluorescent staining and flow cytometric analysis).

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 ***Chapters 7 and 8***.)

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 10***).

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 2×10^7/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 FcBlock™, CD16/CD32, Cat. No. 553141 and 553142) and rat FcγIII Receptor (Rat BD FcBlock, CD32, Cat. No. 550270 and 550271) respectively, can be used to block nonspecific staining due to FcR. To block FcR with BD FcBlock reagents, preincubate the cells with 10 µg/ml of BD FcBlock 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 × *g* for 5 min). Wash the cells 1× with 200 µl of staining buffer, pellet by centrifugation (250 × *g* for 5 min), and remove supernatant.

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

   For staining in tubes, wash the cells 1× 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 23***) or fluorescent 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 1× with 200 µl of staining buffer, pellet by centrifugation (250 × *g* for 5 min), and remove supernatant.

   For staining in tubes, wash the cells 1× 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 23***) 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 fluorescent 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.

Alternative Protocol: Staining Cell Surface Receptors in Whole Blood.

1. Dilute whole blood 1:10 with (1×) BD Pharm Lyse™ (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 S***tain for Receptors and Other Cell Surface Antigens, page 14***).

   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 14***).
   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, 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 14 and 15***).

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.

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 15, 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, two fluorescent antibodies such as FITC- and PE-conjugated antibodies) 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***.

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 PharmingenStain™ (FBS) (Cat. No. 554656) and BD PharmingenStain™ (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).[17] 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***).

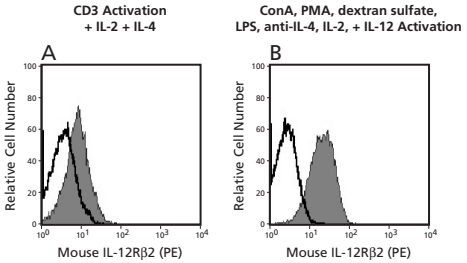

Figure 5. Mouse IL-12Rβ2 expression on in vitro-activated cells. C57BL/6 mouse splenocytes were treated to lyse erythrocytes and were cultured for 5 days with either plate-bound anti-mouse CD3 antibody (Cat. No. 553057) plus recombinant mouse IL-2 (Cat. No. 554578) and IL-4 (Cat. No. 550067) (Panel A) 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 FcBlock (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 IgG_1, κ Isotype control (Cat. No. 553951, both Panels, open histograms). Histograms were derived from gated events of viable (7-AAD negative) lymphocytes.

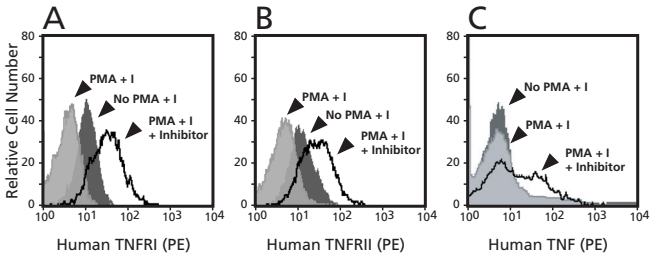

Figure 6. TACE (Tumor Necrosis Factor-Alpha Converting Enzyme) inhibitors block activation-induced shedding of TNFRs and membrane TNF. Human PBMCs isolated by density gradient centrifugation (Ficoll-Paque™) were stimulated with plate-bound anti-human CD3 antibody (10 µg/ml, Cat. No. 555336) and soluble 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 IgG_1 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 phycoerythrin- or allophycocyanin-conjugated antibodies. Phycoerythrin (PE) and allophycocyanin (APC) have high extinction coefficients (the efficiency of conversion of excitation energy to fluorescence energy) and therefore give better quantum yields than most other commercially available fluorochromes.[11,13] Therefore, in multicolor flow cytometric analysis for cytokine receptors and other cell surface antigens, it is recommended that PE- and APC-labeled antibodies be used for staining antigens that are expressed at relatively low levels. 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.[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).

   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.

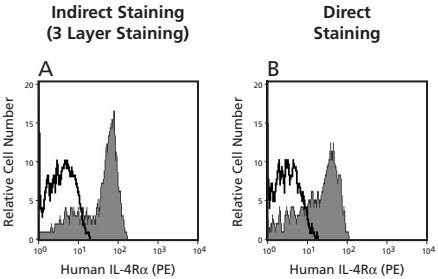

Figure 7. Analysis of IL-4Rα chain expression on human B cells. Human PBMCs were isolated by density gradient centrifugation (Ficoll-Paque™) and were treated with human IgG (5 µg/10^6 cells) to block Ig Fc receptors. The cells were subsequently stained with either purified anti-human IL-4R (clone hIL4R-M57, Cat. No. 551850, Panel A) antibody followed by biotinylated anti-mouse IgG_1 (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 µl/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_1, κ 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.

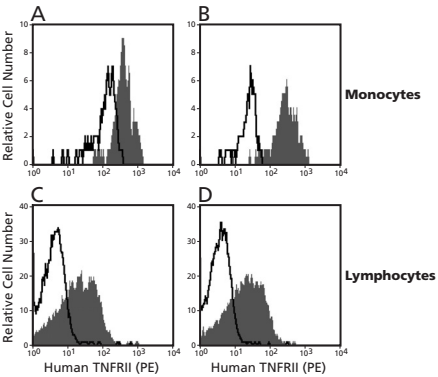

Figure 8. Effect of FcR blocking on the analysis of TNFRII expressed by human PBMCs. Human PBMCs were isolated by density gradient centrifugation (Ficoll-Paque™) and were treated with with human IgG (5 µg/10^6 cells, Panels B and D) to block FcR. The cells were subsequently stained with purified anti-human 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 IgG_{2b}, κ 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***).[18] 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 $F(ab')_2$ 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.

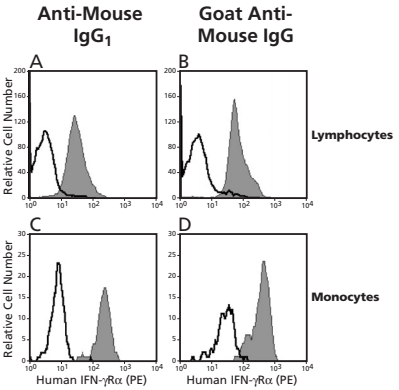

Figure 9. Analysis of IFN-γRα chain expression on human PBMCs. Human PBMCs were isolated by density gradient centrifugation (Ficoll-Paque™) and were stained with purified anti-human IFN-γRα (clone GIR-208, Cat. No. 558932, all Panels) followed with either biotinylated anti-mouse IgG_1 (Cat. No. 553441, 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-γRα antibody (filled histograms) is compared to staining obtained using a Mouse IgG_1, κ 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™, 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™, 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 (eg, 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]

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Cytokine, Chemokine, and Inflammatory Mediator Receptor Antibodies for Flow Cytometry

Description	Clone	Isotype	Format	Cat. No.
Human Cytokine Receptors				
Common β chain (CDw131)	3D7	Mouse IgG_1	Purified	554534
			Biotin	554535
Common γ chain (CD132)	TUGh4	Rat IgG_{2b}	Purified	555896
			Biotin	555897
			PE	555898
Common γ chain (CD132)	AG184	Mouse IgG_1	PE	555900
EGFR	EGFR.1	Mouse IgG_{2b}	Purified	555996
			PE	555997
Endoglin (CD105)	266	Mouse IgG_1	Purified	555690
G-CSFR (CD114)	LMM741	Mouse IgG_1	Purified	554536
			Biotin	554537
			PE	554538
GM-CSFRα (CDw116)	hGMCSFR-M1	Mouse IgG_1	Purified	551284
			Biotin	551412
			PE	551373
GM-CSFRα (CDw116)	M5D12	Mouse IgM	Purified	554530
			Biotin	554531
			FITC	554532
gp130 (CD130)	AM64	Mouse IgG_1	Purified	555756
			PE	555757
IFN-γRα (CD119)	GIR-208	Mouse IgG_1	Purified	558932
			PE	558934
IFN-γRα (CD119)	GIR-94	Mouse IgG_{2b}	Purified	558935
			PE	558937
IGF-I Rα (CD221)	1H7	Mouse IgG_1	Purified	555998
			PE	555999
IGF-I Rα (CD221)	3B7	Mouse IgG_1	Purified	556000
IL-2Rα (CD25)	M-A251	Mouse IgG_1	Purified	555430
			FITC	555431
			PE	555432
			PE-Cy5	555433
			APC	555434
IL-2Rβ (CD122)	Mik-β2	Mouse IgG_{2a}	Purified	554520
			Biotin	554521
			PE	554522
IL-2Rβ (CD122)	Mik-β3	Mouse IgG_1	Purified	554523
			Biotin	554524
			PE	554525
IL-3Rα (CD123)	9F5	Mouse IgG_1	Purified	555642
			Biotin	555643
			PE	555644
IL-3Rα (CD123)	7G3	Mouse IgG_{2a}	Purified	554527
			Biotin	554528
			PE	554529
IL-4Rα (CD124)	hIL4R-M57	Mouse IgG_1	Purified	551894
			Biotin	552120
			PE	552178
IL-5Rα (CD125)	A14	Mouse IgG_1	Purified	555901
			PE	555902

Cytokine, Chemokine, and Inflammatory Mediator Receptor Antibodies for Flow Cytometry

Description	Clone	Isotype	Format	Cat. No.
Human Cytokine Receptors *(continued)*				
IL-6Rα (CD126)	M5	Mouse IgG_1	Purified	551462
			Biotin	551851
			PE	551850
IL-10R (CD210)	3F9	Rat IgG_{2a}	Purified	556012
			PE	556013
IL-12Rβ1 (CD212)	2.4E6	Mouse IgG_1	Purified	556064
			PE	556065
IL-12Rβ2 (CD212)	2B6/12β2	Rat IgG_{2a}	Purified	550722
			PE	550723
IL-18Rα	H44	Mouse IgG_1	Purified	552951
LIFR	12D3	Mouse IgG_1	Purified	559790
			PE	559791
LIFR	7G7	Mouse IgG_1	Purified	559778
			PE	559779
Lymphotoxin β Receptor (TNFR Related Protein)	hTNFR-RP-M12	Mouse IgG_1	Purified	551359
			Biotin	551861
			PE	551503
	BCG6.AF5	Mouse IgG_1	Purified	552875
NGFR	C40-1457	Mouse IgG_1	Purified	557194
			Biotin	557195
			PE	557196
PDGFRa (CD140α)	αR1	Mouse IgG_{2a}	Purified	556001
			PE	556002
PDGFRβ (CD140β)	28D4	Mouse IgG_{2a}	Purified	558820
			PE	558821
SCFR (CD117, c-kit)	YB5.B8	Mouse IgG_1	Purified	555713
			PE	555714
			PE-Cy5	559879
			APC	550412
TNF Receptor type I (CD120a)	MABTNFR1-B1	Mouse IgG_{2a}	Purified	550514
			Biotin	550900
TNF Receptor type II (CD120β)	hTNFR-M1	Rat IgG_{2b}	Purified	551311
			Biotin	552417
			PE	552418
			APC	552419
4-1BB (CDw137)	4B4-1	Mouse IgG_1	Purified	555955
			PE	555956
			PE-Cy5	551137
			APC	550890
Human Chemokine Receptors				
CCR5 (CD195)	2D7/CCR5	Mouse IgG_1	Purified	555991
			FITC	555992
			PE	555993
			APC	556903
			PE-Cy5	556889
CCR5 (CD195)	3A9	Mouse IgG_{2a}	Purified	556041
			PE	556042
			APC	550856

Cytokine, Chemokine, and Inflammatory Mediator Receptor Antibodies for Flow Cytometry

Description	Clone	Isotype	Format	Cat. No.
Human Chemokine Receptors *(continued)*				
CCR6	119A	Mouse IgG_1	Purified	559560
			Biotin	559561
			PE	559562
CCR7	2H4	Mouse IgM	Purified	550937
CCR7	3D12	Rat IgG_{2a}	Purified	552175
			Biotin	552174
			PE	552176
CXCR1 (CD128a, IL-8RA)	5A12	Mouse IgG_{2b}	Purified	555937
			Biotin	555938
			FITC	555939
			PE	555940
CXCR2 (CDw128b,IL-8RB)	6C6	Mouse IgG_1	Purified	555932
			FITC	551126
			PE	555933
			APC	551127
			PE-Cy5	551125
CXCR3 (CD183)	1C6/CXCR3.1	Mouse IgG_1	Purified	557183
			PE	557185
			APC	550967
			PE-Cy5	551128
CXCR4 (CD184, Fusin)	12G5	Mouse IgG_{2a}	Purified	555972
			Biotin	555973
			PE	555974
			APC	555976
			PE-Cy5	555975
CXCR4 (CD184, Fusin)	1D9	Rat IgG_{2a}	Purified	551413
			Biotin	551970
			PE	551510
CXCR5	RF8B2	Rat IgG_{2b}	Purified	552032
			Biotin	552118
Human Inflammatory Mediators and their Receptors				
CD14	M5E2	Mouse IgG_{2a}	Purified	555396
			FITC	555397
			PE	555398
			APC	555399
CD21	1048	Mouse IgG_1	Purified	552727
C1qRp	R139	Mouse IgG_{2b}	Purified	551087
			FITC	551531
			PE	551509
C1qRp	R3	Mouse IgM	Purified	551454
			Biotin	552117
C3a receptor	8H1	Mouse IgG_1	Purified	557173
C5a receptor	C85-4124	Rabbit IgG	Purified	559159
			PE	inquire
C5a receptor	D53-1473	Mouse IgG_1	Purified	550493
			PE	550494

Cytokine, Chemokine, and Inflammatory Mediator Receptor Antibodies for Flow Cytometry

Description	Clone	Isotype	Format	Cat. No.
Human Inflammatory Mediators and their Receptors *(continued)*				
fMLP receptor	5F1	Mouse IgG_1	Purified	556015
			PE	556016
Leucotriene B4 receptor (hBLTR)	203/14F11	Mouse IgG_1	Purified	552400
			Biotin	552835
			PE	552836
Thioredoxin	polyclonal	Goat IgG	Purified	550483
			PE	556016
Thioredoxin (full length, TRX 1-1)	2G11	Mouse IgG_1	Purified	559969
			FITC	559968
Thioredoxin (full length, TRX 1-8)	7D11(TRX4)	Mouse IgG_1	Purified	559966
			Biotin	550887
Human Cell Surface Cytokines				
Lymphotoxin-α (LTα2β1)	BF7.AE2	Mouse IgG_1	Purified	inquire
Lymphotoxin-α (LTα1β1)	AG9.BD6	Mouse IgG_1	Purified	552873
Lymphotoxin-β	B9.C9	MouseIgG_1	Purified	551359
TNF	MAb11	Mouse IgG_1	Purified	554510
			Purified	559071
			FITC	554512
			PE	554513
			PE	559321
			APC	554514
Toll-like Receptors				
Toll-like Receptor 1 (TLR1)	GD2	Mouse IgG_1	Purified	552033
Toll-like Receptor 4 (TLR4)	HTA125	Mouse IgG_1	Purified	551964
			Biotin	551975

See complete listing for Human Complement Receptors in ***Chapter 12.***

Mouse Cytokine Receptors				
CD131 (bIL-3R, β common)	JORO50	Rat IgG_1	Purified	559918
			Biotin	559919
			PE	559920
Common γ chain (CD132)	4G3	Rat IgG_{2a}	Purified	554455
			Biotin	554456
			PE	554457
Common γ chain (CD132)	TUGm2	Rat IgG_{2b}	Biotin	554470
			PE	554471
IFN-γRα (CD119)	GR20	Rat IgG_{2a}	Purified	558770
			Biotin	558771
IFN-γRα (CD119)	2E2	Arm. Hamster IgG	Purified	559911
			Biotin	550482
IFN-γRβ	MOB-47	Arm. Hamster IgG	Purified	559917
IL-1R I (CD121a)	35F5	Rat IgG_1	Purified	553693
			Biotin	550969
			PE	557489
IL-1R I (CD121a)	12A6	Rat IgG_{2a}	Purified	557490
IL-1R II (CD121b)	4E2	Rat IgG_{2a}	Purified	554448
			Biotin	554449
			PE	554450

Cytokine, Chemokine, and Inflammatory Mediator Receptor Antibodies for Flow Cytometry

Description	Clone	Isotype	Format	Cat. No.
Mouse Cytokine Receptors *(continued)*				
IL-2Rα (CD25)	7D4	Rat IgM	Purified	553068
			Biotin	553069
			Biotin	553070
			FITC	553071
			FITC	553072
IL-2Rα (CD25)	3C7	Rat IgG_{2b}	Purified	557364
			PE	553075
IL-2Rα (CD25)	PC61	Rat IgG_1	Purified	557425
			PE	553866
			APC	557192
			PerCP-Cy5.5	551071
			PE-Cy7	552880
IL-2Rβ (CD122) (IL-2/-15Rβ)	TM-β1	Rat IgG_{2b}	Purified	557461
			Biotin	559884
			FITC	553361
			PE	553362
IL-2Rβ (CD122)	5H4	Rat IgG_{2a}	Purified	554451
			FITC	554452
IL-3Rα (CD123)	5B11	Rat IgG_{2a}	Purified	555069
			Biotin	555070
			PE	555071
IL-4Rα	mIL4R-M1	Hamster IgG	Purified	551853
			Biotin	552508
			PE	552509
IL-6Rα (CD126)	D7715A7	Rat IgG_{2b}	Purified	554460
			Biotin	554461
			PE	554462
IL-7Rα (CD127)	B12-1	Rat IgG_{2a}	Purified	558776
			Biotin	555288
IL-7Rα (CD127)	SB/14	Rat IgG_{2a}	Purified	550425
			PE	550479
IL-7Rα (CD127)	SB/199	Rat IgG_{2b}	PE	552543
IL-10R (CD210)	1B1.3a	Rat IgG_1	Purified	559912
			Biotin	559913
			PE	559914
IL-12Rβ1 (CD212)	114	Mouse IgG_{2a}	Purified	551455
			Biotin	551973
			PE	551974
IL-12Rβ2	HAM10B9	Hamster IgG	Purified	552819
Lymphotoxin β Receptor	AF.H6	Hamster IgG	Purified	552940
Lymphotoxin β Receptor	AC.H6	Hamster IgG	Purified	552939
PDGFRα (CD140a)	APA5	Rat IgG_{2b}	Purified	558774
SCFR (CD117, c-kit)	2B8	Rat IgG_{2b}	Purified	553352
			Biotin	553353
			FITC	553354
			PE	553355
			APC	553356
SCFR (CD117, c-kit)	ACK45	Rat IgG_{2b}	Purified	553868
			PE	553869
TNF receptor type I (CD120a)	55R-286	Arm. Hamster IgG	Purified	559915
TNF receptor type II (CD120b)	TR75-89	Arm. Hamster IgG	Purified	559916
			Biotin	550476
			PE	550086

Cytokine, Chemokine, and Inflammatory Mediator Receptor Antibodies for Flow Cytometry

Description	Clone	Isotype	Format	Cat. No.
Mouse Chemokine Receptors				
CCR3	Polyclonal	Rabbit IgG	Purified	556882
CCR5 (CD195)	C34-3448	Rat IgG_{2c}	Purified	559921
			Biotin	559922
			PE	559923
CXCR4 (CD184, Fusin)	2B11/CXCR4	Rat IgG_{2b}	Purified	551852
			Biotin	551968
			FITC	551967
			PE	551966
CXCR5	2G8	Rat IgG_{2a}	Purified	551961
			Biotin	551960
			PE	551959
Mouse Cell Surface Cytokines				
Lymphotoxin-α	AF.B3	Hamster IgG	Purified	552937
Lymphotoxin-β	BB.F6.F6.BF2	Hamster IgG	Purified	552938
TNF	MP6-XT22	Rat IgG_1	Purified	554416
			Purified	559064
			FITC	554418
			PE	554419
			APC	554420
TNF	TN3-a9.12	Hamster IgG	PE	559503
Mouse Inflammatory Mediator Receptors				
CD14 (LPS Receptor)	rmC5-3	Rat IgG_1	Purified	553738
			FITC	553739
			PE	553740
CR2/CR1 (CD21/CD35)	7G6	Rat IgG_{2b}	Purified	533817
			FITC	553818
			PE	552957
C5a receptor	C1150-32	Polyclonal Rabbit IgG	Purified	552837

See complete listing for Mouse Complement Receptors in ***Chapter 12****.*

Rat Cytokine Receptors				
IL-2Rα (CD25)	OX-39	Mouse IgG_1	Purified	559980
			Biotin	559981
			FITC	554865
			PE	554866
Rat Cell Surface Cytokines				
TNF	TN3-19.12	Hamster IgG	PE	559503

See complete listing for Rat Complement Receptors in ***Chapter 12****.*

Note: Please see the 2003 BD Biosciences Product Catalog for more information concerning reagents that recognize:

- *TNF Superfamily Ligands and Receptors, including Fas, FasLigand, CD40, CD40 Ligand, etc.*
- *BRM molecules expressed by cells from other species including Non-Human Primates, Pigs, Rabbits, and Dogs.*

Reagents for Immunofluorescent Staining of Cell Surface Molecules

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** or contact BD Biosciences Technical Services at **877.232.8995** for a copy of the latest BD Biosciences Catalog.

Notes

Chapter 2

BD™ Cytometric Bead Array (CBA) Multiplex Assays

Introduction

Flow cytometry is a powerful analytical tool that enables the characterization of cells and subcellular organelles as well as particles (eg, plastic 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).[1, 2] Recently, flow cytometry has been applied to the development of multiplex sandwich immunoassays.[3-5] These particle-based, flow cytometric immunoassays are capable of simultaneously identifying the types and measuring the levels of multiple different molecules (aka, antigens, 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 enables 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 (eg, bioactive complement fragments), and their receptors, as well as immunoglobulins, are popular target molecules for study.[3-5] In addition, these assays can be applied to the multiplex analysis of cell signaling molecules that act in complex pathways to orchestrate cellular responses.[6]

The multiplex BD™ Cytometric Bead Array (CBA) Kit employs a series of different particles that are stably labeled with a fluorescent dye whose emission wavelength is read at ~650 nm.[3] Each different group of beads is labeled with a discrete level of fluorescent dye so that it can be distinguished by its mean fluorescence intensity (MFI) upon flow cytometric analysis. 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[5], tears, tissue culture supernatants, or cell lysates. By analogy with the ELISA method (described in ***Chapters 7 and 8***), 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.

The captured analyte is then specifically "detected" by the addition of a fluorescent antibody. Fluorescein isothiocyanate-(FITC)(~530nm) and phycoerythrin (PE)(~585nm) coupled detection antibodies (whose wavelengths are distinguishable from the fluorescence signals emitted by the dyed CBA Capture Beads) are often used. By including serial dilutions of a standard analyte solution (eg, a mixture of cytokine protein standards with known concentrations), the CBA supports the development of standard curves (aka, calibration curves) for

each analyte. With multicolor flow cytometric analysis, the levels of analytes (proportional to the bound detection antibody MFI signals) captured by the different bead groups (distinguished by their MFI signals) are measured. The data is analyzed through use of the BD CBA Software to calculate the concentrations of multiple analytes that may be coexpressed within biological fluid samples. Due to the complexity of the BRM and cell signaling networks that underlie immune function, the BD CBA Kit's capacity to simultaneously measure multiple analytes in a single small-volume sample is highly advantageous.

The list of BD Cytometric Bead Array products is growing. Presently, there are several BD CBA Kits for measuring human and mouse cytokines related to Type 1 and Type 2 Immune Responses and Inflammatory Responses. New BD CBA Human Kits for measuring Active Caspase-3 (involved in apoptosis) and Anaphylatoxic Complement Fragments (C3a, C4a, and C5a) are available. A BD CBA Kit that enables the determination of the heavy and light chain isotypes of mouse immunoglobulins is also offered. Other CBA and accessory products include lyophilized CBA standards, BD CBA Software, and the BD Multiwell™ AutoSampler that can be used to increase the throughput and decrease the hands-on time for performing CBA Assays. For more information concerning BD CBA products, please access the BD Biosciences website, **www.bdbiosciences.com/pharmingen/CBA/**

Principle of the Test

Specific descriptions and instructions are provided with each different BD CBA Kit. In general, BD CBA Kits can simultaneously and quantitatively measure multiple analytes (proteins) in a single sample. Each kit's performance has been optimized for analysis of specific analytes in tissue culture supernatants, EDTA-treated plasma and serum samples, or cell lysates. The BD CBA Capture Bead population(s), each with distinct fluorescence intensities (read at ~650 nm/FL3 by BD FACS™ brand flow cytometers), have been coated with capture antibodies specific for various analytes.

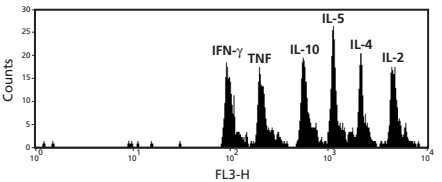

Figure 1. Representative fluorescence (FL3-H) frequency distributions for the Capture Bead populations from the BD CBA Human Th1/Th2 Cytokine Kit.

The BD CBA Capture Beads are mixed with fluorescent (eg, PE-conjugated) detection antibodies and standards, controls, or test samples, to form sandwich complexes (eg, Capture Bead-Ab/analyte/PE-Ab complexes). Following acquisition of sample data using multicolor flow cytometry, the sample results are generated in a graphical and tabular format using the BD CBA Software.

Advantages

The BD CBA Kits provide several advantages when compared with some immunoassay methods. For example, the required sample volume for measuring multiple analytes is smaller than some conventional immunoassays wherein only one analyte can be measured per sample. Due to the BD CBA Kit's capacity to detect six analytes in a single sample, the BD CBA Human Th1/Th2 Cytokine Kit requires approximately one-sixth the sample volume required when compared with a conventional immunoassay. The capacity to use smaller sample volumes is an extremely important feature of the multiplex BD CBA, as precious samples are often available in only limited quantities.[4] The generation of standard curves for multiple analytes is simplified since the analyte standards are often provided as a mixture, thereby requiring no preparation of standard mixtures before making serial dilutions. Moreover, due to the extended dynamic range of BD CBA's when compared with conventional immunoassays, fewer serial dilutions of samples may be required. Altogether, these features can help make BD CBA experiments take less time to perform than individual immunoassays.

Limitations

The BD CBA is not recommended for use with stream-in-air flow cytometers. Fluorescent signal intensities may be reduced with these instruments and adversely affect the assay sensitivity. Stream-in-air instruments include the BD FACStar™ Plus and BD FACSVantage™ (BD Biosciences Immunocytometry Systems, San Jose, CA) flow cytometers.

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.

Materials Required but not Provided

In addition to the reagents provided in a BD CBA Kit, the following items are also required:

a. A flow cytometer equipped with a 488 nm laser capable of detecting and distinguishing fluorescence emissions at 576 and 670 nm (eg, BD FACScan™ or BD FACSCalibur™ systems) and BD CellQuest™ Software.

b. 12 × 75 mm sample acquisition tubes for a flow cytometer (eg, BD Falcon™, Cat. No. 352008).

c. BD CBA Software (Cat. No. 550065).

Note: For use with BD CellQuest Software, Microsoft® Excel and a Macintosh or PC-compatible computer are required to utilize the BD CBA Software. See the BD CBA Software User's Guide for details.

d. BD CaliBRITE™ 3 Beads (Cat. No. 340486).

BD CBA Assay Procedures

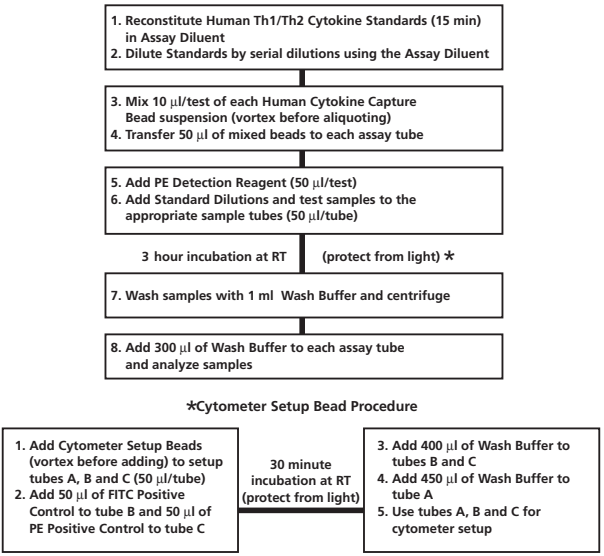

Figure 2. Overview: BD CBA Human Th1/Th2 Cytokine Kit Assay Protocol

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/

Preparation of BD CBA Assay Standards

Each BD CBA Kit contains standard mixtures in an easy-to-use format. The lyophilized standards (once reconstituted) or the standards provided at 4°C, are serially diluted before mixing with the Capture Beads and the Detection Reagent in a given assay.

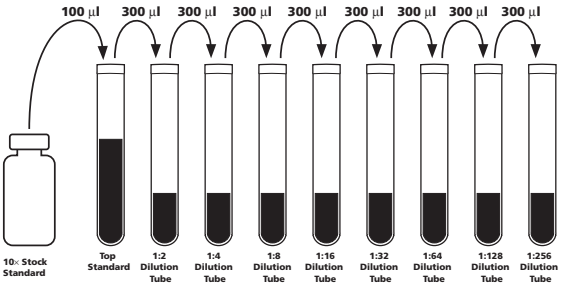

Figure 3. Example standards serial dilutions for the BD CBA Human Th1/Th2 Cytokine Kit.

An example of the approximate concentration (pg/ml) of recombinant protein in each dilution tube in the BD CBA Human Th1/Th2 Cytokine Kit is shown in *Table 1*.

Protein (pg/ml)	Top Standard	1:2 Dilution Tube	1:4 Dilution Tube	1:8 Dilution Tube	1:16 Dilution Tube	1:32 Dilution Tube	1:64 Dilution Tube	1:128 Dilution Tube	1:256 Dilution Tube
Human IL-2	5000	2500	1250	625	312.5	156	80	40	20
Human IL-4	5000	2500	1250	625	312.5	156	80	40	20
Human IL-5	5000	2500	1250	625	312.5	156	80	40	20
Human IL-10	5000	2500	1250	625	312.5	156	80	40	20
Human TNF-α	5000	2500	1250	625	312.5	156	80	40	20
Human IFN-γ	5000	2500	1250	625	312.5	156	80	40	20

Table 1. BD CBA Human Th1/Th2 Cytokine Standard Concentrations after Dilutions.

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. The cytometer setup information in this section is for the BD FACScan and BD FACSCalibur flow cytometers. The BD FACSComp™ Software is useful for setting up the flow cytometer. BD CellQuest Software is required for analyzing samples and formatting data for subsequent analysis using the BD CBA Software.

Instrument Setup with BD FACSComp Software and BD CaliBRITE Beads

1. Add 50 µl of Cytometer Setup Beads to three cytometer setup tubes labeled A, B and C.
2. Add 50 µl of FITC Positive Control Detector to tube B.
3. Add 50 µl of PE Positive Control Detector to tube C.
4. Incubate tubes A, B and C for 30 minutes at room temperature and protect from direct exposure to light.
5. Add 450 µl of Wash Buffer to tube A and 400 µl of Wash Buffer to tubes B and C.

Instrument Setup with BD FACSComp Software and BD CaliBRITE Beads

1. Perform instrument start up.
2. Perform flow check.
3. Prepare tubes of BD CaliBRITE Beads and open BD FACSComp Software.
4. Launch BD FACSComp Software
5. Run BD FACSComp Software in Lyse/No Wash mode.

6. Proceed to ***Instrument Setup with the Cytometer Setup Beads***.

   Note: 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 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 as described in ***Instrument Setup with the Cytometer Setup Beads***, steps 3 – 5. Optimization of the fluorescence parameter settings is still required (ie, PMT and compensation settings, see ***Instrument Setup with the Cytometer Setup Beads***, ***Step 6***).

Instrument Setup with the Cytometer Setup Beads

1. Launch BD CellQuest Software and open the BD CBA Instrument Setup template.

   Note: The BD CBA Instrument Setup template can be found on the BD CBA Software or FACStation CD for Macintosh computers in the BD CBA folder. Following installation on Macintosh computers using BD CBA Software Version 1.0, the template can be found in the BD Applications/BD CBA folder/Sample Files/Mouse Isotyping Files/Instrument Setup folder. For BD CBA Software Version 1.1 or higher, the template can be found in the BD Applications/BD CBA folder. The template is not installed from the CD on PC-compatible computers. This file and instrument setup templates for two-laser and other flow cytometers may also be downloaded via the internet from: **www.bdbiosciences.com/pharmingen/CBA/downloads.shtml**

2. Set the instrument to Acquisition mode.

   Note: The BD CBA Software will evaluate data in five parameters (FSC, SSC, FL1, FL2 and FL3). Turn off additional detectors.

3. Set SSC (side light scatter) and FSC (forward light scatter) to Log mode.
4. Decrease the SSC PMT voltage by 100 from what BD FACSComp set.
5. Set the Threshold to FSC at 650.
6. In setup mode, run Cytometer Setup Beads tube A. Follow the setup instructions in the CBA manual.

   Note: Pause and restart acquisition frequently during the instrument setup procedure in order to reset detected values after settings adjustments. Adjust gate R1 so that the singlet bead population is located in gate R1 (***Figure 4a***).

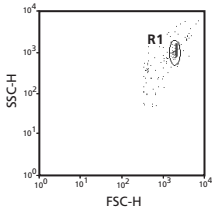

Figure 4a

Adjust gate R1 so that the singlet bead population is located in gate R1 (***Figure 4a***).

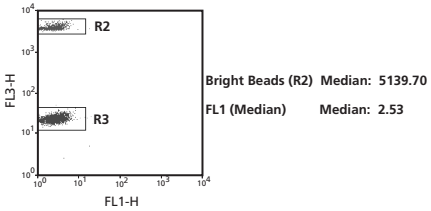

Figure 4b

Adjust the FL3 PMT so that the median of the top FL3 bead population's intensity is approximately 5000 (***Figure 4b***). Adjust gate R3 as necessary so that the dim FL3 bead population is located in gate R3 (***Figure 4b***). Do not adjust the R2 gate.

Adjust the FL1 PMT so that the median of FL1 is approximately 2.0–2.5 (***Figure 4b***).

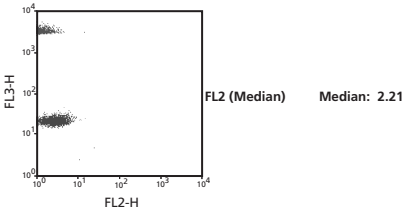

Figure 4c

Adjust the FL2 PMT value so that the median of FL2 is approximately 2.0 – 2.5 (***Figure 4c***).

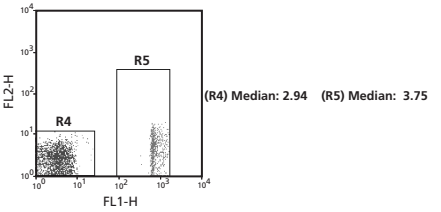

Figure 4d

Run Cytometer Setup Beads tube B to adjust the compensation settings for FL2 – %FL1.

Adjust gate R5 as necessary so that the FL1 bright bead population is located in gate R5 (***Figure 4d***). Using the FL2 – %FL1 control, adjust the median of R5 to equal the median of R4 (***Figure 4d***).

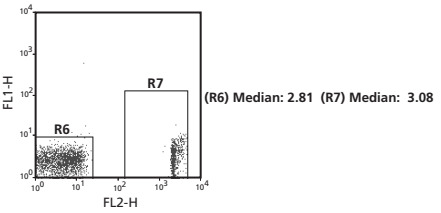

Figure 4e

Run Cytometer Setup Beads tube C to adjust the compensation settings for FL1 – %FL2 and FL3 – %FL2.

Adjust gate R7 so that the FL2 bright bead population is located in gate R7 (***Figure 4e***). Using the FL1 – %FL2 control, adjust the median of R7 to equal the median of R6 (***Figure 4e***).

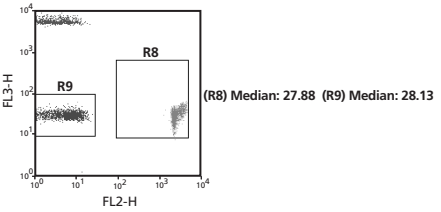

Figure 4f

Adjust gate R9 so that the FL2 bright bead population is located in gate R9 (***Figure 4f***). Using the FL3 – %FL2 control, adjust the median of R9 to equal the median of R8 (***Figure 4f***).

Set the FL2 – %FL3 to 0.1 if necessary. Save and print the optimized instrument settings.

Data Acquisition

1. Open the acquisition template on the BD CBA Software.

   Note: Following installation of the BD CBA Software, the Acquisition template is located in the BD Applications/ BD CBA Folder/Sample Files/Mouse Isotyping Files/Instrument Set Up Folder and is labeled "Isotype Kit Acquire Template". Alternatively, the Acquisition template may be downloaded via the internet from: **www.bdbiosciences.com/pharmingen/CBA/downloads.shtml**

2. Set acquisition mode and retrieve the optimized instrument settings as per the manual.
3. In the Acquisition and Storage window, set the resolution to 1024.
4. Set number of events to be counted as described in the BD CBA kit manual. (This will ensure that the sample file contains approximately 300 events per Capture Bead).
5. Set number of events to be collected to "all events". Saving all events collected will ensure that no true bead events are lost due to incorrect gating.
6. In setup mode, run tube No. 1 and using the FSC vs. SSC dot plot, place the R1 region gate around the singlet bead population (see ***Figure 4a***).
7. Samples are now ready to be read and data acquired.
8. Begin sample acquisition with the flow rate set at HIGH.

   Note: Run the negative control tube (0 pg/ml standards) before any of the recombinant standard tubes. Run the control assay tubes before any unknown test assay tubes.

   To facilitate analysis of data files using the BD CBA 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).

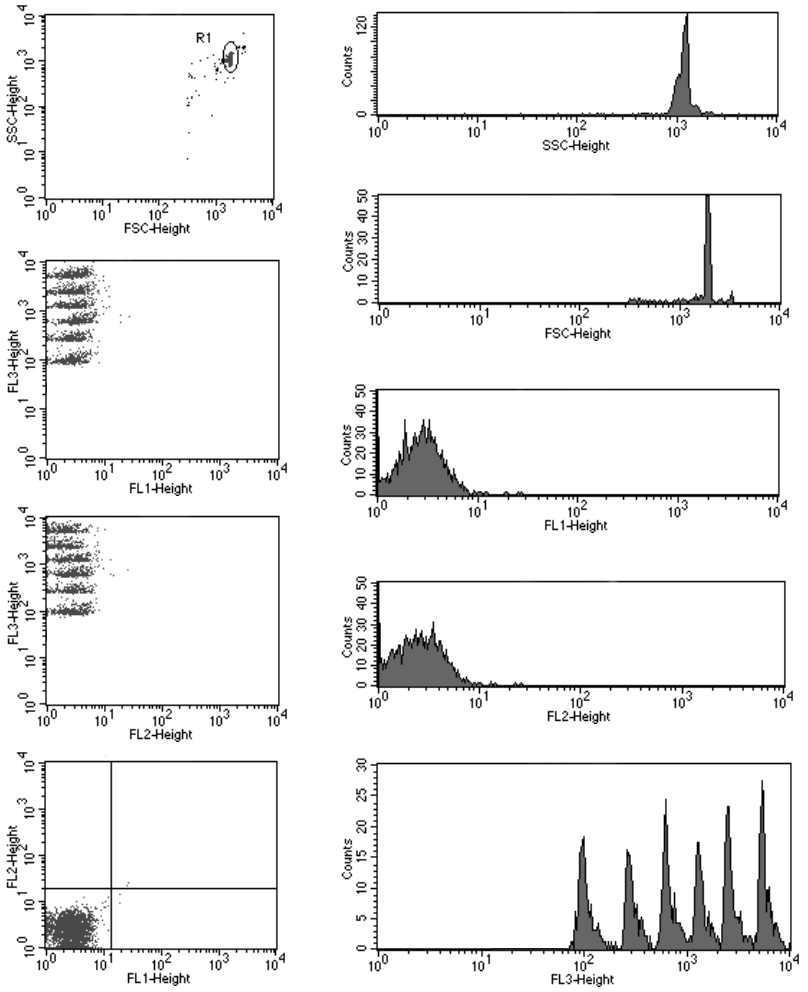

Figure 5. Example Acquisition Template depicting sample data using the BD CBA Human Th1/Th2 Cytokine Kit.

Analysis of Sample Data

The analysis of BD CBA data is optimized when using the BD CBA Software. Install the software according to the instructions in the Software User's Guide. Refer to the manual for each BD CBA Kit for more information on data analysis.

Typical Data

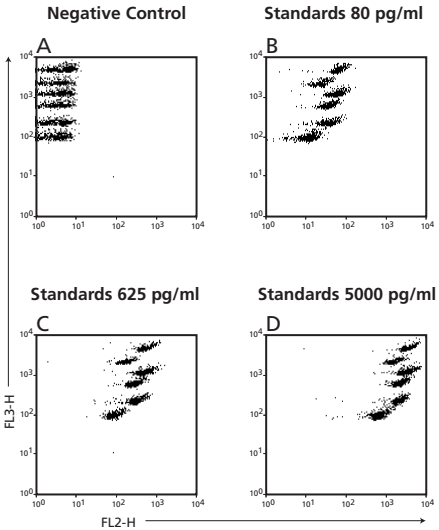

Figure 6. Example BD CellQuest dot plots of various standard dilutions analyzed in the BD CBA Human Th1/Th2 Cytokine Kit.

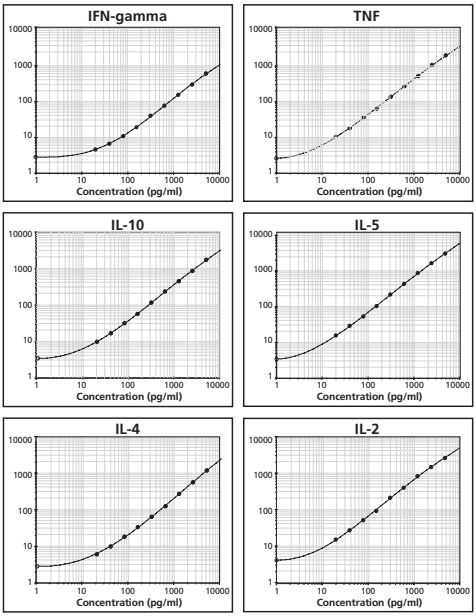

Figure 7. Example standard curves generated using the BD CBA Human Th1/Th2 Cytokine Kit and plotted with the BD CBA Software.

Results

BD Cytometric Bead Array Analysis

8/24/00

					IFN-gamma			TNF		
	Filename	SampleID	Acq Date	Dilut Factor	FL2 MFI	Tube pg/ml	Sample pg/ml	FL2 MFI	Tube pg/ml	Sample pg/ml
1	081500Katy.017	110 supe,8/9/00,neat	15-Aug-00	1	716.9	>5000		3337.6	>5000	
2	081500Katy.018	110 supe,8/9/00,neat	15-Aug-00	1	736.5	>5000		3278.1	>5000	
3	081500Katy.019	110 supe,8/9/00,1/4	15-Aug-00	4	1263.5	>5000		1065.0	3200.3	12801.4
4	081500Katy.020	110 supe,8/9/00,1/4	15-Aug-00	4	1263.5	>5000		1263.5	3863.6	15454.4
5	081500Katy.021	110 supe,8/9/00,1/16	15-Aug-00	16	813.1	>5000		250.3	687.4	10998.1
6	081500Katy.022	110 supe,8/9/00,1/16	15-Aug-00	16	881.7	>5000		324.9	901.5	14424.5
7	081500Katy.023	110 supe,8/9/00,1/64	15-Aug-00	64	271.4	4880.9	312380.6	69.2	182.8	11697.6
8	081500Katy.024	110 supe,8/9/00,1/64	15-Aug-00	64	283.9	5082.7	325295.8	73.7	195.0	12482.7
9	081500Katy.025	110 supe,8/9/00,1/256	15-Aug-00	256	66.1	1442.1	369173.8	18.8	45.9	11748.2
10	081500Katy.026	110 supe,8/9/00,1/256	15-Aug-00	256	65.5	1431.1	366356.1	16.7	40.3	10316.0
11										
12										
13										
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15										

Page 1

BD Biosciences

Figure 8. Example sample data analysis using the BD CBA Software.

BD CBA Assay Performance

For more information concerning performance characteristics (eg, sensitivity, spike recovery, dilution linearity, specificity, and intra- and inter-assay precision), please consult the manual for each specific BD CBA Kit. All current BD CBA product manuals are available on the BD Biosciences website at: **www.bdbiosciences.com/pharmingen/CBA/**

Summary

In summary, the BD Cytometric Bead Arrays represent exciting new technology for analyzing the expression of multiple analytes (eg, cytokines, chemokines, inflammatory mediators, immunoglobulins, and cell signaling molecules) that are often found present together in complex mixtures within biological fluids. For more information concerning BD CBA products, please read the references cited below and access the BD Biosciences website, **www.bdbiosciences.com/pharmingen/CBA/**

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The BD Cytometric Bead Array System. BD Biosciences Brochure.

BD Cytometric Bead Array Product List

Description	Contains	Apps	Format	Size	Cat. No.
Human					
Anaphylatoxin Kit	C3a, C4a, C5a	FCM	Kit	50 tests	552363
Chemokine Kit	IL-8, RANTES, MIG, MCP-1, IP-10	FCM	Kit	50 tests	552990
Inflammation Kit	IL-8, IL-1β, IL-6, IL-10, TNF, IL-12p70	FCM	Kit	50 tests	551811
Inflammation Standards	IL-8, IL-1β, IL-6, IL-10, TNF, IL-12p70	FCM	Lyophilized	1 vial	552932
Th1/Th2 Cytokine Kit	IL-2, IL-4, IL-5, IL-10, TNF, IFN-γ	FCM	Kit	50 tests	550749
Th1/Th2 Cytokine Kit II	IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ	FCM	Kit	50 tests	551809
Th1/Th2 Cytokine Standards	IL-2, IL-4, IL-5, IL-6, IL-10, TNF, IFN-γ	FCM	Lyophilized	1 vial	551810
Apoptosis Kit	Bcl-2, cleaved PARP Active Caspase-3	FCM	Kit	50 tests	inquire
Active Caspase-3	Caspase-3	FCM	Kit	100 tests	552124
Mouse					
Immunoglobulin Isotyping Kit	Heavy and light chain isotypes of mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgA, IgM, and IgE	FCM	Kit	1 kit	550026
Inflammation Kit	IL-6, IL-10, MCP-1 IFN-γ, TNF, IL-12p70	FCM	Kit	50 tests	552364
Th1/Th2 Cytokine Kit	IL-2, IL-4, IL-5, TNF, IFN-γ	FCM	Kit	50 tests	551287
Th1/Th2 Cytokine Standards	IL-2, IL-4, IL-5, TNF, IFN-γ	FCM	Lyophilized	1 vial	552967
Other					
Phosphorylated STAT1 Kit	Phosphorylated STAT1	FCM	Kit	100 tests	557740
BD CBA Software	Mac and PC compatible CDROM and User's Guide	FCM		1 CD	550065

Notes

Chapter 3

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 alpha chain is an MHC-encoded, transmembrane molecule containing three extracelluar domains $\alpha1$, $\alpha2$, and $\alpha3$, while the β chain is a non-MHC encoded small protein called β_2 microglobulin (β_2M) with a molecular weight of 12kDa. Peptide-binding is mainly carried out by the $\alpha1$ and $\alpha2$ domains of the heavy chain. The β_2M is not a membrane protein and associates with the heavy chain in a non-covalent fashion. Although β_2M 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 $\alpha1$ and $\beta1$ 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 cannonical TCR. The TCR primarily expressed by mouse NK cells is Vα 14 J281 and by human NK cells, is Vα 24.

BD DimerX reagents, from BD Biosciences Pharmingen, 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 IgG_1 through recombinant DNA technology (***Figure 1***). The expression vector containing the fusion protein is then co-transfected with genes containing human β_2M 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 β_2M molecule (***Figure 1***, and SDS-PAGE).

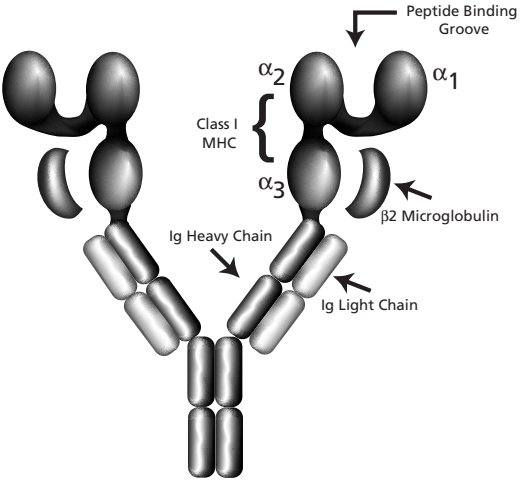

Figure 1. Schematic representation of the MHC class I:Ig dimeric protein.

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 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.

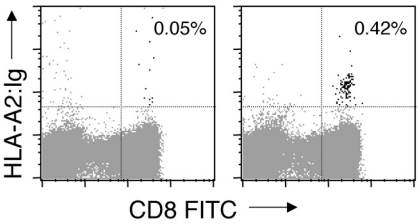

Figure 2. Flow cytometric analysis of normal human lymphocytes from a cytomegalovirus (CMV) seropositive donor. BD DimerX HLA-A2:Ig was loaded with a CMV pp65-derived, HLA-A2-binding peptide (NLVPMVATV) at 640 molar excess. PBMC were stained with unloaded (left panel) or loaded (right panel) purified HLA-A2:Ig, then stained with PE-conjugated anti-mouse IgG_1 (BD Biosciences Pharmingen, Cat. No. 550083), anti-CD14-APC and HLA-DR-APC (BD Biosciences Immunocytometry Systems, Cat. No. 340691), and anti-CD8-FITC (BD Biosciences Pharmingen, Cat. No. 551347). Antibody conjugates were chosen to be non-IgG_1 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.

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.

Standard Protocol

Using BD DimerX MHC:Ig Proteins for flow cytometry involves a two-step approach:

First, ligand loading followed by immunofluorescent staining with flow cytometric analysis. Note, for most BD DimerX the ligands are peptides, however, for CD1d:Ig the ligands are glycolipids. Below we describe the peptide-loading.

Ligand loading

1. Material:

   a. 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.

   b. BD DimerX products contain azide and two molar excess of human β_2M. (In the case of the mouse and human CD1d:Ig fusion proteins, the β_2M is co-expressed with the fusion protein and no molar excess of β_2M is added. The β_2M expressed with the mouse and human CD1d:Ig is mouse in origin).

      Since the BD DimerX molecules are produced from a mammalian cell line, it is generally believed that during the intracelluar transport, the MHC binding site of the BD DimerX is bound with endogenous peptides. It is necessary to replace these endogenous peptides with a single antigenic peptide for flow cytometry applications. In most cases, this can be accomplished by passive loading with a high molar excess of target peptide at 37°C. For low affinity peptides, alkaline or acidic stripping of the endogenous peptides may help to improve loading efficiency.

2. Passive loading

   Co-incubate the target peptides with the BD DimerX reagent at 40, 160 and 640 molar excess. For very high-affinity peptides, loading at 40 molar

excess is sufficient For low affinity peptides, the peptide to BD DimerX molecular ratio should be increased to achieve better loading. Researchers should determine the optimal specific peptide loading conditions for their experimental systems.

The following calculation, using an 8–amino–acid peptide as an example, may be used:

D_p= Molecular weight of peptide

D_{dimer} = 250,000 daltons

R = desired excess of molar ratio: eg, 160

Mp = microgram peptide of interest

M_{dimer} = 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.

$$Mp = \frac{M_{dimer} \times R \times D_p}{D_{dimer}}$$

a. Mix the peptide and BD DimerX reagent together in BD FACS™ buffer (eg, BD Pharmingen™ Stain with BSA, Cat. No. 554657) and incubate at various temperatures.

   Usually the incubation is overnight at 37°C, which will facilitate peptide-exchange and improve 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 3***). 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.

3. Active peptide-loading by alkaline or acid stripping.[2]

   Alkaline Stripping

   a. Incubate BD DimerX reagent with 5 volume equivalents of high pH peptide-stripping buffer (150 mM NaCl, 15 mM Na_2CO_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 mMNa_2PO_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

a. Resuspend target cells in staining buffer (eg, BD Pharmingen Stain with BSA, Cat No. 554657) at a concentration of approximately 10^6 cells per 50 µl.

Note: It may be necessary to block Fc Receptors (FcR) on cells in a mixed cell population prior to staining cells with the BD DimerX reagent in order to reduce non-specific staining of FcR bearing cells.

b. Add loaded BD DimerX to cell suspension. Incubate for 60 minutes at 4°C. (It is not recommended to incubate target cells with the reagent at 37°C). Since the affinity of cognate TCR and peptide/BD DimerX varies, the optimal concentration needs to be determined empirically. Titrate the BD DimerX reagent to determine the optimal concentration for your experimental system (suggested titration range: 0.06, 0.25, 1, and 4 µg).

c. Wash once with 200 µl of staining buffer.

d. Resuspend the cells in 100 µl BD FACS buffer containing appropriately diluted fluorescent secondary reagent. We typically use PE-conjugated A85-1 mAb (anti-mouse IgG_1, Cat No. 550083) at 1 µg for each sample. Incubate 30 to 60 minutes at 4°C.

Note: For multicolor staining, it is important that all antibodies are non-IgG_1 isotypes, unless blocking procedure are employed to avoid cross-reactivity.

e. Wash cells 2× with 120 µl buffer, resuspend cell pellet in approximately 0.5 ml of staining buffer in a tube appropriate for flow cytometry. Use immediately, as fixation of the cells is not recommended.

For more information please review the individual ***BD DimerX Technical Data Sheets*** packaged with the reagent or available on line at **www.bdbiosciences.com**

Troubleshooting

Background on non-T cells

Background staining on non-T cells is a common occurance with the use of both tetramer and BD DimerX reagents. Background can be reduced by preincubating the mouse cells with Mouse BD FcBlock™ CD16/CD32 (FcγIII/II Receptor (Cat. No. 553141 or 553142) or human PBL with 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 IgG_1 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 37ºC or 42.5ºC 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.

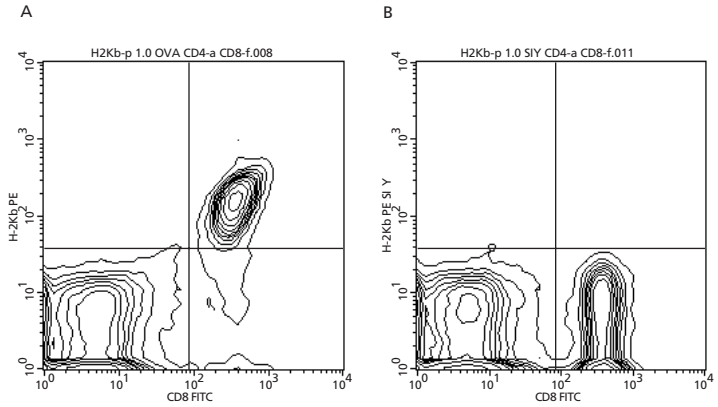

Figure 3. Antigen-specific T cell identification of OT-1 TCR transgenic mouse splenocytes. Splenocytes from OT-1 TCR transgenic mice were incubated with Mouse FC Block™ antibody (anti-mouse CD16/32, Cat. No. 553141/553142, Both Panels) and immunofluorescently stained with FITC-conjugated anti-mouse CD8 (clone 53-6.7. Cat. No. 553030/553031, Both panels) and PE-conjugated Mouse BD DimerX H-2kb:Ig fusion protein (BD Biosciences Pharmingen, 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.

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BD DimerX MHC:Ig Product List

Description	Isotype	Apps	Format	Size	Cat. No.
Human DimerX I: Recombinant Soluble Dimeric CD1d:Ig Fusion Protein	Mouse IgG_1, λ	FCM	Purified	0.1 mg	557764
Mouse DimerX I: Recombinant Soluble Dimeric CD1d:Ig Fusion Protein	Mouse IgG_1, λ	FCM	Purified	0.25 mg	552947
Human DimerX I: Recombinant Soluble Dimeric HLA-A2:Ig Fusion Protein	Mouse IgG_1, λ	FCM	Purified	0.05 mg	551263
Mouse DimerX I: Recombinant Soluble Dimeric H-2D^b:Ig Fusion Protein	Mouse IgG_1, λ	FCM	Purified	0.25 mg	551323
Mouse DimerX I: Recombinant Soluble Dimeric H-2K^b:Ig Fusion Protein	Mouse IgG_1, λ	FCM	Purified	0.25 mg	550750
Mouse DimerX I: Recombinant Soluble Dimeric H-2K^b:Ig Fusion Protein	Mouse IgG_1, λ	FCM	PE	0.1 mg	552944
Mouse DimerX I: Recombinant Soluble Dimeric H-2L^d:Ig Fusion Protein	Mouse IgG_1, λ	FCM	Purified	0.25 mg	550751
Recombinant human β_2 microglobulin		FCM	Purified	0.1 mg	551089

Chapter 4

Immunofluorescent Staining of Intracellular Molecules for Flow Cytometric Analysis

Introduction

New methods and reagents have been developed that enable high-resolution, multiparameter flow cytometric analysis of the nature and frequency of cells that produce antibodies, cytokines, chemokines, and inflammatory mediators such as perforin or granzymes. Flow cytometry is a powerful analytical technique in which individual cells can be simultaneously analyzed for several parameters (***Figure 1***). These include size and granularity, as well as the coexpressed patterns of cell surface (described in ***Chapter 1***) and intracellular molecules defined by fluorescent antibodies and dyes (described in ***Chapter 9***).[1–5] Fluorescent antibodies directed against cytokines, chemokines and inflammatory mediators have become very useful for intracellular staining and multiparameter flow cytometric analysis to determine the cellular mechanisms that underlie immunological and inflammatory responses.[1, 3–8] For example, activated cell populations can be stained by multicolor immunofluorescence 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.[9, 10] 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.[2]

Staining of intracellular antigens depends on the identification of antigen-specific monoclonal antibodies that are compatible with a fixation and permeabilization procedure.[11–13] 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. BD Biosciences offers a large array of fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, allophycocyanin (APC)-and other fluorochrome conjugated antibodies directed against intracellular effector molecules that can be used in single- or multi-color flow cytometric

analyses of immune function. We also offer reagents for fixation and permeabilization, positive control cells, protein transport inhibitors, and Ig isotype controls specifically tested for intra-cellular staining.

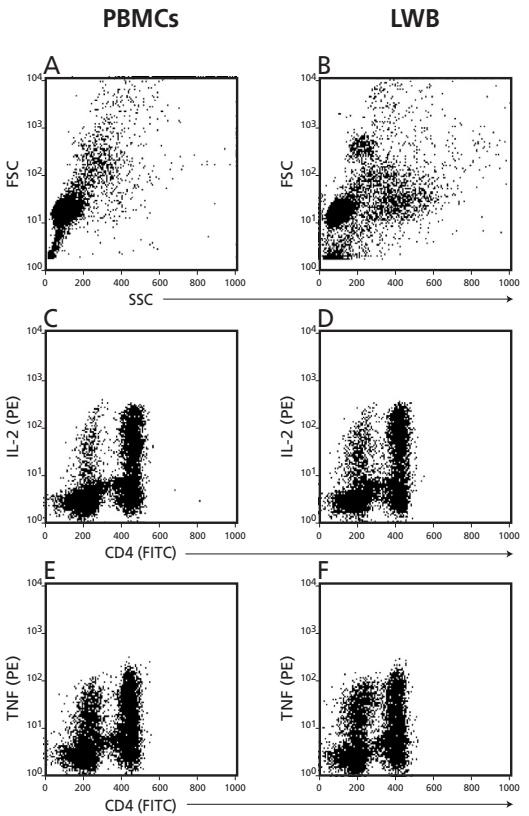


Figure 1. Analysis of IL-2 and TNF production in activated human PBMCs and lysed whole blood cells. Heparinized blood was either passed over Ficoll-Paque™ to isolate human PBMCs or treated with ammonium chloride buffer to lyse erythrocytes and obtain "lysed whole blood cells" (LWB). Both blood cell preparations 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.

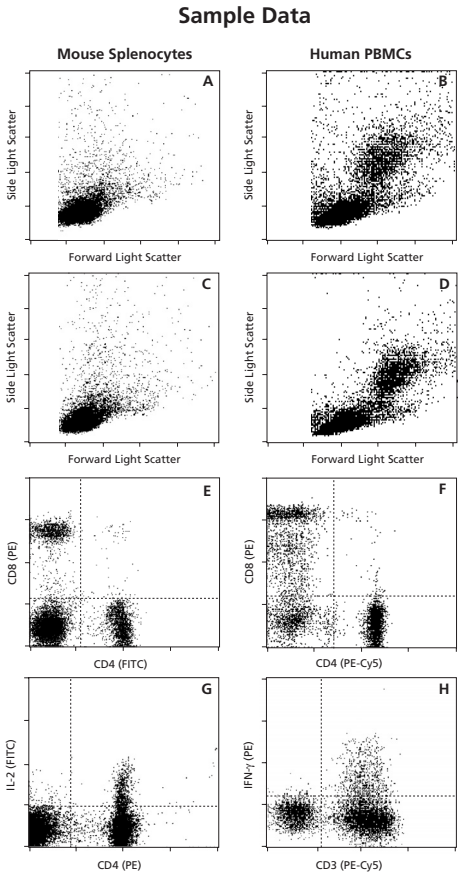


Figure 2. Effects of the BD Cytofix/Cytoperm Solution on cell light scattering properties, cell surface antigen staining, and intracellular cytokine staining. Panels A and B show the forward light scatter and side light scatter profiles for freshly-prepared, untreated mouse splenocytes and Ficoll-Hypaque-isolated 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.

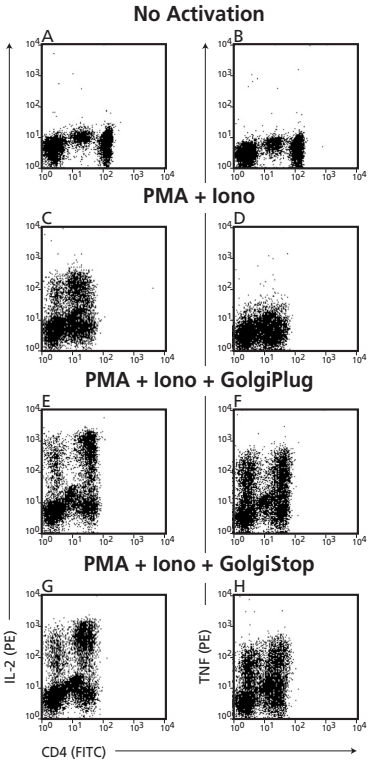


Figure 3. The effect of protein transport inhibitors on intracellular cytokine staining. Human PBMCs were isolated from heparinized whole blood by density centrifugation on Ficoll Paque™ and were either cultured for 4 hrs with no activators and no protein transport inhibitors (A, B) or were stimulated 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.

General Methods

Stimulation of Cells

Various *in vitro* methods (***Figure 4***) have been reported for generating cytokine-producing cells.[1, 3–15] 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.[8]

BD Biosciences Pharmingen 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.

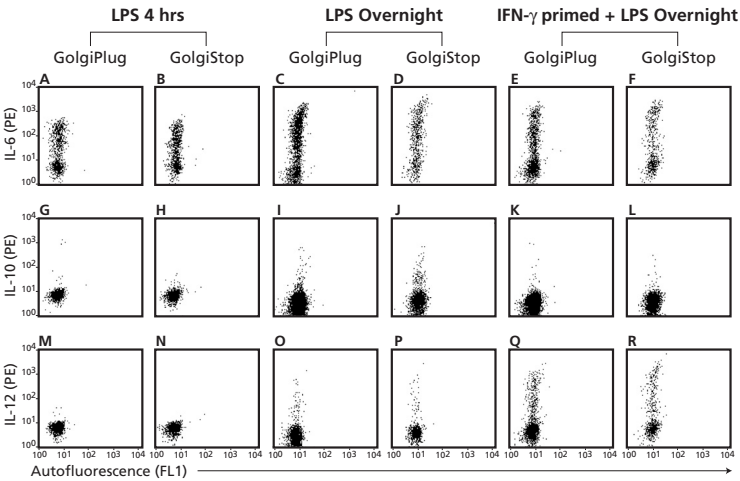


Figure 4. The effect of various activation conditions and various protein transport inhibitors on intracellular cytokine staining. Human PBMCs were isolated from heparinized whole blood by density gradient centrifugation (Ficoll-Paque™). The cells were stimulated with either LPS (1 µg/ml, Sigma, Cat. 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 differention. 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.

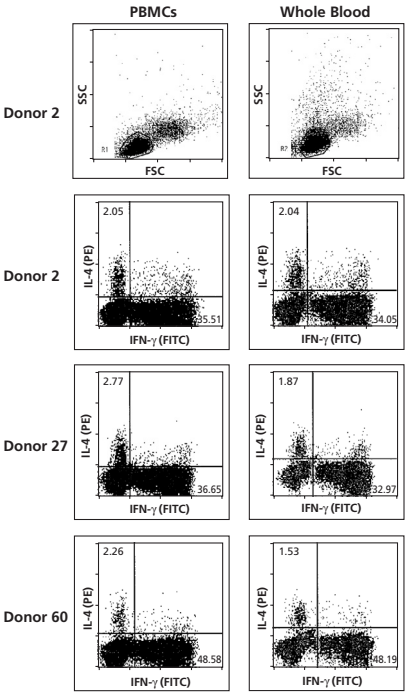
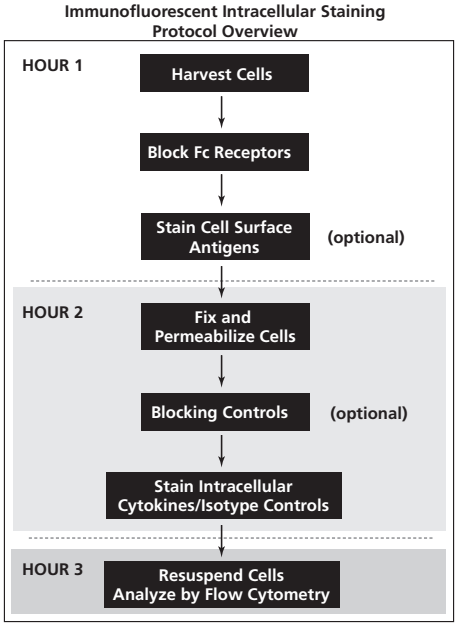


Figure 5. Frequencies of detectable cytokine-producing cells are comparable when staining activated PBMCs or activated whole blood from the same donor. Ficoll-Hypaque™ purified PBMCs (left panels) and whole blood (right panels) from each of the three donors were activated with PMA (50 ng/ml) and ionophore 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 Pharmingen 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.

Protocol: Multicolor Staining for Intracellular Cytokines and Cell Surface Antigens

Harvest Cells



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.[14]

1. In the mouse and rat systems, purified 2.4G2 and D34–485 antibodies directed against FcγII/III (mouse BD FcBlock™; Cat. No. 553142 and 553141) and Fcγ receptors (rat BD FcBlock 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 FcBlock, preincubate cell suspension with 1 µg BD FcBlock/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/10^6 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 ~10^6 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 2× 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 1× 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/10^7 cells for bulk fixing) of Cytofix Buffer at 4°C for 10 – 20 min.

   b. Wash cells 2× 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.

2. Permeabilizing Fixed Cells

   a. For frozen cells, after thawing, gently wash 2× in 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

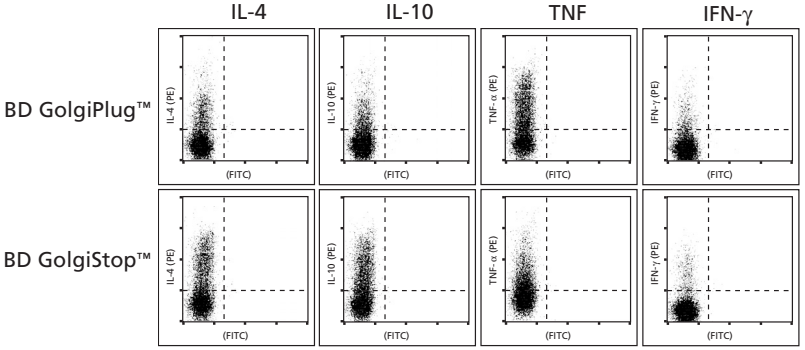
1. Thoroughly resuspend fixed/permeabilized cells in 50 µl of BD Perm/Wash Buffer (100 µl for staining in tubes) containing a pre-determined optimal concentration of a fluorescent anti-cytokine antibody or appropriate negative control. Incubate at 4°C for 15 – 30 min in the dark.
2. Wash cells 2 times 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, Cat. No. 555496) 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% CO_2 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 2 ml of BD Pharm Lyse™ (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.



	IL-2	IL-3	IL-4	IL-5	IL-10	GM-CSF	TNF	IFN-γ
GolgiPlug™ 1 µg/ml	12.9	5.7	14.5	5.2	12.7	10.1	38.2	5.2
GolgiStop™ 2 µM	15.2	8.5	29.1	7.2	30.7	13.3	22.4	5.6

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-γ.

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.[5, 6]

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.[2] 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.[2]

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 ELISPOT 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).[1, 12, 13]

1. Positive control cells

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

Cell Set	Cytokines Measured	Cat. No.
Mouse		
MiCK-1	IL-2, TNF, IFN-γ	554652
MiCK-2	IL-3, IL-4, IL-10, GM-CSF	554653
MiCK-3	IL-6, IL-12p40, TNF, MCP-1	554654
Human		
HiCK-1	IL-2, TNF, IFN-γ	555061
HiCK-2	IL-3, IL-4, IL-10, IL-13, GM-CSF	555062
HiCK-3	IL-1α, IL-1β, IL-6, IL-12, TNF	555063
HiCK-4	IL-8, GROα, IP-10, MCP-1, MCP-3, MIG, MIP-1α	555064
Rat		
RiCK-2	IL-4, IL-10, IFN-γ, GM-CSF	555094

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/10^6 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 Pharmingen Staining Buffers

   BD Biosciences Pharmingen offers two buffers, BD PharmingenStain (FBS) (Cat. No. 554656) and BD PharmingenStain (BSA) (Cat. No. 554657), that are rigorously pretested for their ability to optimize immunofluorescent staining and maintain cell viability.

Fixation and Permeabilization Reagents

BD Biosciences Pharmingen 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.

Reagents for Analyzing Other Intracellular Molecules

BD Biosciences Pharmingen also offers fluorescent nucleic acid dyes and a variety of antibodies for the immunofluorescent staining and flow cytometric analysis of other intracellular (and cell surface) molecules. These molecules are involved in areas of research such as cell proliferation and cell cycling (described in ***Chapter 9***), cell signaling and apoptosis [see ***Apoptosis Manual*** and ***Brochure*** for ***Intracellular Flow Cytometry*** at **www.bdbiosciences.com**]. A variety of antibody formats are available to maximize multiparameter analysis capabilities. Reagents for examining the nature and frequencies of mouse cells that express cytoplasmic immunoglobulin have also been developed (refer to brochure for ***Intracellular Flow Cytometry*** for a list of available reagents).

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Immunofluorescent Staining Product List

Description	Clone	Isotype	Format	Cat. No.
Human				
IL-1α	364-3B3-14	Mouse IgG_1	PE	554561
			Blocking Control/Unlabeled	554558
			Recombinant Cytokine	551838
IL-2	MQ1-17H12	Rat IgG_{2a}	FITC	554565
			PE	554566
			PE	559334*
			APC	554567
			Blocking Control/Unlabeled	554563
			Recombinant Cytokine	554603
IL-3	BVD3-1F9	Rat IgG_1	PE	554676
			Blocking Control/Unlabeled	554673
			Recombinant Cytokine	554604
IL-4	MP4-25D2	Rat IgG_1	FITC	554484
			PE	554485
			APC	554486
			Blocking Control/Unlabeled	554482
IL-4	8D4-8	Mouse IgG_1	PE	554516
			PE	559333*
			Blocking Control/Unlabeled	556917
IL-5	TRFK5	Rat IgG_1	PE	554395
			PE	559335*
			APC	554396
			Blocking Control/Unlabeled	554392
			Recombinant Cytokine	554606
IL-5	JES1-39D10	Rat IgG_{2a}	PE	554489
			PE	559332*
			Blocking Control/Unlabeled	554487
			Recombinant Cytokine	554606
IL-6	MQ2-13A5	Rat IgG_1	FITC	554544
			PE	554545
			Blocking Control/Unlabeled	554542
			Recombinant Cytokine	550071
IL-6	MQ2-6A3	Rat IgG_{2a}	FITC	554696
			PE	554697
			PE	559331*
			Blocking Control/Unlabeled	554694
			Recombinant Cytokine	550071
IL-8	G265-8	Mouse IgG_{2b}	FITC	554719
			PE	554720
			Blocking Control/Unlabeled	554717
			Recombinant Cytokine	554609
IL-10	JES3-9D7	Rat IgG_1	PE	554498
			PE	559337*
			Blocking Control/Unlabeled	554496
			Recombinant Cytokine	554611
IL-10	JES3-19F1	Rat IgG_{2a}	PE	554706
			APC	554707
			Blocking Control/Unlabeled	554704
			Recombinant Cytokine	554611

*** PE format available in 100 test size.**

Immunofluorescent Staining Product List

Description	Clone	Isotype	Format	Cat. No.
Human *(continued)*				
IL-12 (p40/p70)	C11.5.14	Mouse IgG_1	FITC	554574
			PE	554575
			PE	559329*
			APC	554576
			Blocking Control/Unlabeled	554573
IL-12 (p70)	20C2	Rat IgG_1	PE	557020
			PE	559325*
			Blocking Control/Unlabeled	555065
IL-13	JES10-5A2	Rat IgG_1	PE	554571
			PE	559328*
			Blocking Control/Unlabeled	555457
			Recombinant Cytokine	554614
IL-16	14.1	Mouse IgG_{2a}	PE	554736
			Blocking Control/Unlabeled	554734
			Recombinant Cytokine	554637
GM-CSF	BVD2-21C11	Rat IgG_{2a}	FITC	554506
			PE	554507
			Blocking Control/Unlabeled	554503
			Recombinant Cytokine	550068
GRO–α	10G4	Mouse IgG_1	PE	555042
			Blocking Control/Unlabeled	555041
IFN–γ	B27	Mouse IgG_1	FITC	554700
			PE	554701
			PE	559327*
			APC	554702
			Blocking Control/Unlabeled	554699
IFN–γ	4S.B3	Mouse IgG_1	FITC	554551
			PE	554552
			PE	559326*
			Blocking Control/Unlabeled	554549
IP-10	6D4/D6/G2	Mouse IgG_{2a}	PE	555049
			Blocking Control/Unlabeled	556886
MCP-1	5D3-F7	Mouse IgG_1	PE	554666
			PE	559324*
			Blocking Control/Unlabeled	554662
MCP-3	9H11	Mouse IgG_1	PE	555033
			Blocking Control/Unlabeled	555031
MIP-1α	11A3	Mouse IgG_{2a}	FITC	554729
			PE	554730
			PE	559323*
			Blocking Control/Unlabeled	554728
			Recombinant Cytokine	554622
RANTES	2D5	Mouse IgG_1	PE	554732
			PE	559322*
			Blocking Control/Unlabeled	556859

*** PE format available in 100 test size.**

Immunofluorescent Staining Product List

Description	Clone	Isotype	Format	Cat. No.
Human *(continued)*				
TNF	MAb11	Mouse IgG_1	FITC	554512
			PE	554513
			PE	559321*
			APC	554514
			Blocking Control/Unlabeled	554510
			Recombinant Cytokine	554618
LT-α (TNF-β)	359-81-11	Mouse IgG_1	PE	554556
			Blocking Control/Unlabeled	554554
			Recombinant Cytokine	554619
Mouse				
IL-2	JES6-SH4	Rat IgG_{2b}	FITC	554427
			PE	554428
			APC	554429
			Blocking Control/Unlabeled	554425
			Recombinant Cytokine	550069
IL-3	MP2-8F8	Rat IgG_1	PE	554383
			Blocking Control/Unlabeled	554381
			Recombinant Cytokine	554579
IL-4	BVD4-1D11	Rat IgG_{2b}	PE	554389
			Blocking Control/Unlabeled	554386
			Recombinant Cytokine	550067
IL-4	11B11	Rat IgG_1	PE	554435
			APC	554436
			Blocking Control/Unlabeled	554433
			Recombinant Cytokine	550067
IL-5	TRFK5	Rat IgG_1	PE	554395
			APC	554396
			Blocking Control/Unlabeled	554392
			Recombinant Cytokine	554581
IL-6	MP5-20F3	Rat IgG_1	PE	554401
			Blocking Control/Unlabeled	554400
			Recombinant Cytokine	554582
IL-10	JESS-16E3	Rat IgG_{2b}	FITC	554466
			PE	554467
			APC	554468
			Blocking Control/Unlabeled	554464
			Recombinant Cytokine	550070
IL-12 (p40/p70)	C15.6	Rat IgG_1	PE	554479
			APC	554480
			Blocking Control/Unlabeled	554477
IL-17	TC11-18H10	Rat IgG_1	PE	559502
			Blocking Control/Unlabeled	559501
			PE	554406
			Blocking Control/Unlabeled	554404
			Recombinant Cytokine	554586

*** PE format available in 100 test size.**

Immunofluorescent Staining Product List

Description	Clone	Isotype	Format	Cat. No.
Mouse *(continued)*				
IFN–γ	XMG1.2	Rat IgG_1	FITC	554411
			PE	554412
			APC	554413
			Blocking Control/Unlabeled	554409
MCP-1	2H5	Hamster IgG	PE	554443
			Blocking Control/Unlabeled	551217
TNF	MP6-XT22	Rat IgG_1	FITC	554418
			PE	554419
			APC	554420
			Blocking Control/Unlabeled	554416
			Recombinant Cytokine	554589
TNF	TN3-19.12	Hamster IgG	PE	559503
			Blocking Control/Unlabeled	557516
			Recombinant Cytokine	554589
Rat				
IL-4	OX-81	Mouse IgG_1	PE	555082
			Blocking Control/Unlabeled	555080
			Recombinant Cytokine	555107
IL-10	A5-4	Mouse IgG_{2b}	PE	555088
			Blocking Control/Unlabeled	555087
			Recombinant Cytokine	555113
GM-CSF	B61-5	Mouse IgG_1	PE	555092
			Blocking Control/Unlabeled	556885
IFN–γ	DB-1	Mouse IgG_1	FITC	559498
			PE	559499
			Blocking Control/Unlabeled	559650
MCP-1	2H5	Hamster IgG	PE	554443
			Blocking Control/Unlabeled	554441
			Recombinant Cytokine	555110
TNF	TN3-19.12	Hamster IgG	PE	559503
			Blocking Control/Unlabeled	557516
Isotype Controls				
Mouse IgG_1	MOPC-21		FITC	554679
			PE	554680
			PE	559320*
			APC	554681
Mouse IgG_{2a}	G155-178		FITC	554647
			PE	554648
			PE	559319*
Mouse IgG_{2b}	27-35		FITC	555057
			PE	555058
Rat IgG_1	R3-34		FITC	554684
			PE	554685
			PE	559318*
			APC	554686

*** PE format available in 100 test size.**

Immunofluorescent Staining Product List

Description	Clone	Format	Cat. No.
Isotype Controls *(continued)*			
Rat IgG_{2a}	R35-95	FITC	554688
		PE	554689
		PE	559317*
		APC	554690
Rat IgG_{2b}	A95-1	FITC	556923
		PE	556925
		APC	556924
Hamster IgG	G235-2356	PE	554711

Related Reagents	
BD Cytofix/Cytoperm Kit	554714
BD Cytofix/Cytoperm Plus - (with BD GolgiStop)	554715
BD Cytofix/Cytoperm Plus - (with BD GolgiPlug)	555028
BD GolgiStop (containing monensin)	554724
BD GolgiPlug (containing brefeldin A)	555029
BD Cytofix/Cytoperm	554722
Leukocyte Activation Cocktail	550583
BD Perm/Wash Buffer	554723
BD Cytofix Buffer	554655
BD PharmingenStain Buffer (FBS)	554656
BD PharmingenStain Buffer (BSA)	554657
BD BrdU Flow Kit (FITC)	559619
Starter Kit for Intracellular Cytokine Staining - Human	559302
Starter Kit for Intracellular Cytokine Staining - Mouse	559311
BD Pharm Lyse	555899

Intracellular Cytokine-Positive Control Cells

Description	Cytokines Expressed	Cat. No.
Human		
HiCK 1	IL-2, IFN–γ, TNF	555061
HiCK 2	IL-3, IL-4, IL-10, IL-13, GM-CSF	555062
HiCK 3	IL-1–α, IL-1β, IL-6, IL-12, TNF	555063
HiCK 4	IL-8, GRO, IP-10, MCP-1, MCP-3, MIG, MIP-1α, RANTES	555064
Mouse		
MiCK 1	IL-2, IFN–γ, TNF	554652
MiCK 2	IL-3, IL-4, IL-10, GM-CSF	554653
MiCK 3	IL-6, IL-12, MCP-1, TNF	554654
Rat		
RiCK 2	IL-4, IL-10, GM-CSF	555094

*** PE format available in 100 test size.**

Notes

Chapter 5

BD FastImmune™ Cytokine Flow Cytometry

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 tetramers 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 cooling[6] 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]

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. BD Biosciences has developed such assays, using a number of different antigens that include viral lysates, recombinant viral proteins, and peptides. In this protocol, we describe the preparation and use of certain antigens with which we have experience, including a superantigen, staphylococcal enterotoxin B (SEB), used as a positive control. 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.

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 37°C. 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 FACS™ 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***).

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.

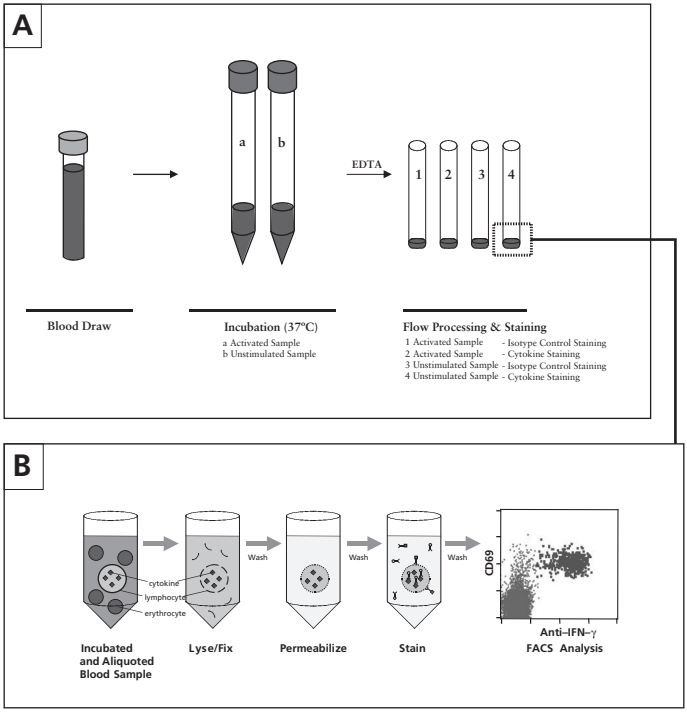

Figure 1. Schematic of whole blood FastImmune antigen-specific assay: Part A From blood draw to sample activation to flow cytometric sample processing; Part B Staining and processing of samples for flow cytometric analysis, applies to tubes 1 to 4 from Part A.

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.

Activation Agent	Source	Stock Solution	Use in Assay
SEB positive control	Sigma Catalog No. S4881 (1 mg)	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.	Use 20 μL of stock solution for stimulation of 1 mL blood at a final concentration of 1 μg/mL.
CMV Lysate	Advanced Biotechnologies (ABI) Catalog No. 10-144-000 (1 mg) Catalog No. 10-144-100 (0.1 mg)	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.	Use 20 μL of stock solution for stimulation of 1 mL blood at a final concentration of 1 μg/mL.
CMV pp65 protein	Austral Biotechnologies Catalog No. CMA-1420-4 (50 μg)	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.	Use 20 μL of stock solution for stimulation of 1 mL blood at a final concentration of 0.5 μg/mL.
Peptides		Most peptides can be dissolved in DMSO at a concentration of 2 mg/mL. Aliquots of 5 μL each are frozen at –80°C.	Use 5 μL of stock solution for stimulation of 1 mL blood at a final concentration of 10 μg/mL.

Table 1. Antigens in this assay

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
- IgG_{2a} FITC/IgG_1 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
- IgG_{2a} FITC/IgG_1 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
- IgG_{2a} FITC/IgG_1 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
- IgG_{2a} FITC/IgG_1 PE/CD4 PerCP-Cy5.5
- Activation and processing solutions

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 flow cytometer
  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.§
  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% NaN_3 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 (eg, 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.[6]

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.[6] 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 AutoCOMP™ 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 FLl 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.

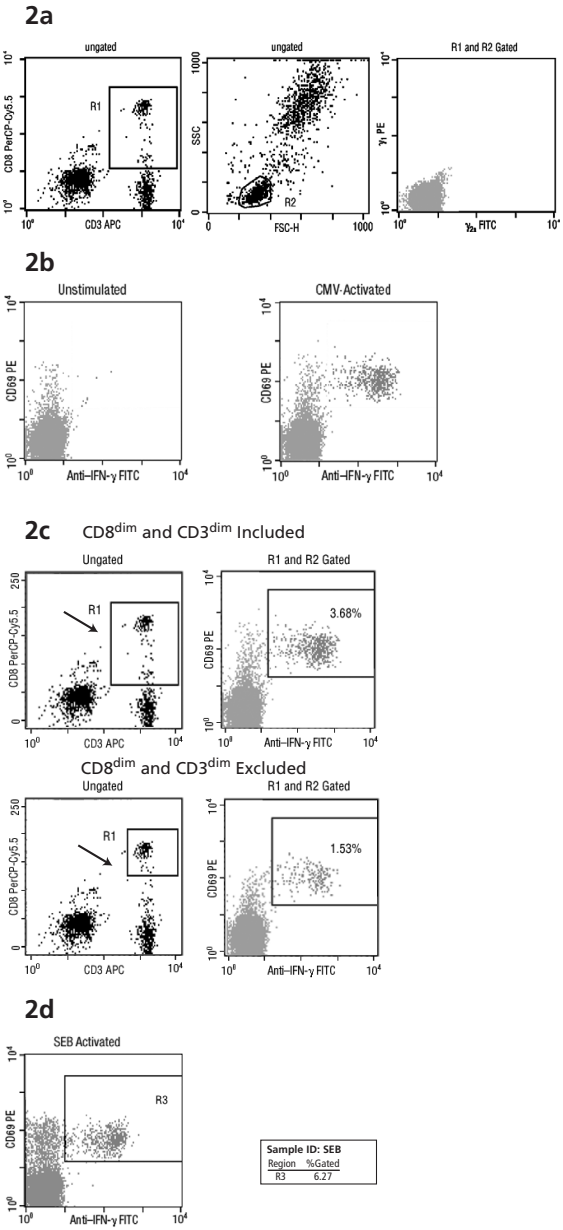

Figure 2. BD FastImmune CD8 Kit: 2a Gating strategy on isotype control, 2b Unstimulated and CMV-activated Anti–IFN-γ vs CD69 dot plots, 2c Importance of including $CD8^{dim}$/$CD3^{dim}$ cells for maximum detection of cytokine-positive cells, and 2d SEB-activated positive control.

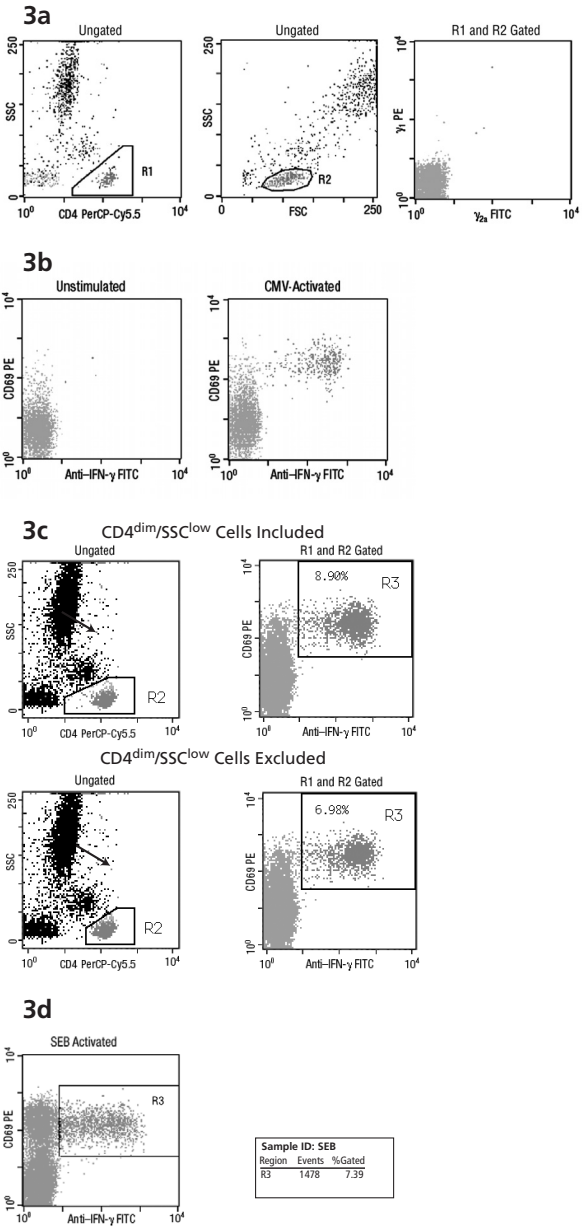

Figure 3. BD FastImmune CD4 Kits: 3a Gating strategy on isotype control, 3b Unstimulated and CMV-activated anti-cytokine vs CD69 dot plots, 3c SEB-activated positive control, and 3d Importance of including $CD4^{dim}$ and SSC^{low} cells for maximum detection of cytokine-positive cells.

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 $CD3^{dim}$ and $CD8^{dim}$ 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 dim lymphocytes (SSC^{low}) 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 $CD4^{dim}$ but SSC^{high}. 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.[6]

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.[13] 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***.

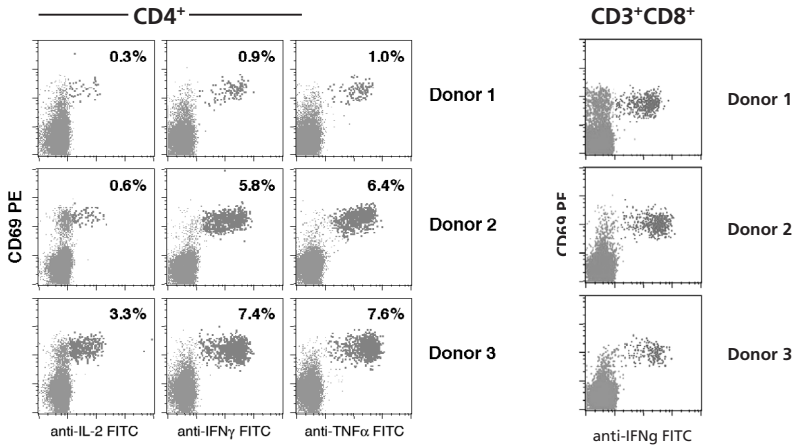

Figure 4. Biological variation among CMV-seropositive donors in response to CMV.

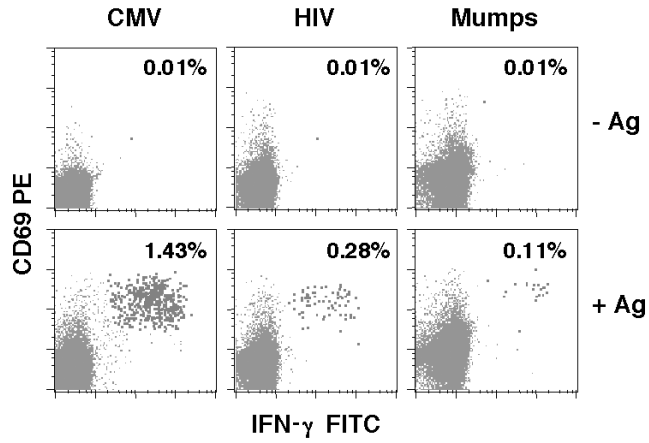

Figure 5. Typical CD4 IFN-γ responses to three different antigens.

Troubleshooting

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

Problem	Possible Cause	Solution	Comments
Poor cell recovery	Inadequate centrifugation	Perform all spins at 500 x g for at least 5 minutes.	Fixed and permeabilized cells are more buoyant than live cells; therefore, they require higher centrifugal force to pellet.
	Loss of pellet on aspiration	Decant supernatants.	Cell pellets are loose and easily disturbed by aspiration.
	Low CD4 count (eg, in HIV-infected samples)	Stain 200µL or more blood per sample.	Increase volume of BD FACS Lysing Solution accordingly; other reagent volumes do not need adjusting. Validate assay performance on larger sample volumes
No cytokine-positive cells	Inadequate activation, permeabilization, or staining as necessary	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.
	Lack of immune competence in the donor	Use a positive control, such as SEB activation, to assess the immune competence of the donor in question.	
	Wrong anticoagulant used for blood collection	Use only sodium heparin anticoagulant. Do not use lithium heparin. Do not use ACD, EDTA, or other calcium-chelating anticoagulants.	Calcium is required for lymphocyte activation; calcium-chelating anticoagulants prevent activation.
Low numbers of cytokine-positive cells	Inadequate activation	Titrate antigen to find the optimal dose for stimulation.	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.
		Use a freshly diluted aliquot of BFA, and store aliquots of BFA at –20°C.	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 exogeneous addition of peptide(s) or peptide mixes to whole blood and PBMCs.[47,48]
Low intensity of cytokine staining	Inadequate permeabilization or staining or both	Dilute BD FACS Lysing Solution and BD FACS Permeabilizing Solution 2 to 1× with DI water, and use at room temperature.	Do NOT dilute BD FACS Lysing Solution or BD FACS Permeabilizing Solution 2 in PBS or other buffers.
		Minimize residual volume after each wash by shaking the tube once or twice after decanting supernatant.	A low residual volume of about 100 µL is needed to avoid excessive dilution of BD FACS Permeabilizing Solution 2 or staining mAb.
		Use 500 µL/sample of BD FACS Permeabilizing Solution 2 for a full 10 minutes at room temperature.	BD FACS Lysing Solution and BD FACS Permeabilizing Solution 2 should be used at room temperature, and all incubations should be at room temperature.
		Vortex thoroughly to resuspend cells in BD FACS Permeabilizing Solution 2.	
High background in unstimulated samples	Poor compensation	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.
	Imprecise gating	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.
		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.	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: Use an exclusion channel, such as CD33 APC + CD62P APC, to simplify exclusion of monocytes and activated platelets.	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.
Long run time needed to acquire adequate number of $CD4^{+}$ events	Excessive dilution of samples in fixative before acquisition	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.
	Poor cell recovery or limited number of $CD4^{+}$ cells in sample	See *Poor cell recovery* in this table.	

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***.

* 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

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BD FastImmune™ Product List

Description	Format	Size	Cat. No.
BD FastImmune Cytokine Kits			
CD8 Anti-Hu–IFN-γ Intracellular Detection Kit	FITC/PE/PerCP-Cy5.5/APC†	25 each•	346049*
Kits contain:			
BD FastImmune Anti-Hu–IFN-γ/CD69/CD8/CD3			
BD FastImmune IgG_{2a}/IgG_1/CD8/CD3 Isotype Control			
BD FastImmune Brefeldin A			
BD FastImmune EDTA Solution			
BD FastImmune CD28/CD49d Costimulatory Reagent			
BD FACS™ Lysing Solution†			
BD FACS Permeabilizing Solution 2			
CD4 Anti-Hu–IFN-γ Intracellular Detection Kit	FITC/PE/PerCP-Cy5.5	25 each•	340970
CD4 Anti-Hu–IL-2 Intracellular Detection Kit	FITC/PE/PerCP-Cy5.5		340971
CD4 Anti-Hu–TNF-α Intracellular Detection Kit	FITC/PE/PerCP-Cy5.5		340972
Kits contain:			
BD FastImmune Anti-Hu–cytokine/CD69/CD4			
BD FastImmune IgG_{2a}/IgG_1/CD4 Isotype Control			
BD FastImmune Brefeldin A			
BD FastImmune EDTA Solution			
BD FastImmune CD28/CD49d Costimulatory Reagent			
BD FACS Lysing Solution			
BD FACS Permeabilizing Solution 2			

• 25 each for stimulated and resting samples

BD FastImmune Multicolor Reagents			
BD FastImmune Anti-Hu–IFN-γ/CD69/CD8/CD3	FITC/PE/PerCP-Cy5.5/APC	50 tests	346048
BD FastImmune Isotype Control IgG_{2a}/IgG_1/CD8/CD3	FITC/PE/PerCP-Cy5.5/APC	50 tests	346047
BD FastImmune Anti-Hu–IFN-γ/CD69/CD4	FITC/PE/PerCP-Cy5.5	50 tests	340962
BD FastImmune Anti-Hu–IL-2/CD69/CD4	FITC/PE/PerCP-Cy5.5	50 tests	340963
BD FastImmune Anti-Hu–TNF-α/CD69/CD4	FITC/PE/PerCP-Cy5.5	50 tests	340964
BD FastImmune Isotype Control IgG_{2a}/IgG_1/CD4	FITC/PE/PerCP-Cy5.5	50 tests	340965
BD FastImmune Anti-Hu–IFN-γ/Anti-Hu–IL-4	FITC/PE	50 tests	340456
BD FastImmune Isotype Control IgG_{2a}/ IgG_1	FITC/PE	50 tests	340458
BD FastImmune Cytokine Value Bundle No. 1‡	FITC/PE/PerCP	50 tests	340460
Bundle contains:			
BD FastImmune Anti-Hu–IFN-γ FITC/Anti-Hu–IL-4 PE			
BD FastImmune IgG_{2a} FITC/IgG_1 PE Isotype Control			
CD3 PerCP (100 tests)			
Proliferation Reagent—Intracellular			
BD FastImmune Anti-BrdU with DNase FITC[a]		50 tests	340649

[a] Based on the incorporation of bromodeoxyuridine to replace thymidine during DNA replication, Anti-BrdU fluorescent conjugated–antibody binding is used to identify the dividing cells. Developed for use with the BD FastImmune intracellular cytokine assay.

BD FastImmune™ Product List

Description	Size	Cat. No.
Single-Color Reagents—Intracellular		
BD FastImmune Anti-Hu–IL-1ra PE	50 tests	340525
BD FastImmune Anti-Hu–IL-1α FITC	50 tests	340513
BD FastImmune Anti-Hu–IL-1α PE	50 tests	340514
BD FastImmune Anti-Hu–IL-1β FITC	50 tests	340515
BD FastImmune Anti-Hu–IL-1β PE	50 tests	340516
BD FastImmune Anti-Hu–IL-2 FITC	50 tests	340448
BD FastImmune Anti-Hu–IL-2 APC		
BD FastImmune Anti-Hu–IL-2 PE	50 tests	340450
BD FastImmune Anti-Hu–IL-4 PE	50 tests	340451
BD FastImmune Anti-Hu–IL-6 FITC	50 tests	340526
BD FastImmune Anti-Hu–IL-6 PE	50 tests	340527
BD FastImmune Anti-Hu–IL-8 FITC	50 tests	340509
BD FastImmune Anti-Hu–IL-8 PE	50 tests	340510
BD FastImmune Anti-Hu–IL-13 PE	50 tests	340508
BD FastImmune Anti-Hu–TNF-α FITC	50 tests	340511
BD FastImmune Anti-Hu–TNF-α PE	50 tests	340512
BD FastImmune Anti-Hu–TNF-α APC	100 tests	340534
BD FastImmune Anti-Hu–IFN-γ FITC	50 tests	340449
BD FastImmune Anti-Hu–IFN-γ PE	50 tests	340452
BD FastImmune Anti-Hu–IFN-γ APC		
CD69 PE (intracellular formulation)	50 tests	341652
CD69 APC	100 tests	340560
CD3 PerCP	100 tests	347344
CD3 PerCP-Cy5.5	50 tests	340949
CD3 APC	100 tests	340440
CD4 PerCP	100 tests	347324
CD4 PerCP-Cy5.5	50 tests	341654
CD4 APC	100 tests	340443
CD8 PerCP-Cy5.5	50 tests	341051
CD8 APC	100 tests	340584
Isotype Control IgG_1 FITC	100 µg	349041
Isotype Control IgG_1 PE (intracellular formulation)*	50 µg	340013
Isotype Control IgG_{2a} FITC	100 µg	349051
Isotype Control IgG_{2a} PE (intracellular formulation)*	25 µg	340459

Accessory Sample Activation and Sample Processing Products*

For Whole Blood Activation:

BD FastImmune CD28/CD49d Costimulatory Reagent, 1×[a]	300 µL	347690
BD FastImmune Brefeldin A Solution, 10×[b]	250 µL	347688
BD FastImmune EDTA Solution, 1×[c]	2.50 mL	347689

[a] Use at 5 mL/0.5 mL whole blood.

[b] Dilute 1:10 with sterile PBS and use at 1× concentration, 10 mL/0.5 mL whole blood.

[c] Use at 1× concentration at 50 mL/0.5 mL whole blood.

BD FastImmune™ Product List

Description	Size	Cat. No.
Use for Lysis and Permeabilization Post Whole Blood Activation:		
BD FACS Lysing Solution, 10× (150 tests standard protocol)	30 mL	347691
BD FACS Lysing Solution, 10× (500 tests standard protocol)	100 mL	349202
BD FACS Permeabilizing Solution 2, 10× (200 tests)	10 mL	347692
BD FACS Permeabilizing Solution 2, 10× (500 tests)	25 mL	340973

‡ Not available in all countries; contact your local BD Biosciences representative for availability.

* Use of these products to measure activation antigens expressed on mononuclear cell subsets for purpose of monitoring immunoregulatory status can fall under one or more claims of the following patents: US 5,445,939; 5,656,446; 5,843,689; Europe 319,543; Canada 1,296,622; Australia 615,880; and Japan 2,769,156.

Notes

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.[5–7] 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]

In collaboration with CTL Analyzers LLC (**www.immunospot.com**; Cleveland, OH), BD Biosciences has identified a panel of novel, optimized antibody pair combinations that are specially formulated for performing ELISPOT assays. CTL's ImmunoSpot Series 1 and Series 2 Analyzers allow for state-of-the-art video

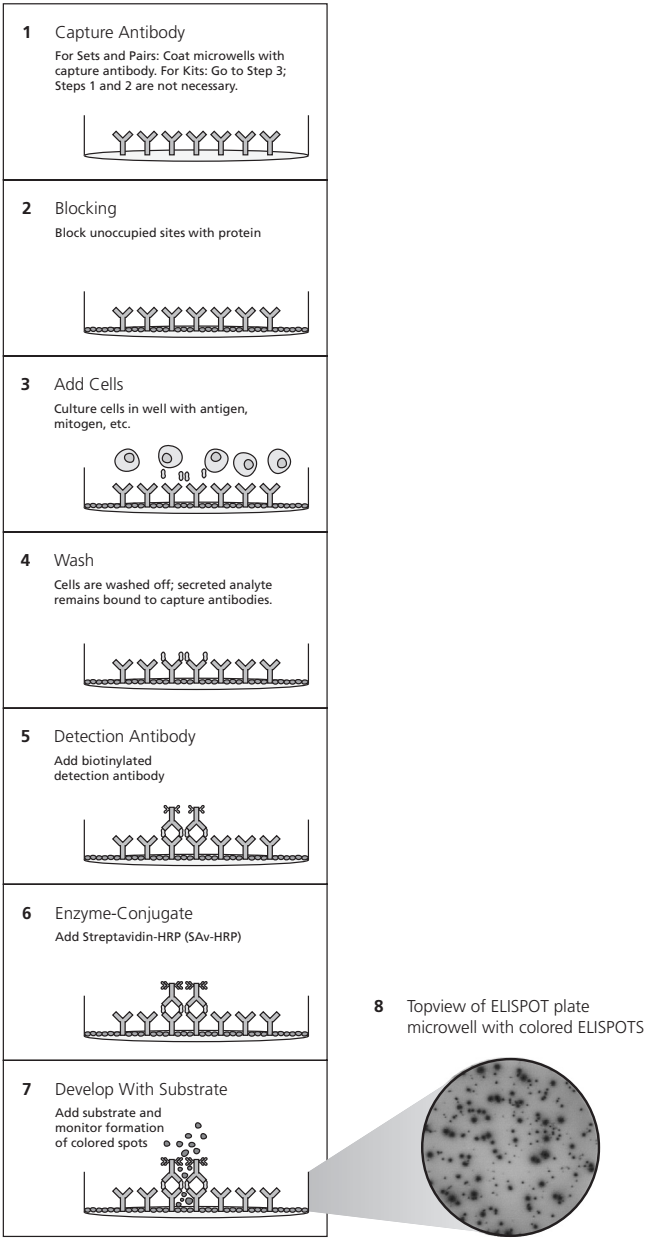
capture and computer-assisted image analysis of ELISPOT plate microwell membranes. During analysis, images of the individual, developed ELISPOT plate microwell membranes are first captured and stored as either high-resolution TIF or compressed JPEG files. These image data files can then be used for manual or automatic spot counting and spot size analysis after setting up the proper counting parameters with the ImmunoSpot Software™. 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 ELISPOT assay. ELISPOT-compatible antibody clones (ie, paired capture and detection antibodies) were selected based on their ability to generate discreet, densely-colored "spots" (ie, ELISPOTs). In some cases, the best performing ELISPOT 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 ELISPOT 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 ELISPOT 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 ELISPOT assay include its high sensitivity, its capacity for high-throughput analyses, its minimal cell number requirement, and its ability to analyze cryopreserved lymphocytes. ELISPOT analysis is also compatible with other assays. For example, cells characterized by ELISPOT analysis can be subsequently transferred for cloning, proliferation assays, flow cytometric analysis, or other methods of analysis.

Granzyme B ELISPOT assay

Granzyme B ELISPOT assay is a non-radioactive alternative to Chromium-release assays. Detection of Granzyme B-secreting cells in ELISPOT assays correlates with cytolytic responses measured by classic radioactive ^{51}Cr-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 ELISPOT assay directly measures the frequencies of Granzyme-B-producing cells.

ELISPOT Assay Protocol



Note: Use ELISPOT plates and reagents under aseptic conditions (eg, 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 114***.

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, therefore, omit steps 1 and 2 for Kits.
   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 ***Immunofluorescent Staining of Intracellular Molecules for Flow Cytometry*** (see ***Chapter 4***). 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 which is diluted in complete tissue culture medium. Add 100 µl/well to ELISPOT plate.
   c. Prepare cell suspensions at different densities, (eg, 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, (ie, 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% CO_2 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 2× with 200 µl/well of distilled water (dH_2O). Allow wells to soak for 3 – 4 min at each wash step.
    b. Wash wells 3× 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) (BD Biosciences Pharmingen Cat. No. 557630):
    a. Discard Detection Antibody solution. Wash wells 3× 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 4× 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 2× 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 dH_2O.
    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 the ImmunoSpot® Analyzer. With the ImmunoSpot® Analyzer and Software it is possible to generate data in several formats including, unprocessed and processed well membrane images, spot counts per well, mean spot size per well, and spot size histograms for each well.

*** 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 $Na_2HPO_4 \bullet 7H_2O$; 0.24 g KH_2PO_4; add dH_2O 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 5×10^{-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_2O_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.
8. When scanning a plate in the ImmunoSpot® Analyzer, make sure the plate is completely inserted into the base.

Available Formats for ELISPOT Products

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

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

ELISPOT Reagent Sets

- 10 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

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

For a complete listing of ELISPOT reagents currently available, please refer to the Product List at the end of this chapter or visit the ELISPOT Homepage at **www.bdbiosciences.com/pharmingen/ELISPOT**

Key Features of BD ELISPOT

Optimized Antibody Pairs

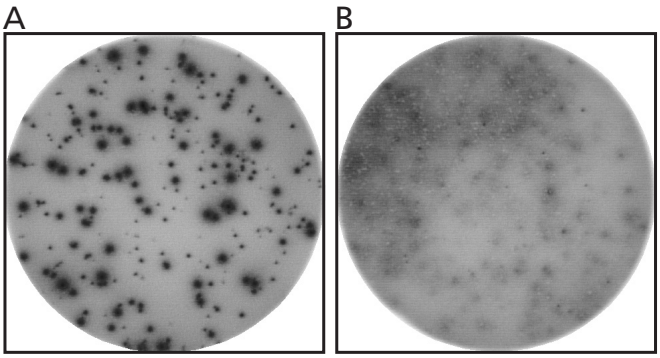


Figure 1. Superior human IL-2 ELISPOTs are obtained using BD ELISPOT Set with a cocktail of capture antibodies. Human PBMCs were stimulated (overnight) with PMA (5 ng/ml; Sigma, Cat. No. P-8139) and ionomycin (500 ng/ml; Sigma, Cat. No. I-0634) in the microwell of a BD ELISPOT plate pre-coated with a 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 (BD Biosciences Pharmingen, Cat. No. 551282).
Panel B: Suboptimal human IL-2 ELISPOT using other antibodies.

BD ELISPOT Kits for Convenient and Consistent Results

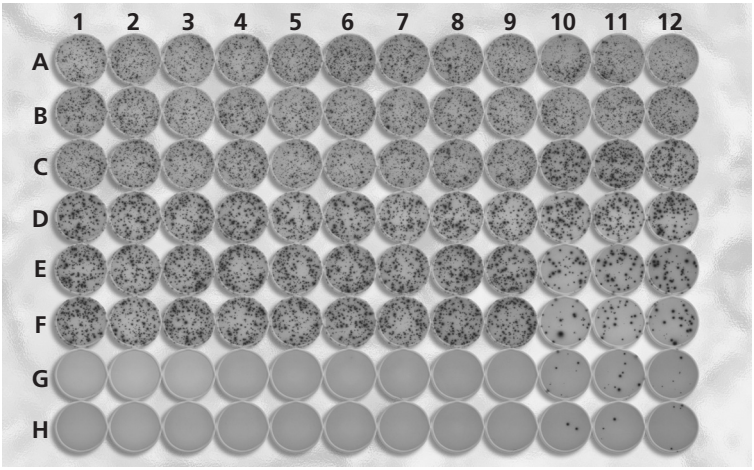


Figure 2. Detection of IFN-γ -secreting cells with a BD ELISPOT Human IFN-γ Kit. (BD Biosciences Pharmingen Cat. No. 552138). Human PBMCs were stimulated with PMA and ionomycin for 18 hours. (blank controls: microwells G 1 – 9: cells without stimuli; microwells H 1 – 9: stimuli without cells)

Consistent Performance of BD ELISPOT Human IFN-γ Kits (with pre-coated plates)				
	Plate	No. of Spots/Well (Mean of 36 wells)	SD	%CV
Exp. 1	1	432	19	4.4
	2	466	18	3.8
	3	432	22	5.0
Exp. 2	4	338	26	7.8
	5	365	19	5.2
	6	363	20	5.6
Exp. 3	7	303	13	4.3
	8	311	13	4.2
	9	301	17	4.1
			Average	4.9

Note: Results of three independent experiments performed at same cell concentration (8×10^4 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	
Features	**Benefits**
Automated BD BioCoat™ coating process	Consistent results (Table 2)
Pre-coated plates	Minimal well-to-well and plate-to-plate variation
High quality antibody pairs (NA/LE™ capture Ab and specially formulated detection Ab)	Crisp, clearly-defined immunospots with low background (see cover photo and at right)
Cross-reactive with NHP*	Broad application including NHP studies
Contains all the necessary reagents	Convenient
Validated in leading edge ImmunoSpot® Analyzer	Complete solution for your ELISPOT research

**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

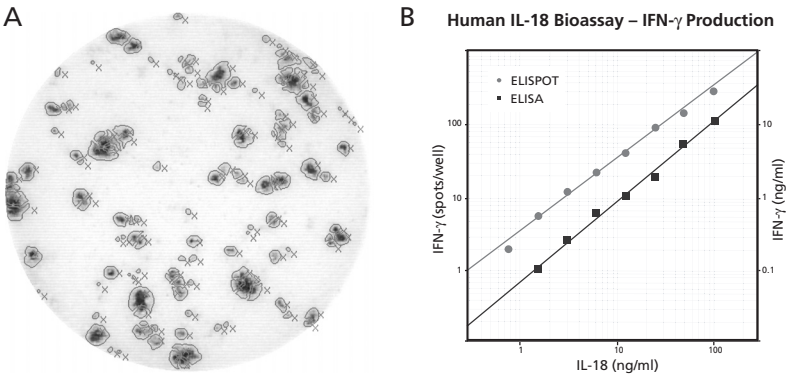


Figure 3: ELISPOT analysis of IL-18-mediated effects on human IFN-γ-producing cells.
Human PBMCs were prepared at 2×10^6 cells/ml in RPMI 1640 complete medium. Recombinant human IL-12 p70 (Cat. No. 554613) was added to the cell suspension at a final concentration of 400 pg/ml. The cell suspension was 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. 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).

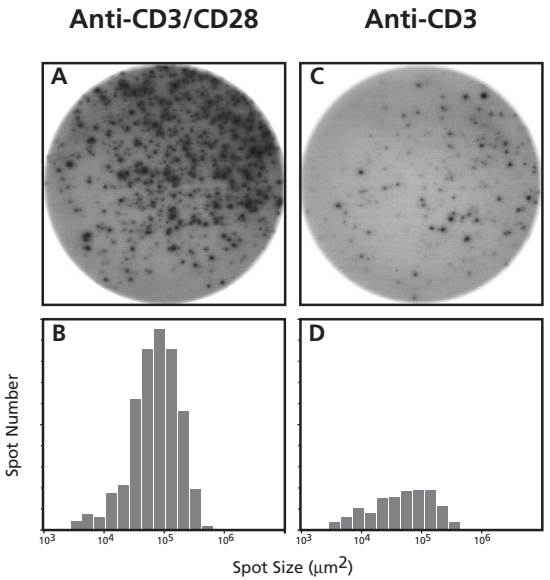


Figure 4. ELISPOT analysis of co-stimulated mouse IL-2 secreting cells. BALB/c mouse spleen cells were incubated in an ELISPOT plate that was hand coated with 5 µg/ml of anti-mouse IL-2 capture antibody (BD Biosciences Pharmingen, Component No. 51-1816KC in the BD ELISPOT Mouse IL-2 Set, Cat. No. 551076) and 1 µg/ml anti-mouse CD3 (BD Biosciences Pharmingen, Cat. No. 553057), with or without 2 µg/ml soluble anti-mouse CD28 (BD Biosciences Pharmingen, Cat. No. 553294) overnight. Biotinylated anti-mouse IL-2 detection antibody was added at 2 µg/ml (BD Biosciences Pharmingen, 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) 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.

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3. Guan, Q., W. Ma, D. Ernst, E. Morgan, and J. Dong. 2002. Analysis of cell-mediated immunity by granzyme B ELISPOT technology. *BD Biosciences HotLines* 7(2):1–3.
4. Immunospot Series 2.0 Analyzer. *BD Biosciences HotLines* 7(2):4.
5. *New ELISPOT Instrumentation and Reagent Sets*. Please see: **www.bdbiosciences.com/product_spotlights/elispot_instrumentation/**
6. *BD™ ELISPOT Research Products. Including Human and Non-Human Primate IFN-γ Kits with Pre-coated Plates.* 2002. BD Biosciences. Please see this brochure at: **www.bdbiosciences.com/pdfs/brochures/02-7900030-53A1.pdf**

BD™ ELISPOT Assays Product List

Description	Apps	Size	Cat. No.
BD ELISPOT Human Reagents			
Human Granzyme B Kit	ELISPOT	2 plates	552573
Human Granzyme B Set	ELISPOT	10 plates	552572
Human GM-CSF Set	ELISPOT	10 plates	552966
Human IFN-γ Kit	ELISPOT	2 plates	552138
Human IFN-γ Set	ELISPOT	10 plates	551849
Human IFN-γ Pair	ELISPOT	Abs for 5 plates	551873
Human IL-2 Kit	ELISPOT	2 plates	552142
Human IL-2 Set	ELISPOT	10 plates	551282
Human IL-2 Pair	ELISPOT	Abs for 5 plates	551884
Human IL-4 Set	ELISPOT	10 plates	551084
Human IL-4 Pair	ELISPOT	Abs for 5 plates	551885
Human IL-5 Kit	ELISPOT	2 plates	552139
Human IL-5 Set	ELISPOT	10 plates	551085
Human IL-5 Pair	ELISPOT	Abs for 5 plates	551886
Human IL-10 Set	ELISPOT	10 plates	551018
Human IL-10 Pair	ELISPOT	Abs for 5 plates	551883
Human IL-12p70 Set	ELISPOT	10 plates	552574
Human TNF Set	ELISPOT	10 plates	551446
Human TNF Pair	ELISPOT	Abs for 5 plates	551882
BD ELISPOT Mouse Reagents			
Mouse IFN-γ Kit	ELISPOT	2 plates	552569
Mouse IFN-γ Set	ELISPOT	10 plates	551083
Mouse IFN-γ Pair	ELISPOT	Abs for 5 plates	551881
Mouse IL-2 Set	ELISPOT	10 plates	551076
Mouse IL-2 Pair	ELISPOT	Abs for 5 plates	551876
Mouse IL-4 Set	ELISPOT	10 plates	551017
Mouse IL-4 Pair	ELISPOT	Abs for 5 plates	551878
Mouse IL-5 Set	ELISPOT	10 plates	551075
Mouse IL-5 Pair	ELISPOT	Abs for 5 plates	551880
Mouse IL-6 Set	ELISPOT	10 plates	552567
Mouse IL-10 Set	ELISPOT	10 plates	551445
Mouse IL-10 Pair	ELISPOT	Abs for 5 plates	551874
Mouse IL-12p70 Set	ELISPOT	10 plates	inquire
Mouse TNF Set	ELISPOT	10 plates	551491
Mouse TNF Pair	ELISPOT	Abs for 5 plates	551875
BD ELISPOT Rat Reagents			
Rat IL-4 Set	ELISPOT	10 plates	552570
BD ELISPOT Reagents - Other			
AEC Substrate Set	ELISPOT	Rgts for 10 plates	551951
Avidin-HRP	ELISPOT	1.0 ml	551950
Streptavidin-HRP	ELISPOT	1.0 ml	557630

Notes

Chapter 7

ELISA for Specifically Measuring the Levels of 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.[1] In 1971, Engvall and Perlmann[2] 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.

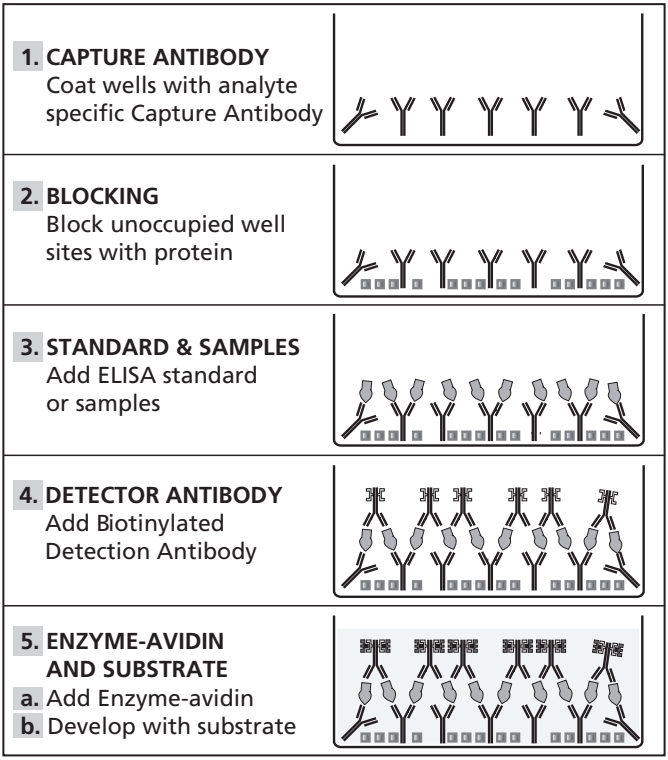

Figure 1. Basic stages of the sandwich ELISA for measuring soluble cytokine and chemokine protein levels (see text for further description).

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]

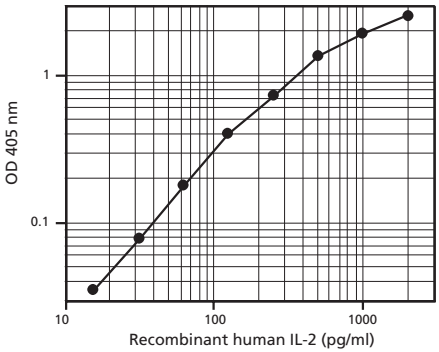

Figure 2. Standard curve from a sandwich ELISA that measures human IL-2 protein levels. A standard curve was generated by a sandwich ELISA using the purified 5344.111 antibody (Cat. No. 555051) as the capture antibody, doubling dilutions of recombinant human IL-2 protein solution, and biotinylated-B33-2 (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 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.[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.[1, 8]

Application Notes

Sandwich ELISAs are exquisitely specific because antibodies directed against two (or more, see ***Figure 1***) distinct epitopes are often used.[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.[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 4)***, the ***BD FastImmune™ Cytokine Flow Cytometry (Chapter 5)***, or ***BD™ ELISPOT Assays for Cells That Secrete Biological Response Modifiers (Chapter 6)*** are required for acquiring this type of information.

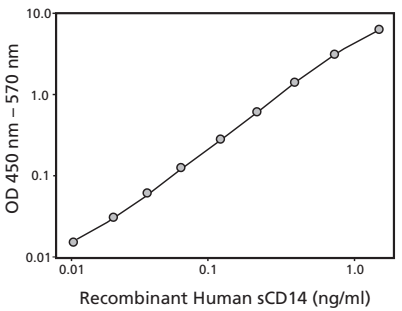

Figure 3. Standard curve from a sandwich ELISA that measures soluble human CD14 protein levels. A standard curve was generated by using the purified 55-3 (Cat. No. 551403) as the capture antibody, doubling dilutions of recombinant soluble human CD14 protein and biotinylated-3-C39 (Cat. No. 551405) as the detection antibody. Avidin-HRP and TMB substrate (Cat. No. 555214) were used to develop the ELISA.

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.[10] 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.[11, 12] Moreover, T cell subsets have been found to produce cytokines differentially in response to different stimuli.[12, 13] 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 the ***BD RiboQuant™ Multi-Probe RNase Protection Assay System (Chapter 11)***, ***Immunofluorescent Staining of Intracellular Molecules for Flow Cytometric Analysis (Chapter 4)***, ***BD FastImmune Cytokine Flow Cytometry (Chapter 5)*** 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.

The concentrations of immunoreactive analyte measured by ELISA may or may not correlate directly with the measured concentrations of bioactive analyte molecules.[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-β1. 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 *in vivo*.[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™ Cytometric Bead Array (CBA) (***Chapter 2***) is another important platform that can perform simultaneous measurement of multiple analytes in a single small-volume sample.

ELISA Protocol — General Procedure

*(For detailed explanations see **Footnotes and References**)*

Capture antibody:

1. Dilute the purified capture antibody to 1 – 4 µg/ml in Coating Buffer[a]. Add 50 – 100 µl of diluted antibody to the wells of an enhanced protein-binding ELISA plate (eg, BD Falcon™, 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[d] 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[c].

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[c].

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 Pharmingen, 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 Pharmingen, 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[i] 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[f] within 20 minutes of use. Add 100 µl of 3% H_2O_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_2HPO_4, adjust pH to 9.0 with 0.1 M NaH_2PO_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_2HPO_4, 2.0 g KH_2PO_4, 2.0 g KCl; mix with deionized (ddH_2O) 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_2O; pH to 4.35 with NaOH. Aliquot 11 ml per vial and store at -20°C. Add 100 µl 3% H_2O_2 prior to use.

g. **3% H_2O_2 Solution:** Add 10 ml of 30% H_2O_2 to 90 ml of H_2O. 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_20, 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 Troubleshooting Tips

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 Pharmingen 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 –80°C.

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 Pharmingen). For best results, use recommended ELISA standards from related BD Biosciences Pharmingen 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.[17]

e. **Optimal Sensitivity:** For optimal sensitivity, incubation of standards and samples for longer periods at room temperature or overnight at 4°C 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.

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ELISA Product List

Matched Antibody Pairs and Protein Standards for Sandwich ELISA

Description	Clone	Isotype	Apps	Format	Size	Cat. No.
Human Cytokines and Chemokines						
IL-1α	364-3B3-14	Mouse IgG_1	Capture	Purified	1.0 mg	551223
	28.9	Mouse IgG_1	Detection	Biotin	0.5 mg	554518
	IL-1α		Standard	Recombinant	5 µg	551838
IL-2	5344.111	Mouse IgG_1	Capture	Purified	0.5 mg	555051
	B33-2	Mouse IgG_1	Detection	Biotin	0.5 mg	555040
	IL-2		Standard	Recombinant	10 µg	554603
IL-3	BVD8-3G11	Rat IgG_1	Capture	Purified	0.5 mg	554672
	BVD3-1F9	Rat IgG_1	Detection	Biotin	0.5 mg	554674
	IL-3		Standard	Recombinant	10 µg	554604
IL-4	8D4-8	Mouse IgG_1	Capture	Purified	0.5 mg	554515
	MP4-25D2	Rat IgG_1	Detection	Biotin	0.5 mg	554483
	IL-4		Standard	Recombinant	5 µg	554605
IL-5	TRFK5	Rat IgG_1	Capture	Purified	0.5 mg	554393
	JES1-5A10	Rat IgG_{2a}	Detection	Biotin	0.5 mg	554491
	IL-5		Standard	Recombinant	5 µg	554606
IL-5	JES1-39D10	Rat IgG_{2a}	Capture	Purified	0.5 mg	554488
	JES1-5A10	Rat IgG_{2a}	Detection	Biotin	0.5 mg	554491
	IL-5		Standard	Recombinant	5 µg	554606
IL-6	MQ2-13A5	Rat IgG_1	Capture	Purified	0.5 mg	554543
	MQ2-39C3	Rat IgG_{2a}	Detection	Biotin	0.5 mg	554546
	IL-6		Standard	Recombinant	10 µg	550071
IL-7	BVD10-40F6	Rat IgG_1	Capture	Purified	0.5 mg	554493
	BVD10-11C10	Rat IgG_{2a}	Detection	Biotin	0.5 mg	554494
	IL-7		Standard	Recombinant	5 µg	554608
IL-8	G265-5	Mouse IgG_{2b}	Capture	Purified	0.5 mg	554716
	G265-8	Mouse IgG_{2b}	Detection	Biotin	0.5 mg	554718
	IL-8		Standard	Recombinant	20 µg	554609
IL-10	JES3-19F1	Rat IgG_{2a}	Capture	Purified	0.5 mg	554705
	JES3-12G8	Rat IgG_{2a}	Detection	Biotin	0.5 mg	554499
	IL-10		Standard	Recombinant	5 µg	554611
IL-12	C8.3	Mouse IgG_1	Capture	Purified	1.0 mg	551227
(p40/p70)	C8.6	Mouse IgG_1	Detection	Biotin	0.5 mg	554660
	IL-12 (p40)		Standard	Recombinant	5 µg	554633
IL-12 (p70)	20C2	Rat IgG_1	Capture	Purified	0.5 mg	555065
	C8.6	Mouse IgG_1	Detection	Biotin	0.5 mg	554660
	IL-12 (p70)		Standard	Recombinant	5 µg	554613
IL-13	JES10-5A2	Rat IgG_1	Capture	Purified	0.5 mg	554570
	B69-2	Mouse IgG_1	Detection	Biotin	0.5 mg	555054
IL-15	G243-935	Mouse IgG_1	Capture	Purified	0.5 mg	554712
	G243-886	Mouse IgG_1	Detection	Biotin	0.5 mg	554713
	IL-15		Standard	Recombinant	5 µg	554630
Eotaxin	3C7	Mouse IgG_1	Capture	Purified	0.5 mg	555035
	10C11	Mouse IgG_{2b}	Detection	Biotin	0.5 mg	555060
	Eotaxin		Standard	Recombinant	5 µg	555102
G-CSF	BVD13-3A5	Rat IgG_1	Capture	Purified	0.5 mg	554669
	BVD11-37G10	Rat IgG_{2a}	Detection	Biotin	0.5 mg	554670

ELISA Product List

Description	Clone	Isotype	Apps	Format	Size	Cat. No.
Human Cytokines and Chemokines *(continued)*						
GM-CSF	BVD2-23B6	Rat IgG_{2a}	Capture	Purified	0.5 mg	554502
	BVD2-21C11	Rat IgG_{2a}	Detection	Biotin	0.5 mg	554505
	GM-CSF		Standard	Recombinant	10 µg	550068
GROα,β,γ	10G4	Mouse IgG_1	Capture	Purified	0.5 mg	550478
	GROα,β,γ		Standard	Recombinant	25 µg	555103
GROα	4C5	Mouse IgG_1	Capture	Purified	0.5 mg	555050
	GROa		Standard	Recombinant	25 µg	555103
IFN-γ	NIB42	Mouse IgG_1	Capture	Purified	1.0 mg	551221
	4S.B3	Mouse IgG_1	Detection	Biotin	0.5 mg	554550
	IFN-g		Standard	Recombinant	25 µg	554616
IP-10	4D5	Mouse IgG_{2a}	Capture	Purified	0.5 mg	555046
	6D4/D6/G2	Mouse IgG_{2a}	Detection	Biotin	0.5 mg	555048
	IP-10		Standard	Recombinant	10 µg	551130
MCP-1	5D3-F7	Mouse IgG_1	Capture	Purified	1.0 mg	551226
	Polyclonal	Rabbit IgG	Detection	Biotin	0.5 mg	554667
	MCP-1		Standard	Recombinant	10 µg	554620
MCP-1	10F7	Mouse IgG_1	Capture	Purified	0.5 mg	555055
	5D3-F7	Mouse IgG_1	Detection	Biotin	0.5 mg	554664
	MCP-1		Standard	Recombinant	10 µg	554620
MCP-3	9H11	Mouse IgG_1	Capture	Purified	1.0 mg	551228
	6H5	Mouse IgG_1	Detection	Biotin	0.5 mg	555034
	MCP-3		Standard	Recombinant	10 µg	554629
MIG	B8-11	Mouse IgG_1	Capture	Purified	0.5 mg	555038
	B8-6	Mouse IgG_1	Detection	Biotin	0.5 mg	555037
	MIG		Standard	Recombinant	5 µg	554636
RANTES			Standard	Recombinant	10 µg	554621
TGF-β1	A75-2.1	Rat IgG_{2a}	Capture	Purified	0.5 mg	555052
	A75-3.1	Rat IgG_{2a}	Detection	Biotin	0.5 mg	555053
	TGF-b1		Standard	Recombinant	1 µg	552438
TNF	MAb1	Mouse IgG_1	Capture	Purified	1.0 mg	551220
	MAb11	Mouse IgG_1	Detection	Biotin	0.5 mg	554511
	TNF		Standard	Recombinant	10 µg	554618
LT-α	359-238-8	Mouse IgG_1	Capture	Purified	1.0 mg	551222
	359-81-11	Mouse IgG_1	Detection	Biotin	0.5 mg	554555
	LT-α		Standard	Recombinant	10 µg	554619
CD40L	Hcd40L-M90.1	Mouse IgG_1	Capture	Purified	0.5 mg	552559
	Hcd40L-M91.2	Mouse IgG_1	Detection	Biotin	0.5 mg	552560
CD120a	MABTNFR1-A1	Mouse IgG_1	Capture	Purified	0.5 mg	552535
(TNF receptor I)	MABTNFR1-B1	Mouse IgG_{2a}	Detection	Biotin	0.5 mg	552536
CD120b	hTNFR-M1	Rat IgG_{2a}	Capture	Purified	0.5 mg	551311
(TNF receptor type II)	hTNFR-M1	Rat IgG_{2a}	Detection	Biotin	0.5 mg	552477
CD121a	HIL1R-M1	Mouse IgG_1	Capture	Purified	0.5 mg	551388
(IL-1 receptor type I)	HIL1R-M8	Rat Ig_{2b}	Detection	Biotin	0.5 mg	551389

ELISA Product List

Description	Clone	Isotype	Apps	Format	Size	Cat. No.
Human Cytokines and Chemokines						
CD124	HIL4R-M10.1	Rat Ig_{2a}	Capture	Purified	0.5 mg	552472
(IL-4 receptor α chain)	HIL4R-M8.2.2	Rat Ig_1	Detection	Biotin	0.5 mg	552473
CD126	M5	Mouse IgG_1	Capture	Purified	0.5 mg	551462
(IL-6 receptor α chain)	M182	Mouse IgG_1	Detection	Biotin	0.5 mg	552503
CD130	A1/gp130	Mouse IgG_1	Capture	Purified	0.5 mg	552426
(gp130)	D2	Mouse IgG_1	Detection	Biotin	0.5 mg	552403
CDw137	H41BB-M127	Mouse IgG_1	Capture	Purified	0.5 mg	552532
ligand	4B4-1	Mouse IgG_1	Detection	Biotin	0.5 mg	552533
(4-1BB ligand)						
IL-1 receptor II (IL-1 RII)	MNC2	Mouse IgG_1	Capture	Purified	0.5 mg	552401
Soluble CD14	55-3	Mouse IgG_{2a}	Capture	Purified	0.5 mg	551403
	3-C39	Mouse IgG_{2a}	Detection	Biotin	0.5 mg	551405
Mouse Cytokines and Chemokines						
IL-1α	ALF-161	Hamster IgG	Capture	Purified	0.5 mg	550604
	C1150-27	Rabbit Ig	Detection	Biotin	0.5 mg	550606
	IL-1α		Standard	Purified	5 µg	559612
IL-1β	B122	Hamster IgG	Capture	Purified	0.5 mg	550605
	C1150-3	Rabbit IgG	Detection	Biotin	0.5 mg	550623
	IL-1β		Standard	Recombinant	5 µg	554577
IL-2	JES6-1A12	Rat IgG_{2a}	Capture	Purified	0.5 mg	554424
	JES6-5H4	Rat IgG_{2b}	Detection	Biotin	0.5 mg	554426
	IL-2		Standard	Recombinant	20µg	550069
IL-3	MP2-8F8	Rat IgG_1	Capture	Purified	0.5 mg	554381
	MP2-43D11	Rat IgG_{2a}	Detection	Biotin	0.5 mg	554384
	IL-3		Standard	Recombinant	10 µg	554579
IL-4	BVD4-1D11	Rat IgG_{2b}	Capture	Purified	0.5 mg	554387
	11B11	Rat IgG_1	Capture	Purified	0.5 mg	554434
	BVD6-24G2	Rat IgG_1	Detection	Biotin	0.5 mg	554390
	IL-4		Standard	Recombinant	10 µg	550067
IL-5	TRFK5	Rat IgG_1	Capture	Purified	0.5 mg	554393
	TRFK4	Rat IgG_{2a}	Detection	Biotin	0.5 mg	554397
	IL-5		Standard	Recombinant	5 µg	554581
IL-6	MP5-20F3	Rat IgG_1	Capture	Purified	0.5 mg	554400
	MP5-32C11	Rat IgG_{2a}	Detection	Biotin	0.5 mg	554402
	IL-6		Standard	Recombinant	5 µg	554582
IL-9	D8402E8	Rat IgG_{2a}	Capture	Purified	1.0 mg	551218
	D9302C12	Hamster IgG	Detection	Biotin	0.5 mg	554473

ELISA Product List

Description	Clone	Isotype	Apps	Format	Size	Cat. No.
Mouse Cytokines and Chemokines *(continued)*						
IL-10	JES5-2A5	Rat IgG_1	Capture	Purified	1.0 mg	551215
	SXC-1	Rat IgM	Detection	Biotin	0.5 mg	554423
	JES5-16E3	Rat IgG_{2b}	Detection	Biotin	0.5 mg	554465
	IL-10		Standard	Recombinant	10 µg	550070
IL-12 (p40)	C15.6	Rat IgG_1	Capture	Purified	1.0 mg	551219
	C17.8	Rat IgG_{2a}	Detection	Biotin	0.5 mg	554476
	IL-12 (p40)		Standard	Recombinant	2 µg	554594
IL-12 (p70)	9A5	Rat IgG_{2b}	Capture	Purified	0.5 mg	554658
	C17.8	Rat IgG_{2a}	Detection	Biotin	0.5 mg	554476
	IL-12 (p70)		Standard	Recombinant	5 µg	554592
IL-17	TC11-18H10.1	Rat IgG_1	Capture	Purified	0.5 mg	555068
	TC11-8H4.1	Rat IgG_1	Detection	Biotin	0.5 mg	555067
CRG-2	A102-6	Rat IgG_1	Capture	Purified	0.5 mg	559601
GM-CSF	MP1-22E9	Rat IgG_{2a}	Capture	Purified	0.5 mg	554404
	MP1-31G6	Rat IgG_1	Detection	Biotin	0.5 mg	554407
	GM-CSF		Standard	Recombinant	10 µg	554586
IFN-γ	R4-6A2	Rat IgG_1	Capture	Purified	1.0 mg	551216
	XMG1.2	Rat IgG_1	Detection	Biotin	0.5 mg	554410
	IFN-γ		Standard	Recombinant	10 µg	554587
MCP-1	2H5	Ham IgG	Capture	Purified	1.0 mg	551217
	4E2/MCP	Ham IgG	Detection	Biotin	0.5 mg	554444
	MCP-1		Standard	Recombinant	5 µg	554590
TNF	TN3-19.12	Ham IgG	Capture	Purified	0.5 mg	557516
	Polyclonal	Rabbit Ig	Detection	Biotin	0.5 mg	557432
	TNF		Standard	Recombinant	10 µg	554589
TNF	G281-2626	Rat IgG_1	Capture	Purified	1.0 mg	551225
	MP6-XT3	Rat IgG_1	Detection	Biotin	0.5 mg	554415
	TNF		Standard	Recombinant	10 µg	554589
M-CSF-1	5A1	Rat IgG_1	Capture	Purified	0.5 mg	552513
	D24	Rat IgG_1	Detection	Biotin	0.5 mg	552514
			Standard	Recombinant	200 ng	552515
Mouse Immunoglobulins						
IgA	C10-3	Rat (LOU) IgG_1, κ	Capture	Purified	0.5 mg	556969
	C10-1	Rat (LOU) IgG_1, κ	Detection	Biotin	0.5 mg	556978
	M18-254	Mouse IgA, k	Standard	Purified	0.5 mg	553476
IgE	R35-72	Rat (LOU) IgG_1, κ	Capture	Purified	0.5 mg	553413
	R35-118	Rat (LOU) IgG_1, κ	Detection	Biotin	0.5 mg	553419
	C38-2	Mouse IgE, k	Standard	Purified	0.5 mg	557079
IgG_1	A85-3	Rat (LOU) IgG_3, κ	Capture	Purified	0.5 mg	553445
	A85-1	Rat (LOU) IgG_1, κ	Detection	Biotin	0.5 mg	553441
	MOPC-31C	Mouse Ig_1, κ	Standard	Purified	0.5 mg	557273
IgG_{2a}	R11-89	Rat (LOU) IgG_1, κ	Capture	Purified	0.5 mg	553446
	R19-15	Rat (LOU) IgG_1, κ	Detection	Biotin	0.5 mg	553388
	G155-178	Mouse IgG_{2a}, κ	Standard	Purified	0.5 mg	553454

ELISA Product List

Description	Clone	Isotype	Apps	Format	Size	Cat. No.
Mouse Immunoglobulins *(continued)*						
IgG_{2b}	R9-91	Rat (LOU) IgG_1, κ	Capture	Purified	0.5 mg	553396
	R12-3	Rat (LOU) IgG_{2a}, κ	Detection	Biotin	0.5 mg	553393
	MPC-11	Mouse IgG_{2b}, κ	Standard	Purified	0.5 mg	557351
IgG_3	R2-38	Rat (LOU) IgG_1, κ	Capture	Purified	0.5 mg	553404
	R40-82	Rat (LOU) IgG_{2a}, κ	Detection	Biotin	0.5 mg	553401
	A112-3	Mouse IgG_3, κ	Standard	Purified	0.5 mg	553486
IgM	II/41	Rat IgG_{2a}, κ	Capture	Purified	0.5 mg	553435
	R6-60.2	Rat (LOU) IgG_{2a}, κ	Detection	Biotin	0.5 mg	553406
Rat Cytokines and Chemokines						
IL-2	Polyclonal	Rabbit IgG	Capture	Purified	0.5 mg	555076
	A38-3	Mouse IgG_1	Detection	Biotin	0.5 mg	555077
	IL-2		Standard	Recombinant	5 µg	555106
IL-4	OX-81	Mouse IgG_1	Capture	Purified	0.5 mg	555080
	Polyclonal	Rabbit IgG	Detection	Biotin	0.5 mg	555089
	IL-4		Standard	Recombinant	5 µg	555107
IL-4	OX-81	Mouse IgG_1	Capture	Purified	0.5 mg	555080
	B11-3	Mouse IgG_1	Detection	Biotin	0.5 mg	555090
	IL-4		Standard	Recombinant	5 µg	555107
IL-6	C3-4	Mouse IgG_1	Capture	Purified	0.5 mg	550644
	G307-2	Mouse IgG_1	Detection	Biotin	0.5 mg	550642
	IL-6		Standard	Recombinant	5 µg	557008
IL-10	A5-7	Mouse IgG_1	Capture	Purified	0.5 mg	555083
	A5-6	Mouse IgG_1	Detection	Biotin	0.5 mg	555084
	IL-10		Standard	Recombinant	5 µg	555113
GM-CSF	B61-5/B61-9	Mouse IgG_1	Capture	Purified	0.5 mg	555099
	B61-10	Mouse IgG_1	Detection	Biotin	0.5 mg	555098
	GM-CSF		Standard	Recombinant	5 µg	555111
MCP-1	C4	Mouse IgG_1	Capture	Purified	0.5 mg	555072
	B4	Mouse IgGv	Detection	Biotin	0.5 mg	555074
	MCP-1		Standard	Recombinant	5 µg	555110
TNF	TN3-19.2	Ham IgG	Capture	Purified	0.5 mg	557516
	Polyclonal	Rabbit Ig	Detection	Biotin	0.5 mg	557432
	TNF		Standard	Recombinant	5 µg	555109
Rat Immunoglobulins						
IgA	A93-3	Mouse (BALB/c) IgG_1, κ	Capture	Purified	0.5 mg	553913
	A93-2	Mouse (BALB/c) IgG_1, κ	Detection	Biotin	0.5 mg	553912
	R3-30	Rat IgA, κ	Standard	Purified	0.5 mg	553945
IgE	B41-1	Mouse (BALB/c) IgG_1, κ	Capture	Purified	0.5 mg	553914
	B41-3	Mouse (BALB/c) IgG_1, κ	Detection	Biotin	0.5 mg	553916
	C38-2	Mouse IgE, κ	Standard	Purified	0.5 mg	557079
	27-74	Mouse IgE, κ	Standard	Purified	0.5 mg	553481

ELISA Product List

Description	Clone	Isotype	Apps	Format	Size	Cat. No.
Rat Immunoglobulins *(continued)*						
IgG_1	RG11/39.4	Mouse (SJL) IgG_{2b}, κ	Detection	Biotin	0.5 mg	553890
	R3-34	Rat IgG_1, κ	Standard	Purified	0.5 mg	553922
IgG_{2a}	B46-7	Mouse (BALB/c) IgG_1, κ	Capture	Purified	0.5 mg	553918
	RG7/1.30	Mouse (SJL) $IgG_{2\beta}$, κ	Detection	Biotin	0.5 mg	553894
	A110-2	Rat $IgG_{2\alpha}$, κ	Standard	Purified	0.5 mg	553992
IgG_{2b}	G15-337	Mouse $IgG_{2\beta}$, κ	Capture	Purified	0.5 mg	553882
	RG7/11.1	Mouse (SJL) $IgG_{2\beta}$, κ	Detection	Biotin	0.5 mg	553898
	A95-1	Rat $IgG_{2\beta}$, κ	Standard	Purified	0.5 mg	553986
IgG_{2c}	A92-3	Mouse (BALB/c) IgG_1, κ	Capture	Purified	0.5 mg	553910
	A92-1	Mouse (BALB/c) IgG_1, κ	Detection	Biotin	0.5 mg	553909
	A23-1	Rat, IgG_{2c}, κ	Standard	Purified	0.5 mg	553982
IgM	G53-238	Mouse IgG_1, κ	Capture	Purified	0.5 mg	553885
	G53-238	Mouse IgG_1, κ	Detection	Biotin	0.5 mg	553886
	R4-22	Rat IgM, κ	Standard	Purified	0.5 mg	553940
Armenian Hamster Immunoglobulins*						
IgG_1	HIG-632	Mouse $IgG_{2\beta}$, κ	Capture	Purified	0.5 mg	550637
	G94-56	Mouse (BALB/c) $IgG_{2\beta}$, κ	Detect	Biotin	0.5 mg	554007
	A19-3	Hamster IgG_1	Standard	Purified	0.5 mg	553969
IgG_2	HIG-65	Mouse IgG_1, κ	Capture	Purified	0.5 mg	550638
	G192-1	Mouse (BALB/c) IgG_1, κ	Detect	Biotin	0.5 mg	554025
	B81-3	Hamster IgG_2	Standard	Purified	0.5 mg	559277
IgG_3	G70-204	Mouse IgG_1, κ	Capture	Purified	0.5 mg	554003
	HIG-88	Mouse IgG_1, κ	Detect	Biotin	0.5 mg	550640
	A19-4	Hamster IgG_3	Standard	Purified	0.5 mg	553977
IgM	G188-1	Mouse (BALB/c) IgG_1, κ	Capture	Purified	0.5 mg	554031
	G188-9	Mouse (BALB/c) IgG_1, κ	Detect	Biotin	0.5 mg	554035
	G235-1	Hamster IgM	Standard	Purified	0.5 mg	553958

* Although hamster immunoglobulin isotypes have not been well defined, BD Biosciences Pharmingen has grouped Armenian hamster IgG monoclonal antibodies according to their reactivity with a panel of mouse anti-hamster IgG mAbs. Please refer to the "Reactivity of Mouse Anti-Hamster Ig mAbs" chart on our website at **www.bdbiosciences.com**.

ELISA Product List

Description	Clone	Isotype	Apps	Format	Size	Cat. No.
Rabbit Cytokines						
TNF	Polyclonal	Goat Ig	Capture	Purified	1.0 mg	551214
	Polyclonal	Goat Ig	Detection	Biotin	1.0 mg	551213
	TNF		Standard	Cond. Media	80 ng	553645
Rabbit Immunoglobulins						
IgA	102		Capture	Purified	0.5 mg	551290
	102		Detect	Biotin	0.2 mg	551291
IgG	C101-359		Capture	Purified	0.5 mg	550326
	C101-167		Detect	Biotin	0.5 mg	550346
		Rabbit Ig	Standard	Purified	0.1 mg	550875
IgM	367.2		Capture	Purified	0.5 mg	550939
	367.2		Detect	Biotin	0.2 mg	550938
Pig Cytokines						
IL-1β	4B2.1	Mouse IgG_1	Capture	Purified	0.5 mg	550823
	6E8.10.4	Mouse IgG_1	Detection	Biotin	0.5 mg	550824
IFN-γ	P2G10	Mouse IgG_1, κ	Capture	Purified	0.5 mg	559961
	P2C11	Mouse IgG_{2a}, κ	Detection	Biotin	0.5 mg	559958
TGF-β	CA75-2.1	Rat IgG_{2a}	Capture	Purified	0.5 mg	555052
	A75-3.1	Rat IgG_{2a}	Detection	Biotin	0.5 mg	555053
IL-2	6.6.1.1	Mouse IgG_1	Capture	Purified	0.5 mg	552290
	6.2.1.1	Mouse IgG_1	Detection	Biotin	0.5 mg	552404
Ancillary ELISA reagents						
TMB Substrate Reagent Set	Assay development system for 40 plates (300ml)					555214

Notes

Chapter 8

BD OptEIA™ ELISA Sets and Kits for Quantitation of Analytes in Serum, Plasma, and Cell Culture Supernatants

BD OptEIA™ ELISA Sets

Introduction

ELISA-based immunoassay systems provide researchers with the tools to assess a variety of antigens (analytes) within serum, plasma, and other biological fluids from different species (see ***ELISAs for Specifically Measuring the Levels of Cytokines, Chemokines, Inflammatory Mediators and Their Receptors, Chapter 7***). However, accurate quantitation of analytes within serum and plasma samples has proven to be a particular challenge. Serum and plasma contain a myriad of carrier proteins and binding factors that can interfere with specific analyte measurements. Sandwich ELISA systems must be developed and validated to limit interference by binding proteins or soluble receptors such as heterophilic antibodies[1] and autoantibodies [eg, rheumatoid factors (RF)].[2,3]

The BD OptEIA™ ELISA Sets were developed to measure the levels of biological response modifiers (BRMs) in cell culture supernatants, cell lysates, serum, plasma, and other biological fluids. Several species are represented, including human, mouse, rat, rabbit, pig, and non-human primates (NHP). Cytokines, chemokines, inflammatory mediators, soluble CD markers, immunoglobulin isotypes, adhesion molecules, apoptosis-related molecules, soluble receptors and other molecules pertinent to immune function research are included in the BD OptEIA product line. The Sets utilize specially-formulated $F(ab')_2$ detection antibodies to reduce backgrounds caused by nonspecific binding. In addition, BD OptEIA ELISA Sets have demonstrated quantitation of expected baseline analyte levels in various biological fluids including serum and plasma samples from healthy normal donors.

BD OptEIA ELISA Set Contents

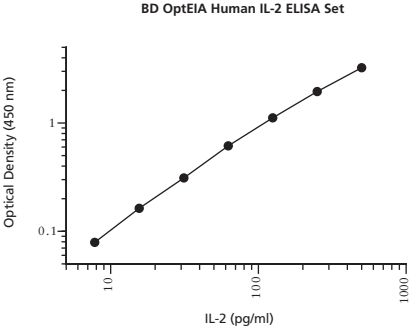
- Capture Antibody, pre-titrated
- Detection Antibody, biotinylated, pre-titrated
- Recombinant Standard Protein
- Avidin-Horseradish Peroxidase (HRP) Conjugate, pre-titrated

Ancillary reagents, TMB Substrate Reagent Set (Cat. No. 555214), Assay Diluent (Cat. No. 555213), BD OptEIA Reagent Set A (containing all necessary buffers and diluents, Cat. No. 550536), and BD OptEIA Reagent Set B (containing all necessary buffers and diluents, Cat. No. 550534) are recommended for use with BD OptEIA ELISA Sets.

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)



BD OptEIA ELISA Set Assay Protocol (see ***Set Insert*** or visit **www.bdbiosciences.com/bd_opteia_elisa** for a detailed specific 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

BD OptEIA ELISA Kits

Introduction

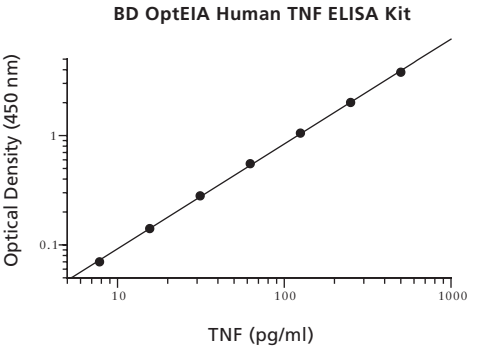
BD OptEIA ELISA Kits contain pre-coated, breakable, 96-well ELISA plates with all of the necessary reagents to specifically and accurately measure analyte levels. Extensive testing is performed on each lot of kits and kit components to ensure quality. Thus, the time and resources required by researchers to develop and optimize an assay are eliminated. BD OptEIA ELISA Kits have been developed for the highest accuracy in measuring analytes present within serum, plasma, and other biological fluids. BD OptEIA ELISA Kits show optimal spike recovery performances for measuring analytes within complex biological fluids such as serum and plasma.

BD OptEIA ELISA Kit Contents

- Precoated, breakable, 96-well ELISA plates
- Lyophilized Standards
- Detection Antibody
- Avidin-HRP Conjugate
- Standard/Sample Diluent
- ELISA Diluent
- Wash Concentrate
- TMB One-Step Substrate Reagent
- Stop Solution

BD OptEIA ELISA Kit Features

- Designed for superior spike recovery and linearity with serum, plasma, and culture supernatant samples.
- $F(ab')_2$ detection antibodies and specially-formulated buffer and diluent system to minimize background caused by non-specific binding.
- Sensitivity: detection of picogram levels of analyte (as low as 1 pg/ml)
- Precision: low inter- and intra-assay variation
- Outstanding Value: pre-coated plates and all necessary reagents
- Ease of Use: 3-1/2 hour incubation time at room temperature with no shaking



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:

Sample Type	Spike Concentration (pg/ml)	Average % Recovery	Average Range
Serum	250	89	81 – 95
(n = 9)	125	86	75 – 93
	62.5	93	78 – 109
Plasma	250	96	95 – 100
(n = 5)	125	88	81 – 99
	62.5	88	74 – 104
Cell Culture	250	98	88 – 106
Media (n = 3)	125	106	101 – 109
	62.5	105	100 – 110

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:

Dilution		Serum (n = 9)	Plasma (n = 5)	Cell Culture Media (n = 3)
1:2	Average % of Expected	104	106	104
	Range	95 – 116	100 – 111	101 – 110
1:4	Average % of Expected	108	105	99
	Range	90 – 124	97 – 114	95 – 108
1:8	Average % of Expected	116	104	93
	Range	95 – 143	93 – 108	90 – 97
1:16	Average % of Expected	107	102	86
	Range	85 – 140	89 – 133	78 – 98

BD OptEIA ELISA Kit Assay Protocol (see ***Kit Booklet*** or visit **www.bdbiosciences.com/bd_opteia_elisa** for a detailed specific 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

BD OptEIA™ CL Chemiluminescent ELISA Kits

Introduction

Chemiluminescent (CL) ELISA systems provide a broader dynamic assay range, superior low-end sensitivity, and a faster protocol than the conventional colorimetric ELISA methods.

The new BD OptEIA™ CL ELISA Kits utilize a luminol-based chemiluminescent substrate and enhancer that result in rapid kinetic light output and high signal intensity. The $F(ab')_2$ detection antibodies and optimized diluents utilized in the BD OptEIA CL ELISA Kits enable quantitation of cytokines and other soluble proteins in serum, plasma, or cell culture supernatant samples. The kits contain a pre-coated 96-well ELISA plate and all necessary reagents to measure analyte levels.

The BD OptEIA CL ELISA Kits have been validated using the new BD Monolight 3096 Microplate Luminometer, an easy-to-use, ultra-sensitive, photon-counting luminometer (Cat. No. 551280).

BD OptEIA CL ELISA Kit Contents

- One Pre-coated 96-well ELISA plate
- Lyophilized Standards: 3 vials
- Detection Antibody
- Avidin-HRP Conjugate
- Standard/Sample Diluent
- ELISA Diluent
- Wash Concentrate
- BD OptEIA CL Substrate A
- BD OptEIA CL Substrate B

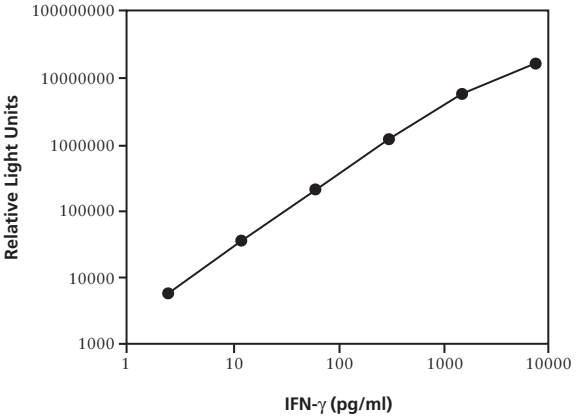
BD OptEIA CL ELISA Kit Features (in addition to the features of the colorimetric BD OptEIA ELISA Kits)

- Superior Low-end Sensitivity (as low as 0.3 pg/ml)
- Broader Assay Range: in most cases, no sample dilution required
- Ease of Use: faster protocol, pre-coated plates, room temperature incubations, no shaking

BD OptEIA CL ELISA Kit Data (Human IFN-γ)

The BD OptEIA CL Human IFN-γ ELISA Kit provides a dynamic assay range of 2.4 to 7,500 pg/ml. The standard curve is shown below.

BD OptEIA CL Human IFN-γ Chemiluminescent ELISA Kit



BD OptEIA CL™ ELISA Kit Assay Protocol (see ***Kit Booklet*** for detailed specific protocol)

1. Add 50 µl of ELISA Diluent per well
2. Add 100 µl of standard or sample per well
3. Incubate 1 hr at RT, wash
4. Add 100 µl of prepared Working Detector per well
5. Incubate 1 hr at RT, wash
6. Add 50 µl of Substrate (mixture of Substrates A + B) per well
7. Read relative light units using a 96-well plate luminometer.

References

1. Boscato, L.M., and M. C. Stuart. Heterophilic antibodies: a problem for all immunoassays. *Clin. Chem.* 34:27-33 (1988).
2. Boscato, L.M., and M. C. Stuart. Incidence and specificity of interference in two-site immunoassays. *Clin. Chem.* 32:1491-1495 (1986).
3. Levinson, S.S. Antibody multispecificity in immunoassay interference. *Clin. Chem.* 25:84-87 (1992).

BD OptEIA™ ELISA Product List

Description	Assay Range	Apps	Size	Cat. No.
BD OptEIA ELISA Sets				
Human				
Active Caspase-3	15.6-1000 U/ml	ELISA	Reagents for 5 Plates	inquire
Cleaved PARP	3.9-250 U/ml	ELISA	Reagents for 5 Plates	inquire
Eotaxin	6.3-400 pg/ml	ELISA	Reagents for 20 Plates	555175
sFas	31.3-2000 pg/ml	ELISA	Reagents for 20 Plates	555224
GM-CSF	4.7-300 pg/ml	ELISA	Reagents for 20 Plates	555126
sICAM-1	1.6-100 ng/ml	ELISA	Reagents for 20 Plates	551424
IFN-γ	4.7-300 pg/ml	ELISA	Reagents for 20 Plates	555142
IL-1α	15.6-1000 pg/ml	ELISA	Reagents for 20 Plates	inquire
IL-1β	15.6-1000 pg/ml	ELISA	Reagents for 20 Plates	558848
IL-2	7.8-500 pg/ml	ELISA	Reagents for 20 Plates	555190
IL-2 sRα	7.8-500 pg/ml	ELISA	Reagents for 20 Plates	559104
IL-3	31.3-2000 pg/ml	ELISA	Reagents for 20 Plates	558979
IL-4	7.8-500 pg/ml	ELISA	Reagents for 20 Plates	555194
IL-5	7.8-500 pg/ml	ELISA	Reagents for 20 Plates	555202
IL-6	4.7-300 pg/ml	ELISA	Reagents for 20 Plates	555220
IL-6R	15.6-1000 pg/ml	ELISA	Reagents for 20 Plates	inquire
IL-7	15.6-1000 pg/ml	ELISA	Reagents for 20 Plates	inquire
IL-8	3.1-200 pg/ml	ELISA	Reagents for 20 Plates	555244
IL-10	7.8-500 pg/ml	ELISA	Reagents for 20 Plates	555157
IL-12 (p40)	31.3-2000 pg/ml	ELISA	Reagents for 20 Plates	555171
IL-12 (p70)	7.8-500 pg/ml	ELISA	Reagents for 20 Plates	555183
IL-15	7.8-500 pg/ml	ELISA	Reagents for 20 Plates	559268
IP-10	7.8-500 pg/ml	ELISA	Reagents for 20 Plates	550926
MCP-1	7.8-500 pg/ml	ELISA	Reagents for 20 Plates	555179
MIG	31.3-2000 pg/ml	ELISA	Reagents for 20 Plates	550998
sCD4	3.1-200 pg/ml	ELISA	Reagents for 20 Plates	inquire
sCD14	15.6-1000 pg/ml	ELISA	Reagents for 20 Plates	inquire
sCD23	0.8-50 ng/ml	ELISA	Reagents for 20 Plates	555208
sFasL	31.3-2000 pg/ml	ELISA	Reagents for 20 Plates	inquire
TGF-β1[a]	62.5-4000 pg/ml	ELISA	Reagents for 20 Plates	559119
TNF	7.8-500 pg/ml	ELISA	Reagents for 20 Plates	555212
TNF Receptor I	15.6-1000 pg/ml	ELISA	Reagents for 20 Plates	550996
LT-α (TNF-β)	15.6-1000 pg/ml	ELISA	Reagents for 20 Plates	550995
TRAIL	62.5-4000 pg/ml	ELISA	Reagents for 20 Plates	550948
Mouse				
GM-CSF	15.6-1000 pg/ml	ELISA	Reagents for 20 Plates	555167
IFN-γ	31.3-2000 pg/ml	ELISA	Reagents for 20 Plates	555138
IFN-γ (AN 18)	3.1-200 pg/ml	ELISA	Reagents for 20 Plates	551866
IgE	1.6-100 ng/ml	ELISA	Reagents for 20 Plates	555248
IgG_1	0.16-10 ng/ml	ELISA	Reagents for 20 Plates	inquire
IgG_{2a}	3.1-200 ng/ml	ELISA	Reagents for 20 Plates	inquire
IL-1α	31.3-2000 pg/ml	ELISA	Reagents for 20 Plates	550347
IL-1β	31.3-2000 pg/ml	ELISA	Reagents for 20 Plates	559603
IL-2	3.1-200 pg/ml	ELISA	Reagents for 20 Plates	555148
IL-3	7.8-500 pg/ml	ELISA	Reagents for 20 Plates	555228

BD OptEIA™ ELISA Product List

Description	Assay Range	Apps	Size	Cat. No.
Mouse *(continued)*				
IL-4	7.8-500 pg/ml	ELISA	Reagents for 20 Plates	555232
IL-5	15.6-1000 pg/ml	ELISA	Reagents for 20 Plates	555236
IL-6	15.6-1000 pg/ml	ELISA	Reagents for 20 Plates	555240
IL-10	31.3-2000 pg/ml	ELISA	Reagents for 20 Plates	555252
IL-12 (p40)	15.6-1000 pg/ml	ELISA	Reagents for 20 Plates	555165
IL-12 (p70)	62.5-4000 pg/ml	ELISA	Reagents for 20 Plates	555256
IL-18	31.3-2000 pg/ml	ELISA	Reagents for 20 Plates	558853
MCP-1	15.6-1000 pg/ml	ELISA	Reagents for 20 Plates	555260
MIP-1β	62.5-4000 pg/ml	ELISA	Reagents for 20 Plates	559753
TNF (Mono/Mono)	15.6-1000 pg/ml	ELISA	Reagents for 20 Plates	555268
TNF (Mono/Poly)	15.6-1000 pg/ml	ELISA	Reagents for 20 Plates	558874
TNF RII	7.8-500 pg/ml	ELISA	Reagents for 20 Plates	558857
Monkey				
IFN-γ	15.6-1000 U/ml	ELISA	Reagents for 20 Plates	551492
IL-2	15.6-1000 U/ml	ELISA	Reagents for 20 Plates	551494
IL-4	7.8-500 U/ml	ELISA	Reagents for 20 Plates	551495
IL-6	4.7-300 U/ml	ELISA	Reagents for 20 Plates	551496
TNF	7.8-500 U/ml	ELISA	Reagents for 20 Plates	551493
Rat				
IFN-γ	31.3-2000 pg/ml	ELISA	Reagents for 20 Plates	558861
IL-2	31.3-2000 pg/ml	ELISA	Reagents for 20 Plates	557044
IL-4	1.6-100 pg/ml	ELISA	Reagents for 20 Plates	555198
IL-6	78-5000 pg/ml	ELISA	Reagents for 20 Plates	550319
IL-10	15.6-1000 pg/ml	ELISA	Reagents for 20 Plates	555134
MCP-1	31.3-2000 pg/ml	ELISA	Reagents for 20 Plates	555130
TNF	31.3-2000 pg/ml	ELISA	Reagents for 20 Plates	558870
Other				
TMB Substrate Reagent Set		ELISA	1 Set (300 ml each of 2 reagents)	555214
BD OptEIA Reagent Set A (pH 6.5 buffer)[b]		ELISA	Reagents for 20 Plates	550536
BD OptEIA Reagent Set B (pH 9.5 buffer)[b]		ELISA	Reagents for 20 Plates	550534
Assay Diluent		ELISA	500 mls	555213

[a] cross-reactive with mouse, rat, pig

[b] Contains: Coating buffer, Assay diluent, Wash concentrate, Stop solution, and TMB Substrate reagents

BD OptEIA™ ELISA Product List

Description	Assay Range	Sensitivity	Apps	Size	Cat. No.
BD OptEIA ELISA Kits					
Human					
C3a	80-5000 pg/ml	7.3 pg/ml	ELISA	1 plate	550499
C4a	31.3-2000 pg/ml	6.2 pg/ml	ELISA	1 plate	550947
C5a	0.63-40 ng/ml	0.06 ng/ml	ELISA	1 plate	550500
IFN-γ	4.7-300 pg/ml	1 pg/ml	ELISA	2 plates	550612
IL-1β	7.8-500 pg/ml	3.9 pg/ml	ELISA	2 plates	559111
IL-2	7.8-500 pg/ml	1 pg/ml	ELISA	2 plates	550611
IL-4	7.8-500 pg/ml	2 pg/ml	ELISA	2 plates	550614
IL-6	4.7-300 pg/ml	2.2 pg/ml	ELISA	2 plates	550799
IL-8	3.1-200 pg/ml	0.8 pg/ml	ELISA	2 plates	550999
IL-10	7.8-500 pg/ml	2 pg/ml	ELISA	2 plates	550613
IL-12 (p40)	31.3-2000 pg/ml	3.9 pg/ml	ELISA	2 plates	551116
IL-12 (p70)	7.8-500 pg/ml	4 pg/ml	ELISA	2 plates	559258
MCP-1	15.6-1000 pg/ml	1 pg/ml	ELISA	2 plates	559017
TNF	7.8-500 pg/ml	2 pg/ml	ELISA	2 plates	550610
Mouse					
IFN-γ	31.3-2000 pg/ml	14 pg/ml	ELISA	2 plates	550582
IL-4	7.8-500 pg/ml	4.4 pg/ml	ELISA	2 Plates	inquire
IL-6	15.6-1000 pg/ml	3.8 pg/ml	ELISA	2 plates	550950
IL-12 p40	15.6-1000 pg/ml	7.8 pg/ml	ELISA	2 Plates	inquire
TNF	31.3-2000 pg/ml	5 pg/ml	ELISA	2 plates	559732
Rat					
TNF	31.3-2000 pg/ml	13 pg/ml	ELISA	2 plates	550734
BD OptEIA CL Chemiluminescent ELISA Kits					
Human					
IFN-γ	2.4-7500 pg/ml	0.7 pg/ml	ELISA	1 plate	inquire
IL-2	2.2-7000 pg/ml	0.7 pg/ml	ELISA	1 plate	inquire
TNF	1.6-5000 pg/ml	0.3 pg/ml	ELISA	1 plate	inquire

Chapter 9

BrdU Staining and Multiparameter Flow Cytometric Analysis of the Cell Cycle

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 (eg, 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.[1] 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.

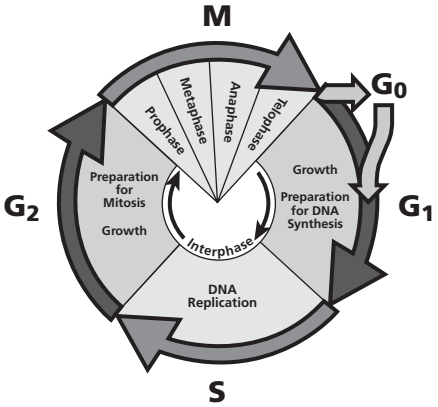


Figure 1. Cell cycle phases.

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 (eg, CD69 and IL-2Rα/CD25, see ***Chapter 5***) 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), a topic featured in ***BD Biosciences Apoptosis Instruction Manual***. 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.[1] 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 (ie, 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 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

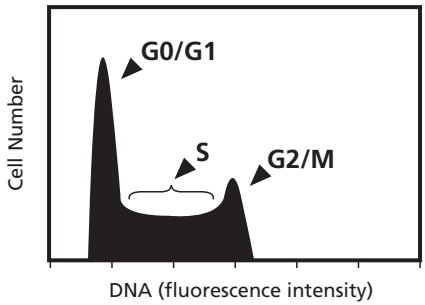


Figure 2. Analysis of relative cellular DNA content using a fluorescent DNA dye and flow cytometry. This illustration depicts a typical data histogram that can be obtained from the relative DNA content analysis of cells within an actively-cycling cell population. The amount of DNA dye that is bound and 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× and 2× DNA levels. Within the G2 or M phases (G2/M), cells have a 2× amount of DNA (ie, a tetraploid chromosomal DNA content).

Table 1. Commonly-used Fluorescent Dyes that Stain DNA for Cell Cycle Analysis by Flow Cytometry

Dyes	Excitation Wavelength	Compatibility-Viable Cells	Compatibility-Fixed Cells	DNA Profile	Multicolor Analysis
Propidium Iodide	488 nm	No	Yes	Good	Yes*
7-AAD	488 nm	No	Yes	High CV	Yes
DAPI	350 nm	No	Yes	Good	Yes
Hoechst 33342	350 nm	Yes	Yes	Good	Yes

* 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 Pharmingen 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 Pharmingen 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 (≥ 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 ~10^6 cells (400 × *g*, 5 min) in tubes and wash 1× in staining buffer [Dulbecco's PBS (DPBS) with 2% FCS and 0.01% NaN_3, 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 H_2O) 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.

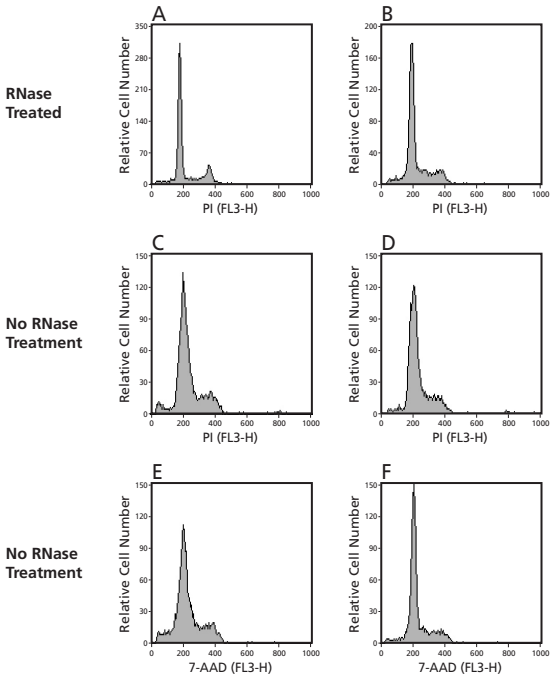


Figure 3. DNA content histograms: PI versus 7-AAD. Cells from two mouse T cell lines, MGG3 (panels A, C, and G) and C20.4 (panels B, D, and F), were harvested, washed 1× with staining buffer, and fixed with ice-cold 70% ethanol (4°C, 1 hr). Cells were then washed to remove the ethanol. Panels A and B: 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.[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).[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 Pharmingen 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.[2] 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.[2]

a. Protocol for Staining DNA with DAPI or Hoechst 33342 for Cell Cycle Analysis[2]

   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% NaN_3, 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 H_20.

Representative data showing DAPI or PI staining and flow cytometric analysis of cells is shown in ***Figure 4***.

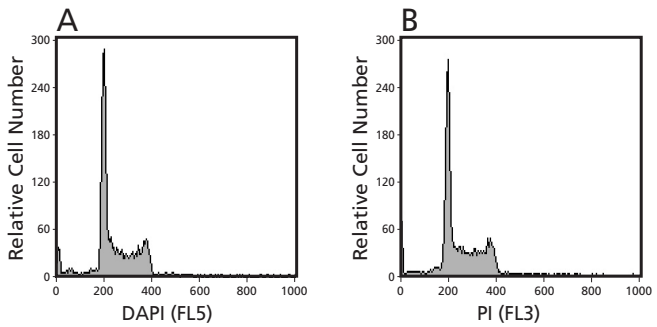


Figure 4. Comparison of DNA staining profiles using DAPI or PI. MGG3 T cells were fixed with ice-cold 70% ethanol for 1 hour at 4°C. The cells were either stained with DAPI (Panel A) or they were RNase A-treated and then stained with PI (Panel B). Cells were then analyzed on a BD LSR cytometer. The DNA 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 FACSCalibur 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[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.[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.[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.[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).[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 *in vitro* and *in vivo* labeling systems.

An important feature of BD Pharmingen™ BrdU Flow Kit (Cat. No. 559619) is that it provides 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.[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 kit ensures consistent results by providing detailed instructions and all critical reagents necessary to implement the staining protocol.

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).[16] 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 2×10^6 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 Kit for *in vivo* use. Inject mice i.p. with 100 µl [1 mg] of the BrdU solution.)[17, 18] 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.[19] Prolonged feeding of BrdU can have toxic effects for the animal.[17–19] 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.[20] BrdU incorporation by cells from these animals has been detected past 70 days.[19]

2. BrdU Flow Kit Staining Protocol (Cat. No. 559619)

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/orRNA 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 H_2O) 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 4***, 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.

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.

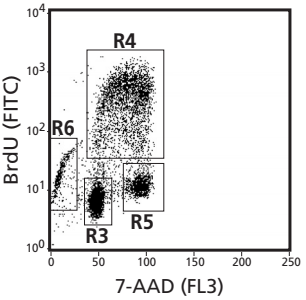


Figure 5. BrdU and 7-AAD coexpression profile for an actively-proliferating cell population. D10.G4.1 T cells were pulsed with 10 µM BrdU for 30 minutes. The cells were then stained for BrdU and 7-AAD using the BrdU Flow Kit and analyzed by flow cytometry. As shown by the boxed region gates, significant 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%).

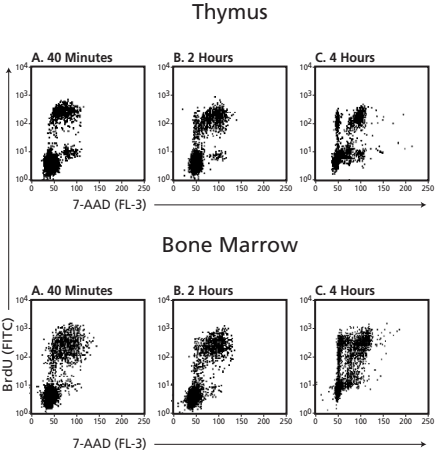


Figure 6. Detection of in vivo-cycling cells. C57BL/6 mice were injected i.p. with 1 mg of BrdU in solution for various time intervals. Animals were sacrificed at 40 minutes, 2 hours, and 4 hours post injection. Thymus and bone marrow cell suspensions were then prepared and stained for incorporated BrdU (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.

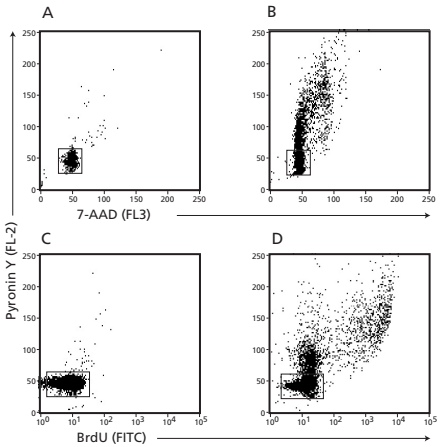


Figure 7. Multiparameter cell cycle analysis of BrdU-pulsed, antigen-stimulated human PBMCs. Human PBMCs were cultured alone (Panels A and C) or with 0.5 µg /ml of tetanus toxoid for 6 days (Panels B and D). 2 cells were pulsed with BrdU (20 µM) for 2 hr prior to harvest. Cells were then stained for 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***).

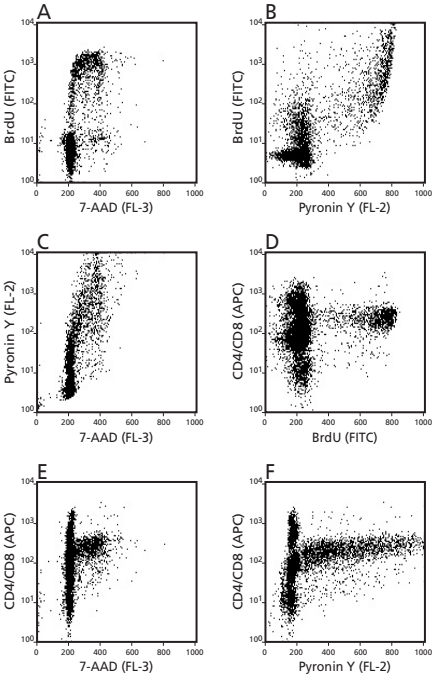


Figure 8. Cell cycle analysis of antigen-activated CD4⁺ and CD8⁺ Human PBMCs. Human PBMCs from a tetanus-vaccinated individual were stimulated with 0.5 µg/ml of Tetanus Toxoid (List Biologicals) for 6 days. During the final 2 hours of culture, the cells were pulsed with 20 µM BrdU. Cells were harvested 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 $BrdU^{-/low}PY^{low}$, $BrdU^{-/low}PY^{+}$ and $BrdU^{+}PY^{bright}$. 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.[21] 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 (eg, 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.

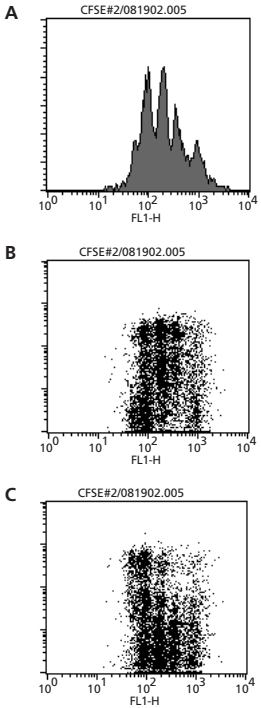


Figure 9. HPBMCs were loaded with 1 µM CFDA SE for 10 minutes at 37°C. Cells were washed twice in 1× PBS then stimulated with 1.5% PHA for 72 hrs. Cells were harvested and then stained with PE anti-human CD4 and allophycocyanin (APC)-anti-human CD8 then analyzed on a BD FACScalibur™. Panel A is the CFSE 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 (eg, 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[22] or to express cytokines such as Interleukin-4.[23] 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.[1]

CFDA SE Labeling Protocol

Dilute CFDA SE in dimethysulfoxide (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 1× 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

In conclusion, 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. Kits as well as individual reagents are available from BD Biosciences Pharmingen for staining cells that have been exposed to and incorporated BrdU. Additional reagents, including reagents that utilize propidium iodide and 7-AAD are also listed. 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.

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Related BD Biosciences Literature

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3. Elia, J., D. Ernst, and J. Waters. 2000. New BrdU Flow Kit. Maximize your multiparameter analysis capabilities with the new BrdU Flow Kit. *BD Biosciences HotLines* 5:4.
4. Kodukula, P., L.-P. Dong, and J. Voland. 2000. New IHC staining for BrdU and more. *BD Biosciences HotLines* 5:1–3.

BrdU Staining Product List

Description	Clone	Isotype	Apps	Format	Size	Cat. No.
Kits						
FITC BrdU Flow Kit			IC/FCM	FITC	50 tests	559619
APC BrdU Flow Kit			IC/FCM	APC	50 tests	552598
Sets						
BrdU	3D4	Mouse IgG_1, κ	IC/FCM, IHC(Fr)	Purified	0.1 mg	555627
			FCM, IC/FCM	FITC Set	100 tests	556028
			FCM, IC/FCM	PE Set	100 tests	556029
BrdU *In-Situ* Kits						
BrdU *In-Situ* Detection Kit			IHC(F), IHC(Fr), IHC(Zn)		50 tests	550803
BrdU *In-Situ* Detection Kit II			IHC(F), IHC(Fr), IHC(Zn)		200 tests	551321

Chapter 10

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 cells 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, ***page 179*** for further details).

The availability of high concentrations of purified cytokine allowed researchers to establish that cytokines could act in a pleiotropic fashion.[1] 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.[2] 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.[3] 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 (see ***Chapter 7***).

BD Biosciences Pharmingen has put in place, with minor modifications, quantitative cytokine bioassays that have been previously reported in the literature. ***Table 1*** provides a brief description of the cytokine bioassays currently used to evaluate cytokine biological activity at BD Biosciences Pharmingen.These bioassays are used to quantitate 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 Pharmingen 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.[4, 5]

Cell growth, cytostasis, or cytolysis can be measured indirectly by measuring the conversion of a redox sensitive tetrazolium salt (MTT).[6] 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 ([^{3}H]-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 CO_2 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 [^{3}H]-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 CO_2 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 [^{3}H]-TdR into DNA is measured by harvesting the labeled indicator cells onto glass fiber filters followed by extensive washing to remove free [^{3}H]-TdR. The filters are dried and the incorporated [^{3}H]-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***.

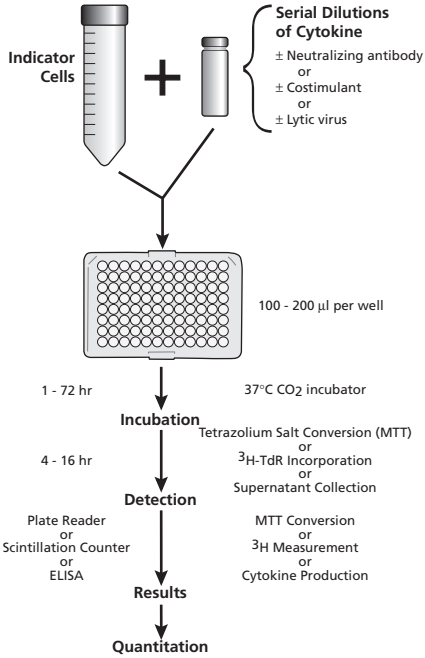

Figure 1. BD Biosciences Pharmingen cytokine bioassay scheme.

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.

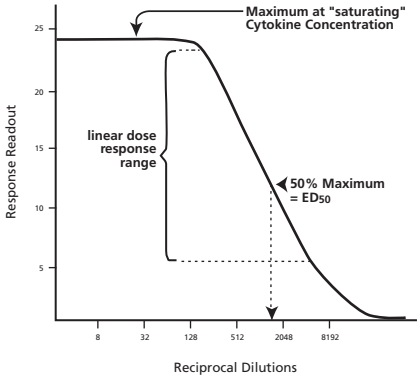

Figure 2. Idealized cytokine bioassay results. At high concentrations, the cytokine induces a saturating maximal response. As the cytokine is serially diluted, a dose-response relationship is observed. We have generally found the range where we observe a biological effect to be 10 – 100 fold.* The dilutions 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.

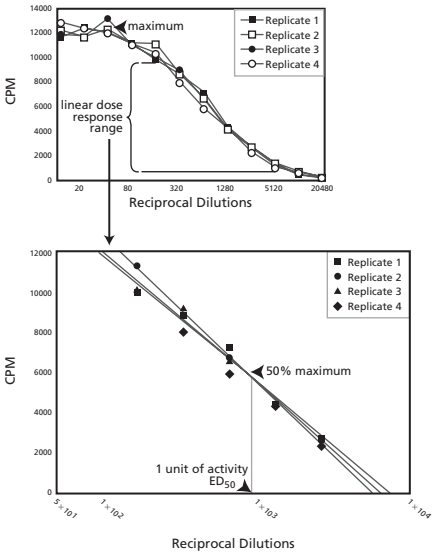

Figure 3. Proliferative response of the CTLL-2 indicator line to recombinant mouse IL-4 as measured by [³H]-TdR incorporation. In the upper half of the figure, the raw data minus background incorporation is plotted versus the reciprocal IL-4 sample dilution. The curve is used to establish the maximum 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.[9] BD Biosciences Pharmingen neutralizing antibodies contain no sodium azide and possess ≤ 0.01 ng of endotoxin per µg neutralizing antibody. 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.

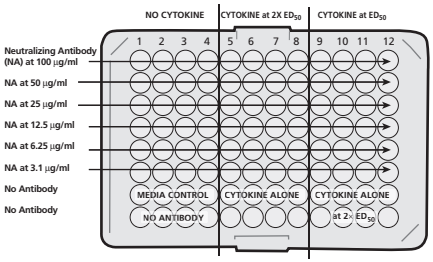

Figure 4. BD Biosciences Pharmingen Cross-Matrix Neutralization Format

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).[10] 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).[11]

Chemokines were originally known to attract mainly granulocytes and monocytes and to be involved in acute and chronic inflammatory responses.[12] 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".[13]

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.[14] 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 Pharmingen utilizes a 48-well chemotaxis chamber (Neuro Probe, Cabin John, MD).[15] ***Figure 5*** illustrates the chemotaxis assay scheme used. Briefly, different

concentrations of 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 filters, 3 µm pore-sized filters for neutrophils and 5 µm pore-sized filters for monocytes and eosinophils, are used. 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.

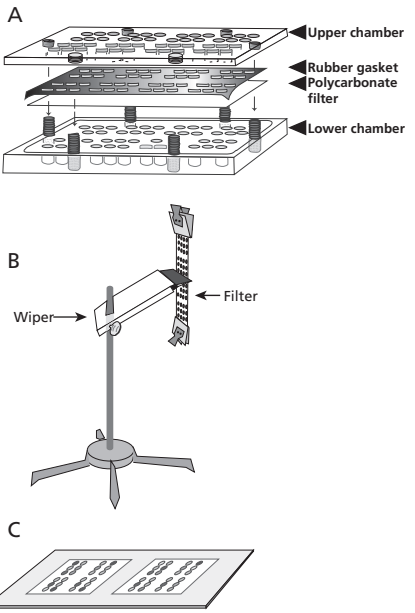

Figure 5. Scheme of chemotaxis assay using a 48-well chemotaxis chamber. (Based on Figure 6.12.1 in *Current Protocols in Immunology*, 1995, Unit 6.12.) (A) Samples (25 – 30 µl) are added to the wells in the lower chamber. A polycarbonate filter is placed over the wells. After chamber assembly, the target 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.

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.

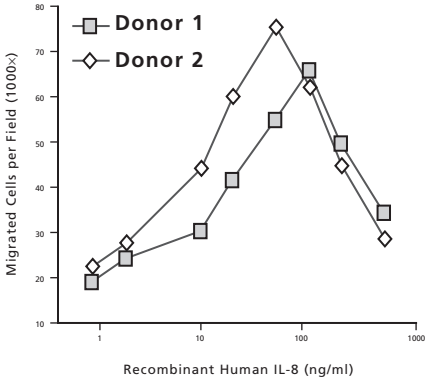

Figure 6. Dose-response curves for chemotaxis of human neutrophils in response to recombinant human IL-8. Each point represents the averaged cell numbers per field (1000×). A 3 µm pore-size polycarbonate filter separated the upper and lower chambers.

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, variation in test results due to donor variation in chemotaxis assays should be anticipated.

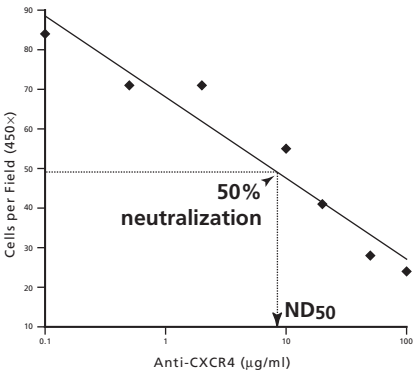

Figure 7. Inhibition of human lymphocyte chemotaxis by monoclonal anti-CXCR4 antibody (Clone 12G5). Chemotaxis was measured by using a 48-well Boyden chamber and a 5 µm pore-size filter precoated with mouse type IV collagen. The concentration of human SDF-1α added in the lower chamber was 200 ng/ml.

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_{50} that is defined as the antibody concentration yielding 50% neutralization of the response.

2. Calcium Mobilization Assay

   Transient increases in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) 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 $[Ca^{2+}]_i$ 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^{2+} from intracellular stores, while diacylglycerol and the increased cytosolic Ca^{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^{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^{2+}-free medium to ~405 nm when the dye is saturated with Ca^{2+}. The ratio of fluorescence of the Ca^{2+}-bound dye and the Ca^{2+}-free dye can be used to determine $[Ca^{2+}]_i$. The cell-permeable acetoxymethyl (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^{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^{2+} from intracellular stores into the cytosol). This response is followed by a decrease of the $[Ca^{2+}]_i$ back to baseline levels. The amplitude of the transient increase of cytosolic free Ca^{2+} is dependent on the stimulatory ligand concentration used to activate the target cells allowing for the determination of an ED_{50} (see ***Figure 8***). Alternatively, the ND_{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***).

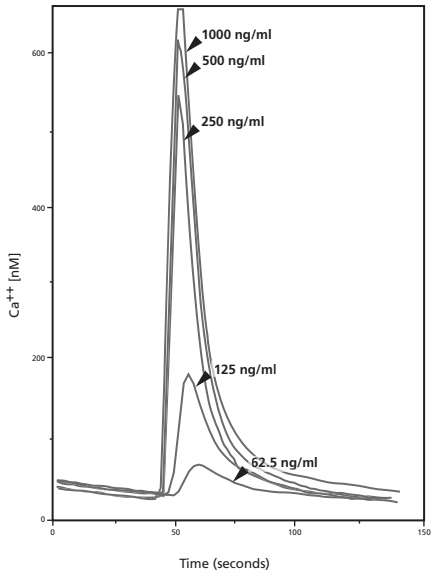

Figure 8. Transient increase in cytoplasmic free calcium induced by various concentrations of recombinant rabbit IL-8. A dose-response titration was performed using human neutrophils.

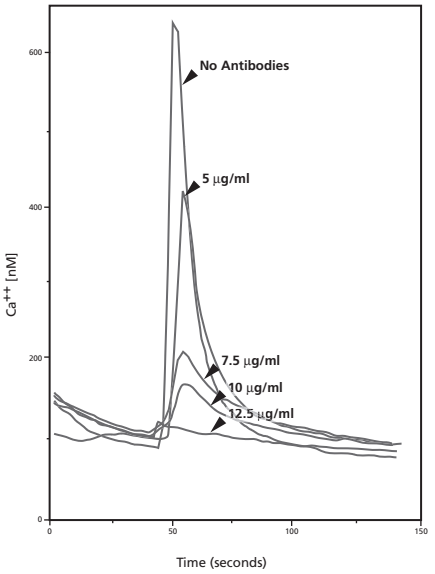

Figure 9. Monoclonal anti-rabbit IL-8 antibody (Clone 2g3) inhibits rabbit IL-8-induced calcium flux in human neutrophils. A dose-response titration of the antibody is shown using recombinant rabbit IL-8 at 125 ng/ml as the stimulant.

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***.

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Cell-based Assays Product List

Description	Apps	Size	Cat. No.
Recombinant Proteins			
Human			
β2 microglobulin	FA	0.1 mg	551089
Eotaxin	ELISA, FA	10 µg	555101
	ELISA, FA	5 µg	555102
GM-CSF	Block, ELISA, FA, IC/FCM	10 µg	550068
GROα	ELISA, FA	25 µg	555103
IFN-γ	ELISA, FA	25 µg	554616
	ELISA, FA	50 µg	554617
IL-1α	Block, ELISA, FA, IC/FCM	5 µg	551838
IL-1β	FA	5 µg	554602
IL-2	Block, ELISA, FA, IC/FCM	10 µg	554603
IL-3	Block, ELISA, FA, IC/FCM	10 µg	554604
IL-4	ELISA, FA	5 µg	554605
IL-5	Block, ELISA, FA, IC/FCM	5 µg	554606
IL-6	Block, ELISA, FA, IC/FCM	10 µg	550071
IL-7	ELISA, FA	5 µg	554608
IL-8	Block, ELISA, FA, IC/FCM	20 µg	554609
IL-10	Block, ELISA, FA, IC/FCM	5 µg	554611
IL-12 (p40)	ELISA	5 µg	554633
IL-12 (p70)	ELISA, FA	5 µg	554613
IL-15	ELISA, FA	5 µg	554630
IL-16	Block, FA, IC/FCM	5 µg	554637
IP-10	ELISA	10 µg	551130
MCP-1	ELISA, FA	10 µg	554620
MCP-3	ELISA, FA	10 µg	554629
MIG	ELISA, FA	5 µg	554636
MIP-1α	Block, IC/FCM	10 µg	554622
RANTES	ELISA, FA	10 µg	554621
SDF-1α	FA	10 µg	555105
TGF-β	ELISA, FA	2 µg	552438
Thioredoxin	Block, IC/FCM, WB	10 µg	550934
TNF	ELISA, FA	10 µg	554618
LT-α	ELISA, FA	10 µg	554619
VEGF	ELISA	10 µg	551515

Cell-based Assays Product List

Description	Apps	Size	Cat. No.
Recombinant proteins *(continued)*			
Mouse			
EPO	FA	5 µg	554597
GM-CSF	Block, ELISA, FA, IC/FCM	10 µg	554586
IFN-γ	ELISA, FA	10 µg	554587
IL-1α	ELISA	5 µg	551778
IL-1β	ELISA, FA	5 µg	554577
IL-2	Block, ELISA, FA, IC/FCM	20 µg	550069
IL-3	Block, ELISA, FA, IC/FCM	10 µg	554579
IL-4	Block, ELISA, FA, IC/FCM	10 µg	550067
IL-5	Block, ELISA, FA, IC/FCM	5 µg	554581
IL-6	Block, ELISA, FA, IC/FCM	5 µg	554582
IL-7	FA	2 µg	554583
IL-9	ELISA	10 µg	551867
IL-10	Block, ELISA, FA, IC/FCM	10 µg	550070
IL-12 (p40)	ELISA	2 µg	554594
IL-12 (p70)	ELISA, FA	5 µg	554592
IL-13	FA	5 µg	554599
MCP-1	ELISA, FA	5 µg	554590
TNF	Block, ELISA, FA, IC/FCM	10 µg	554589
Rat			
GM-CSF	ELISA, FA	5 µg	555111
IFN-γ	FA	5 µg	550072
IL-2	ELISA, FA	5 µg	555106
IL-4	Block, FA, IC/FCM	5 µg	555107
IL-6	ELISA, FA	5 µg	557008
IL-10	Block, ELISA, FA, IC/FCM	5 µg	555113
MCP-1	Block, ELISA, FA, IC/FCM	5 µg	555110
TNF	ELISA, FA	5 µg	555109

Cell-based Assays Table 1

Cytokine*	Assay Type	Indicator Cell	Final Cell Density/ml	Incubation Time (hr)	Detection Method and Length
Cytokines					
Erythropoietin					
Epo, m	Proliferation	TF-1 (ATCC CRL-2003)	1×10^5	42-44	^{3}H-TdR**, 6 hr
Granulocyte Colony Stimulating Factor					
G-CSF, h,m	Proliferation	NFS-60	4×10^4	24	^{3}H-TdR**, 4–5 hr
Granulocyte-Macrophage Colony Stimulating Factor					
GM-CSF, h	Proliferation	TF-1 (ATCC CRL-2003)	1×10^5	42-44	^{3}H-TdR**, 6 hr
GM-CSF, m,r	Proliferation	MC9 (ATCC CRL-8306)	5×10^4	20-24	^{3}H-TdR**, 6–8 hr
Interferon gamma					
IFN-γ, h	EMC virus (4×10^4 pfu/ml) resistance assay	A549 (ATCC CCL-185)	5×10^5	24 + 48***	MTT conversion, 6 hr
IFN-γ, m,r	EMC virus (1×10^4 pfu/ml) resistance assay	L929 (ATCC CCL-1)	5×10^5	24 + 48***	MTT conversion, 6 hr
Interleukin-1α					
IL-1α, h,m	Proliferation	TF-1 (ATCC CRL-2003)	1×10^5	42-44	^{3}H-TdR**, 6 hr
Interleukin-1β					
IL-1β, h	Proliferation	TF-1 (ATCC CRL-2003)	1×10^5	42-44	^{3}H-TdR**, 6 hr
IL-1β, m	Proliferation	TF-1 (ATCC CRL-2003)	5×10^4	42-44	^{3}H-TdR**, 6 hr
Interleukin-2					
IL-2, h,m,r	Proliferation	CTLL-2 (ATCC TIB-214)	2×10^4	22-24	^{3}H-TdR**, 6 hr
Interleukin-3					
IL-3, h	Proliferation	TF-1 (ATCC CRL-2003)	1×10^5	44 - 48	^{3}H-TdR**, 6 hr
IL-3, m	Proliferation	NFS-60	4×10^4	24	^{3}H-TdR**, 4–5 hr

Cell-based Assays Table 1

Cytokine*	Assay Type	Indicator Cell	Final Cell Density/ml	Incubation Time (hr)	Detection Method and Length
Cytokines *(continued)*					
Interleukin-4					
IL-4, h	Proliferation	TF-1 (ATCC CRL-2003)	1×10^5	44 - 48	^{3}H-TdR**, 6 hr
IL-4, m	Proliferation	CTLL-2 (ATCC TIB-214)	4×10^4	22 - 24	^{3}H-TdR**, 4-6 hr
IL-4, r	Proliferation	Rat splenic T cell blasts	2×10^6	48	^{3}H-TdR**, 6 hr
Interleukin-5					
IL-5, h,m	Proliferation	TF-1 (ATCC CRL-2003)	1×10^5	44 - 48	^{3}H-TdR**, 6 hr
Interleukin-6					
IL-6, h	Proliferation	TF-1 (ATCC CRL-2003)	1×10^5	44 - 48	^{3}H-TdR**, 6 hr
IL-6, m,r	Proliferation	NFS-60	4×10^4	22 - 24	^{3}H-TdR**, 4-6 hr
Interleukin-7					
IL-7, h	Proliferation	2 E8 (ATCC TIB-239)	1×10^5	44 - 48	^{3}H-TdR**, 6–8 hr
IL-7, m	Proliferation	2 E8 (ATCC TIB-239)	5×10^5	44 - 48	^{3}H-TdR**, 6–8 hr
Interleukin-8					
IL-8, h	Calcium flux	Human peripheral blood leukocytes	1×10^6	seconds	Calcium flux
Interleukin-9					
IL-9, h	Proliferation	MO-7e	2×10^5	44 - 48	^{3}H-TdR**, 4–5 hr
IL-9, m	Proliferation	MC9 (ATCC CRL-8306)	5×10^4	20-24	^{3}H-TdR**, 6–8 hr
Interleukin-10					
IL-10, h,m,r	mIL-4 (5 ng/ml) Co-stim Proliferation	D36 (DSM ACC 184)	1×10^5	44 - 46	^{3}H-TdR**, 4–5 hr
Interleukin-11					
IL-11, h	Proliferation	7TD1	5×10^3	42 - 44	^{3}H-TdR**, 6 hr

Cell-based Assays Table 1

Cytokine*	Assay Type	Indicator Cell	Final Cell Density/ml	Incubation Time (hr)	Detection Method and Length
Cytokines *(continued)*					
Interleukin-12, p70					
IL-12, h	Proliferation	PHA/IL-2 Activated Human PBMC	1×10^5	40 - 44	^{3}H-TdR**, 7–9 hr
IL-12, m	Proliferation	2D6	1×10^5	44 - 48	^{3}H-TdR**, 6–8 hr
Interleukin-13					
IL-13, h,m	Proliferation	TF-1 (ATCC CRL-2003)	1×10^5	44 - 48	^{3}H-TdR**, 6 hr
Interleukin-15					
IL-15, h,m	Proliferation	CTLL-2 (ATCC TIB-214)	2×10^4	22 - 24	^{3}H-TdR**, 6 hr
Interleukin-16					
IL-16, h,m	T cell chemotaxis	Human peripheral blood T cells	4×10^6	4	Enumerate cells
Interleukin-17					
IL-17, m	Cytokine induction	3T3 Swiss (ATCC CCL-92)	2×10^4	48	IL-6 ELISA
Interleukin-18					
IL-18, h	Costimulated cytokine induction	Human PBMC	2.5×10^6	48	IFN-γ ELISA
IL-18, m	Proliferation	2D6	1×10^5	44 - 48	^{3}H-TdR**, 6–8 hr
Transforming Growth Factor					
TGF-β, h,p	Proliferation Inhibition	TF-1 (ATCC CRL-2003)	1×10^5	42-44	^{3}H-TdR**, 6 hr
Tumor Necrosis Factor					
TNF, h,m,r	Cytolysis	L929 (ATCC CCL-1)	2×10^5	20 - 24 hr	MTT conversion, 6 hr
LT-α, h	Cytolysis	L929 (ATCC CCL-1)	2×10^5	20 - 24 hr	MTT conversion, 6 hr

Cell-based Assays Table 1

Cytokine*	Assay Type	Indicator Cell	Final Cell Density/ml	Incubation Time (hr)	Detection Method and Length
Chemokines					
Eotaxin					
Eotaxin, h	Calcium flux	hCCR3 transfectant cells	1×10^6	seconds	Calcium flux
Growth Regulated Oncogene					
GROα, h	Calcium flux	Human peripheral blood leukocytes	1×10^6	seconds	Calcium flux
Interleukin-8					
IL-8, h	Calcium flux	Human peripheral blood leukocytes	1×10^6	seconds	Calcium flux
Gamma interferon inducible protein-10					
IP-10, h,m	Calcium flux	hCXCR3 transfectant cells	1×10^6	seconds	Calcium flux
Monocyte Chemoattractant Proteins					
MCP-1,2,3 h,m,r	Calcium flux	THP-1 (ATCC TIB-202)	1×10^6	seconds	Calcium flux
Monokine Induced by gamma interferon					
MIG, h,m	Calcium flux	hCXCR3 transfectant cells	1×10^6	seconds	Calcium flux
Regulated upon Activation, Normal T cell Expressed, & Secreted					
RANTES, h	Chemotaxis	THP-1 (ATCC TIB-202)	2.5×10^6	1.5	LDH assay
Stromal Cell-derived Factor					
SDF-1, h	Chemotaxis	Human PBMC	1.5×10^6	3	Enumerate cells

* h = human, m = mouse, r = rat, p = porcine

** 0.5 µCi of tritiated thymidine per well

*** Virus (24 hr) $\rightarrow$ IFNγ (48 hr)

Chapter 11

BD RiboQuant™ Multi-Probe RNase Protection Assay System

Introduction

The ribonuclease protection assay (RPA) is a highly sensitive and specific method for the detection and quantitation of mRNA species. The assay was made possible by the discovery and characterization of DNA-dependent RNA polymerases from the bacteriophages SP6, T7 and T3, and the elucidation of their cognate promoter sequences. These polymerases are ideal for the synthesis of high-specific-activity RNA probes from DNA templates because they exhibit a high degree of fidelity for their promoters, polymerize RNA at a very high rate, efficiently transcribe long segments, and do not require high concentrations of rNTPs. Thus, a cDNA fragment of interest can be subcloned into a plasmid that contains bacteriophage promoters, and the construct can then be used as a template for synthesis of radiolabeled anti-sense RNA probes.

BD Biosciences Pharmingen has developed multi-probe RPA systems that include a series of such templates, each of distinct length and each representing a sequence in a distinct mRNA species. The templates are assembled into biologically relevant sets (***Table 2***) to be used by researchers for the T7 polymerase-directed synthesis of a high-specific-activity, [α–^{32}P]-labeled or biotin-labeled anti-sense RNA probe set. The probe set is hybridized in excess to target RNA in solution, after which free probe and other single-stranded RNA are digested with RNases. The remaining "RNase-protected" probes are purified, resolved on denaturing polyacrylamide gels, and quantified by autoradiography or phosphorimaging when using radiolabeled probes or by chemiluminescent detection when using biotin-labeled probes. The quantity of each mRNA species in the original RNA sample can then be determined based on the intensity of the appropriately-sized, protected probe fragment.

Two distinct advantages of the multi-probe RPA approach are its sensitivity and its capacity to simultaneously quantify several mRNA species in a single sample of total RNA. This allows comparative analysis of different mRNA species within samples and, by incorporating probes for housekeeping gene transcripts, the levels of individual mRNA species can be compared between samples. Moreover, the assay is highly specific and quantitative due to the sensitivity of RNase for mismatched base pairs and the use of solution-phase hybridization driven toward completion by excess probe. Lastly, the multi-probe RPA can be performed on total RNA preparations derived by standard methods from either frozen tissues or cultured cells, without further purification of poly (A)$^+$ RNA.

BD RiboQuant™ Multi-Probe RNase Protection Assay System is a complete system available for detecting and quantifying transcripts. The system includes:

- Multi-Probe Template Sets
- *In Vitro* Transcription Kit
- Non-Rad *In Vitro* Transcription Kit
- RPA Kit
- Non-Rad Detection Kit

Note: Individual components of the system may be purchased separately or together as an RPA Starter Package.

BD RiboQuant Custom Services

Custom Template Sets

Additional templates are available for inclusion in a Custom Multi-Probe Template Set through our BD RiboQuant™ Custom Template Program. Please refer to the complete list of templates on our website at **www.bdbiosciences.com**. If you do not see a template for your gene of interest, BD Biosciences Pharmingen will design, construct, and test, by RPA, templates specific for your research.

Custom RNase Protection Assay Services

The BD Biosciences Pharmingen Custom Products and Services Group (CPSG) provides RPA services for the detection and quantification of multiple mRNA species. Gene regulation in cultured cells and frozen tissue samples can be monitored for basic and clinical research studies. To contact CPSG, please call **888.401.4BDB** (**4232**) or e-mail **cpsg@bdbiosciences.com**.

Custom Services include:

RNA preparation and quantification. RNA is isolated from samples using acid guanidinium thiocyanate-phenol-chloroform extraction. Total RNA is quantitated for use in RPA.

Probe synthesis, hybridization, RNase treatment. [α-^{32}P]-labeled antisense RNA probes are generated from standard and customized BD RiboQuant Multi-Probe Template Sets and hybridized with target RNA. Free probe and other single-stranded RNA are digested with RNases.

Electrophoresis separation and autoradiogram. RNase protected probes are purified and resolved on denaturing polyacrylamide gels and developed on film. The expressed mRNA species are identified by the presence of bands corresponding to the expected fragment length.

Phosphorimaging quantification. Radioactivity of [α-^{32}P]-labeled probes are measured by phosphorimaging. The quantity of each mRNA species is determined based on the housekeeping genes L32 and GAPDH.

Table 1. RPA Starter Packages

Kit	Size	Cat. No.
BD RiboQuant Starter Package includes:	200 RNA samples	556144
In Vitro Transcription Kit	25 transcription reactions	556850
RPA Kit	200 RPA reactions	556134
One Multi-Probe Template Set	10 probe syntheses	
BD RiboQuant Non-Rad Starter Package includes:	200 RNA samples	551919
Non-Rad *In Vitro* Transcription Kit	5 transcription reactions	551917
RPA Kit	200 RPA reactions	556134
One Multi-Probe Template Set	10 probe syntheses	
Non-Rad Detection Kit	10 membranes plus reagents	551918

Template Sets for the Multi-Probe RPA

BD Biosciences Pharmingen currently offers 116 Multi-Probe Template sets for comparative and systematic analysis of cytokines and their respective receptor genes, as well as apoptosis-associated molecules, cell cycle regulators, cell surface antigens, tumor suppressor genes, DNA damage and repair-related molecules, developmental regulators and other receptors, and signaling molecules. The newest Multi-Probe Template Sets detect and measure gene expression for matrix metalloproteinases, integrins, and genes involved in angiogenesis (refer to ***Product Listing*** at the end of the chapter). These Multi-Probe Template Sets can be used with either radiolabeled probes or biotin-labeled probes.

The construction and assembly of templates into biologically relevant multi-probe sets allows for groups of mRNAs with interrelated functions to be analyzed simultaneously. The housekeeping gene probes, L32 and GAPDH, are included in each set for normalizing sampling and technique errors to permit comparison of individual mRNA species between samples.

The RPA, by design, may reveal previously unknown strain-specific polymorphisms in a given gene sequence. This should be considered when interpreting experimental data. Templates are designed considering all sequence data available at the time.

Note: Each template set is supplied in units suitable for ten probe syntheses, with each probe synthesis generating sufficient reagent to analyze more than 40 RNA samples. Store the DNA templates at –20°C.

BD RiboQuant Multiprobe Template Sets are listed on ***pages 222-226***.

Control RNA

BD Biosciences Pharmingen offers mouse, rat, human, and pig Control RNA which serve as integrity controls for the RNA sample (not as markers for protected probes) in the RPA procedure. Each Control RNA does not contain every representative protected band, and the number of representative bands will vary for each probe set. The appropriate Control RNA is packaged with each Multi-Probe Template Set. The Control RNA should be stored at –70°C. For a complete listing of available template sets with probe sizes and Control RNA's, please refer to the ***BD RiboQuant Multiprobe RNase Protection Assay System Instruction Manual, 7th Edition*** or refer to our website at **www.bdbiosciences.com**

BD RiboQuant™ RPA using Radiolabeled Probes

In Vitro Transcription Kit

The *In Vitro* Transcription Kit (***Table 2***) is optimized for the efficient synthesis of high-specific-activity riboprobes from the BD RiboQuant Multi-Probe Template Sets. Each kit contains sufficient reagents for 25 transcription reactions (Cat. No. 556850).

EDTA and ammonium acetate can be stored at room temperature. All other reagents should be stored at –20°C. When the GACU pool and DTT are first thawed, we recommend the solutions be aliquoted (5 µl) prior to re-freezing. RNasin®*, T7 RNA polymerase, and DNase are provided in glycerol-containing solutions; care should be taken to keep solutions on ice and avoid warming of the stock solutions.

Table 2. *In Vitro* Transcription Kit Components (Cat. No. 556850)

Reagent	Concentration	Volume	Reactions	Component No.	Cat. No.
GTP/ATP/CTP/ UTP/ (GACU Pool)	GAC: 2.75 mM ea U: 61 µM	25 µl	25	51-45005Z	556125
DTT	100 mM	50 µl	25	51-45006Z	556126
Transcription Buffer	5×	100 µl	25	51-45007A	556127
RNasin®*	40 U/µl	25 µl	25	51-45008Z	556128
T7 RNA Polymerase	15-20 U/µl	25 µl	25	51-45009Z	556129
RNase-free DNase	1 U/µl	50 µl	25	51-45010Z	556130
Yeast tRNA	2 mg/ml	50 µl	25	51-45011Z	556131
EDTA	20 mM	650 µl	25	51-45012Z	556132
Ammonium Acetate	4 M	1.3 ml	25	51-45013Z	556133

RPA Kit

The RPA Kit (***Table 3***) contains reagents necessary to perform the RPA procedure as outlined in the RPA Protocol. Each kit contains sufficient reagents for the analysis of 200 RNA samples (Cat. No. 556134).

The hybridization buffer, RNase A + T1 mix, Proteinase K, yeast tRNA, and loading buffer should be stored at –20°C. Care should be taken to keep solutions on ice and to avoid excessive warming of the stocks. The RNase buffer, Proteinase

*RNasin® is a registered trademark of Promega Corporation.

K buffer, and ammonium acetate solution can be stored at room temperature after they are first thawed. After thawing, the Proteinase K buffer should be warmed to 37°C to solubilize the SDS in the solution.

Table 3. RPA Kit Components (Cat. No. 556134)

Reagent	Concentration	Volume	Reactions	Component No.	Cat. No.
Hybridization Buffer	1×	3.6 ml	200	51-45015A	556135
RNase Buffer	1×	25 ml	200	51-45016A	556136
RNase A + T1 Mix	A: 80 ng/µl T1: 250 U/µl	60 µl	200	51-45017Z	556137
Proteinase K Buffer	1×	3.9 ml	200	51-45018A	556138
Proteinase K	10 mg/ml	300 µl	200	51-45019Z	556139
Yeast tRNA	2 mg/ml	300 µl	200	51-45020Z	556140
Ammonium Acetate	4M	24 ml	200	51-45021Z	556141
Loading Buffer	1×	1.3 ml	200	51-45022A	556142

Additional Reagents and Supplies Required

Reagents: (all Molecular Biology grade)

- [α-^{32}P]UTP (3000 Ci/mmol, 10 mCi/ml). (BD Biosciences Pharmingen recommends use of [α-^{32}P] UTP which does not contain a commercial dye)

  Note: [α-^{33}P] and [α-^{35}S]UTP can also be substituted for [α-^{32}P]UTP. Please contact Technical Service for usage recommendations.

- Tris-saturated phenol, pH 8.0
- Chloroform:isoamyl alcohol (50:1)
- Ethanol (100% and 90%)
- Mineral oil
- 10× TBE (0.89 M Tris, 0.89 M boric acid, 20 mM EDTA, pH 8.3)
- 40% Acrylamide
- 2% Bis acrylamide
- Urea
- Ultrapure, RNase-free water
- TEMED
- 10% ammonium persulfate

Supplies:

- RNase-free 1.5 ml Eppendorf tubes
- RNase-free pipetor tips
- RNase-free plasticware or glassware
- Pipetors (micro and standard)

Supplies continued

- Gloves, appropriate shielding, and disposal for α-^{32}P
- Microcentrifuges (15,000 × *g* max; room temp and 4°C)
- Heat blocks or water baths
- Vortex mixer
- Vacuum evaporator centrifuge for Eppendorf tubes
- Scintillation counter
- Sequencing gel plates, spacers, and combs
- High-voltage vertical gel electrophoresis system
- Gel dryer
- Autoradiography film and film cassettes with intensifying screens

RPA Protocol using Radiolabeled Probes

All reagents used in this RPA protocol are described in the sections above. In all steps of the protocol, standard precautions should be used to avoid RNase contamination and exposure of personnel to radioactivity. Typically, the probe synthesis is performed during the afternoon of day 1, hybridizations are incubated overnight, and RNase treatments and gel electrophoresis are performed early on day 2 (***Figure 1***).

Note: RNA is very sensitive to RNase contamination. Always use RNase-free supplies and reagents.

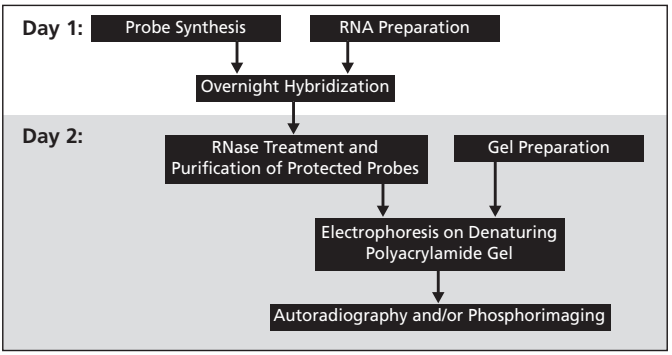

Figure 1. Overview of the RPA Protocol.

Probe Synthesis

1. Bring the [α-^{32}P]UTP, GACU nucleotide pool, DTT, 5× transcription buffer, and RPA template set to room temp. For each probe synthesis, add the following in order to a 1.5 ml Eppendorf tube:

   1 µl RNasin®*

   1 µl GACU pool

   2 µl DTT

   4 µl 5× transcription buffer

   1 µl Multi-Probe Template Set

   10 µl [α-^{32}P]UTP

   1 µl T7 RNA polymerase (keep at –20°C until use; return to –20°C immediately)

2. Mix by pipetting or gently flicking, then quick spin in a microfuge. Incubate at 37°C for 1 hr.
3. Terminate the reaction by adding 2 µl of DNase. Mix by gently flicking and quick spin in a microfuge. Incubate at 37°C for 30 min.
4. Add the following reagents (in order) to each 1.5 ml Eppendorf tube:

   26 µl 20 mM EDTA

   25 µl Tris-saturated phenol

   25 µl chloroform:isoamyl alcohol (50:1)

   2 µl yeast tRNA

5. Mix by vortexing into an emulsion and spin in a microfuge for 5 min at room temp.
6. Transfer the upper aqueous phase to a new 1.5 ml Eppendorf tube and add 50 µl chloroform:isoamyl alcohol (50:1). Mix by vortexing and spin in a microfuge for 2 min at room temp.
7. Transfer the upper aqueous phase to a new 1.5 ml Eppendorf tube and add 50 µl 4 M ammonium acetate and 250 µl ice-cold 100% ethanol. Invert the tubes to mix and incubate for 30 min at –70°C. Spin in a microfuge for 20 – 30 min at 4°C.
8. Carefully remove the supernatant and add 100 µl of ice cold 90% ethanol to the pellet. Spin in a microfuge for 10 – 15 min at 4°C.
9. Carefully remove all of the supernatant and air dry the pellet for 5 to 10 min (do not dry in a vacuum evaporator centrifuge). Add 50 µl of hybridization buffer and solubilize the pellet by gently vortexing for 30 sec. Quick spin in a microfuge.

*RNasin® is a registered trademark of Promega Corporation.

10. Quantitate duplicate 1 µl samples in a scintillation counter. Expect a maximum yield of ~3×10^6 Cherenkov counts/µl (measurement of cpm/µl without the presence of scintillation fluid) with an acceptable lower limit of ~3×10^5 Cherenkov counts/µl. Low probe yield will result in lower signal and reduction in number of reactions. Store the probe at –20°C until needed. Generally, the probe can be used only for two successive overnight hybridizations (when labeled with [α-^{32}P] UTP). For an alternative protocol for purifying the synthesized probe without phenol, please refer to the BD RiboQuant manual.

RNA Preparation & Hybridization

1. For the best results, use procedures that generate total RNA of high quality and purity. RNA should be stored in RNase-free water at –70°C. Add the desired amount of target RNA (generally 1 – 20 µg) to 1.5 ml Eppendorf tubes and include a tube that contains 2 µl of 2 mg/ml yeast tRNA as a background control. In general, 20 – 24 total sample tubes are an easily manageable number for each RPA set up. With the BD control RNA, a 2 µl volume (ie, 2 µg) is recommended.
2. If RNA has been stored in water, freeze the samples for 15 min at –70°C. Dry completely (~1 hr) in a vacuum evaporator centrifuge (no heat). Alternatively, RNA can also be ethanol precipitated prior to the addition of hybridization buffer.
3. Add 8 µl of hybridization buffer to each sample. Solubilize the RNA by gently vortexing for 3 – 4 min and quick spin in the microfuge.
4. Dilute the probe from step 10 of probe synthesis with hybridization buffer to the appropriate concentration. The optimal probe concentration (cpm/µl) for each standard Multi-Probe Template Set is included on the Technical Data Sheet supplied with the set. Add 2 µl of diluted probe to each RNA sample and mix by pipetting. Add a drop of mineral oil to each tube and quick spin in the microfuge.
5. Place the samples in a heat block pre-warmed to 90°C. Immediately turn the temperature to 56°C (allowing the temp to ramp down slowly) and incubate for 12 – 16 hr. Remove samples from heat block 15 min prior to the RNase treatments, and place at RT to allow the temperature to equilibrate slowly. All incubations may also be carried out in a water bath.

RNase Treatment

1. Prepare the RNase cocktail (per 20 samples):

   2.5 ml RNase buffer

   6 µl RNase A + T1 mix
2. Remove the RNA samples from the heat block and pipette 100 µl of the RNase cocktail underneath the oil into the aqueous layer (bubble). Spin in the microfuge for 10 sec and incubate for 45 min at 30°C.

3. During the RNase digestion, prepare the Proteinase K cocktail. Prewarm Proteinase K buffer to 37°C to solubilize SDS prior to using.

   For 20 samples, mix:

   390 µl Proteinase K buffer

   30 µl Proteinase K

   30 µl yeast tRNA

   Mix and add 18 µl aliquots of the cocktail to new, labeled Eppendorf tubes.

4. Using a pipetor, extract the RNase digests from underneath the oil (avoid transfer of the oil) and transfer to the tubes containing the Proteinase K solution. Vortex briefly, quick spin in the microfuge, and incubate for 15 min at 37°C.
5. Add 65 µl Tris-saturated phenol and 65 µl chloroform:isoamyl alcohol (50:1). Vortex into an emulsion and spin in the microfuge for 5 min at RT.
6. Carefully extract the upper aqueous phase (set the pipetor to 120 µl and totally avoid the organic interface) and transfer to a new tube. Add 120 µl 4 M ammonium acetate and 650 µl ice-cold 100% ethanol. Mix by inverting the tubes and incubate for 30 min at –70°C. Spin in the microfuge for 20 – 30 min at 4°C.
7. Carefully remove the supernatant and add 100 µl ice-cold 90% ethanol. Spin in the microfuge for 10 – 15 min at 4°C.
8. Carefully remove the supernatant and air dry the pellet completely (do not dry in a vacuum evaporator centrifuge). Add 5 µl of 1× loading buffer, vortex for 2 – 3 min, and quick spin in the microfuge. Prior to loading the samples on the gel, heat the samples for 3 min at 90°C and then place them immediately in an ice bath.

Gel Resolution of Protected Probes

1. Clean a set of gel plates (>40 cm in length) thoroughly with water followed by ethanol. Siliconize the short plate and clean again. Assemble the gel mold using 0.4 mm spacers.
2. Combine the following to give a final concentration of 4.75% acrylamide (74.5 ml acrylamide solution at final ratio 19:1 acrylamide/bis):

Weigh:	35.82 g of urea
Add:	22.35 ml dH_2O
	7.45 ml of 10× TBE
Heat:	~ 42°C to dissolve urea
Add:	8.85 ml of 40% acrylamide
	9.31 ml of 2% bis acrylamide
Filter:	Using 0.4 – 0.8 µm filter
Add:	450 µl ammonium persulfate (10%)
	60 µl TEMED

   Note: Use recommended acrylamide concentration and acrylamide: bis ratio. It is critical for the correct resolution of unprotected and protected probe bands.

3. Mix acrylamide solution well, pour immediately into the gel cast removing any air bubbles that may form, and insert comb (we recommend using 0.5 cm width well; do not use sharkstooth comb).
4. After polymerization (about 1 h), place gel into a vertical gel rig that has a heat dispenser and add 0.5× TBE running buffer into upper and lower reservoirs. Remove the gel comb and flush the wells thoroughly with 0.5× TBE. Pre-run gel at 55 watts constant power for 45 min before loading samples.
5. Flush the wells with running buffer just prior to loading samples. Load samples and controls (including ^{32}P-labeled probe, diluted to 1000 – 2000 cpm in 10 µl loading buffer). Run the gel at 55 watts constant power until the leading edge of the Bromophenol Blue (BPB, front dye) reaches 30 cm from the bottom of the well.
6. Disassemble the gel mold, remove the short plate, and adsorb the gel to filter paper. Cover the gel with Saran wrap and layer between two additional pieces of filter paper. Place in the gel dryer under vacuum for ~1 hr at 80°C. Place the dried gel on film (Kodak X-AR) in a cassette with an intensifying screen and develop at –70°C (exposure times will vary depending upon application). Alternatively, radioactivity can be quantified by phosphorimaging or other equivalent instruments.
7. Using the undigested probes as markers, plot a standard curve, on semi-log graph paper, of migration distance versus log nucleotide length. Use this curve to establish the identity of "RNase-protected" bands in the experimental samples. Note that the probe lengths are greater than the "protected" fragment lengths; this is due to the presence of flanking sequences in the probes that are derived from the plasmid and do not hybridize with target mRNA.

Troubleshooting

Poor Probe Recoveries

1. Use of [α-^{32}P]UTP that has decayed beyond one half-life may lead to decreased probe labeling and increased lane background. Also, we recommend the use of [α-^{32}P]UTP which does not contain commercial dyes (ie, use the [α-^{32}P]UTP that is stored at –70°C and not at 4°C).
2. Avoid repeated freeze-thaw of the DTT stock solution. We recommend storing small aliquots at –20°C.
3. Make certain the transcription reagents, excluding enzymes, (nucleotides, DTT, 5× transcription buffer, template set, [α-^{32}P]-UTP are at room temp prior to adding the RPA template. Spermidine present in the transcription buffer can precipitate DNA at low temperatures.

4. To maximize recovery of probe, orient hinge of microfuge tube in direction of centifugal force in microfuge, and remove tube from microfuge immediately following spin. Carefully remove ethanol and ethanol washes without touching or dislodging pellet. Following ethanol wash, briefly centrifuge for 10 sec. and remove residual ethanol with a P200 pipette tip.
5. Careful removal of ethanol from the precipitated probe will reduce probe loss (we have included yeast tRNA as a carrier to facilitate precipitation). If this problem is suspected, refreeze and recentrifuge the ethanol supernatant.
6. Check the integrity of the probe set by analyzing it on an acrylamide gel.
7. Quantitate duplicate 1 µl samples in the scintillation counter. Expect a maximum yield of ~3×10^6 Cherenkov counts/µl (measurement of cpm/µl without the presence of scintillation fluid) with an acceptable lower limit of ~3×10^5 Cherenkov counts/µl. Store the probe at –20°C until needed. Generally, the probe can only be used for two successive overnight hybridizations (when labeled with [α-^{32}P] UTP). Probe pellet can be resuspended in 25 µl (vs. 50 µl) to obtain a greater cpm/µl concentration.

High Levels of Certain mRNA Species Obscure the Detection of Other mRNA Species

1. Consult BD Biosciences Pharmingen for the availability of RPA Template Sets customized to omit probes for the highly expressed transcripts in your RNA preparations.

Protected Probe Resolution

1. Use recommended polyacrylamide concentration and bis/acrylamide ratio.
2. Immediately prior to loading, denature sample for a full three min at 90°C in a heat block (not oven), then place samples immediately on ice.

High Levels of Breakdown Products in the Gel Lanes

1. Some protected probe fragmentation is normal because mRNA degradation is a natural occurrence within cells. However, if excessive degradation is observed, check the integrity of your RNA samples by agarose gel electrophoresis.
2. Rigorously adhere to the prescribed RNase digestion conditions. These have been carefully optimized for the BD RiboQuant Multi-Probe Template Sets.
3. Use caution when extracting the aqueous phase from the phenol-chloroform extraction (***Step 6 of RNase Treatment***) because residual RNase may be present in the organic interface. This problem can be remedied by performing a second phenol-chloroform or chloroform-only extraction.
4. Residual phenol or ethanol in the sample will cause the appearance of degradation of the probe and is characterized by narrowing of the lanes in the lower portion of the gel.
5. Radiolysis of labeled probe stored over time will contribute to high background.

BD RiboQuant™ RPA using Biotin-labeled Probes

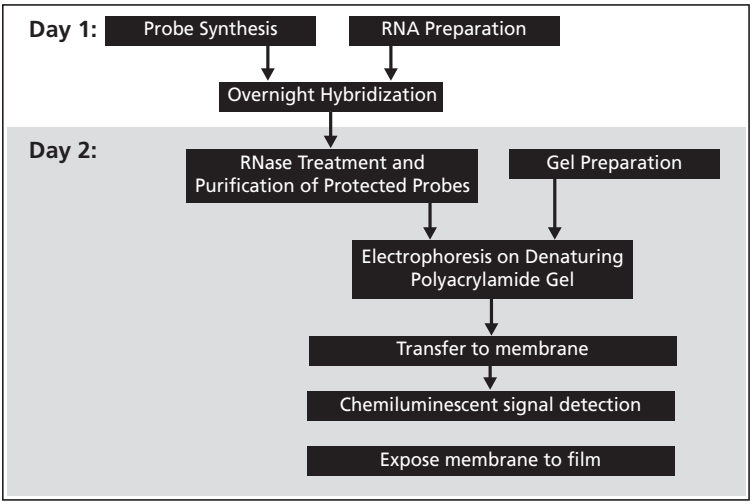

Figure 2. Overview of the Non-Rad RPA Protocol.

Table 4. Non-Rad Starter Package

Kit	Size	Cat. No.
Non-Rad Starter Package includes:	200 RNA samples	551919
Non-Rad *In Vitro* Transcription Kit	5 transcription reactions	551917
RPA Kit	200 RPA reactions	556134
Non-Rad Detection Kit	Reagents for 10 blots	551918
One Multi-Probe Template Set	10 probe syntheses	

Non-Rad *In Vitro* Transcription Kit

The Non-Rad *In Vitro* Transcription (IVT) Kit (***Table 5***) is optimized for the efficient synthesis of high-specific-activity, biotin-labeled riboprobes from the Multi-Probe Template Sets. Each kit contains sufficient reagents for 5 transcription reactions (~10 µg probe yield per reaction) (Cat No. 551917).

EDTA and ammonium acetate can be stored at room temperature. All other reagents should be stored at –20°C. When the GACU pool is first thawed, it must be combined, according to protocol, with biotin-16-UTP. Enzyme Mix and DNase are provided in glycerol-containing solutions; care should be taken to keep solutions on ice and avoid warming of the stock solutions.

Table 5. Non-Rad *In Vitro* Transcription Kit (Cat. No. 551917)

Reagent	Concentration	Volume	Reactions	Component Cat. No.
5× Nucleotide Mix	5 mM A,C,G,* 3.25 mM UTP*	16.5 µl	5	51-46001Z
5× Transcription Buffer	5×	20 µl	5	51-46002A
DTT	100 mM	10 µl	5	51-46003Z
Enzyme Mix RNasin®† T7 RNA Polymerase	 40 U/µl 15-20 U/µl	10 µl	5	51-46004Z
RNase-free DNase	1 U/µl	10 µl	5	51-46005Z
EDTA	20 mM	135 µl	5	51-46006Z
Glycogen	5 µg/µl	5 µl	5	51-46007Z
LiCl	4 M	2.7 ml	5 IVT, 200 RPA	51-46008Z

*Final concentration when mixed with biotin-labeled UTP.

RPA Kit

The RPA Kit (***Table 6***) contains reagents necessary to perform the RPA procedure as outlined in the Non-Rad RPA Protocol. Each kit contains sufficient reagents for the analysis of 200 RNA samples (Cat. No. 556134).

The hybridization buffer, RNase A + T1 mix, Proteinase K, yeast tRNA, and loading buffer should be stored at –20°C. Care should be taken to avoid excessive warming of the stocks. The RNase buffer, Proteinase K buffer, and ammonium acetate solution can be stored at room temperature after they are first thawed. After thawing, the Proteinase K buffer should be warmed to 37°C to solubilize the SDS in the solution. Additional lithium chloride for precipitation of protected RNA samples is included in the Non-Rad *In Vitro* Transcription Kit, (Cat. No. 551917).

Table 6. RPA Kit Components (Cat. No. 556134)

Reagent	Concentration	Volume	Reactions	Component No.	Cat. No.
Hybridization Buffer	1×	3.6 ml	200	51-45015A	556135
RNase Buffer	1×	25 ml	200	51-45016A	556136
RNase A + T1 Mix	A: 80 ng/µl T1: 250 U/µl	60 µl	200	51-45017Z	556137
Proteinase K Buffer	1×	3.9 ml	200	51-45018A	556138
Proteinase K	10 mg/ml	300 µl	200	51-45019Z	556139
Yeast tRNA	2 mg/ml	300 µl	200	51-45020Z	556140
Ammonium Acetate*	4M	24 ml	200	51-45021Z	556141
Loading Buffer	1×	1.3 ml	200	51-45022A	556142

*Use lithium chloride supplied in the Non-Rad *In Vitro* Transcription Kit for precipitation of protected probe.

†RNasin® is a registered trademark of Promega Corporation.

Non-Rad Detection Kit

The Non-Rad Detection Kit (***Table 7***) contains 10 positively-charged nylon membranes and all reagents necessary for the detection of biotin-labeled probes as outlined in the Non-Rad RPA Protocol. Membranes should be stored at room temperature. All other reagents are stored at 4°C.

Table 7. Non-Rad Detection Kit (Cat. No. 551918)

Reagent	Volume	Reactions	Component No.
Nylon Membranes		10 membranes	51-46009Z
Membrane Blocking Buffer	500 ml	10	51-4562KC
Wash Buffer (4×)	500 ml	10	51-4563KC
Substrate Equilibration Buffer	500 ml	10	51-4564KC
Streptavidin-Horseradish Peroxidase	1.5 ml	10	51-4528KC
Stable Peroxide Solution	75 ml	10	51-4531KC
Luminol/Enhancer	75 ml	10	51-4532KC

Additional Reagents and Supplies Required

Reagents (all Molecular Biology grade)

- Biotin-16-UTP, 10 mM (eg, Roche Biochemical, Cat. No. 1388908)
- Tris-saturated phenol, pH 8.0
- Chloroform:isoamyl alcohol (50:1)
- Ethanol (100% and 90%)
- Mineral oil
- 10× TBE (0.89 M Tris, 0.89 M boric acid, 20 mM EDTA, pH 8.3)
- 40% Acrylamide
- 2% Bis acrylamide
- Urea
- Ultrapure, RNase-free water
- TEMED
- 10% Ammonium persulfate

Supplies

- RNase-free 1.5 ml Eppendorf tubes
- RNase-free pipetor tips
- RNase-free plasticware or glassware
- Pipetors (micro and standard)
- Microcentrifuges (15,000 × *g* max; room temp and 4°C)
- Heat blocks or water baths

Supplies continued

- Vortex mixer
- Vacuum evaporator centrifuge for Eppendorf tubes
- Spectrophotometer
- –70°C freezer or dry ice
- Sequencing gel plates, spacers, and combs
- High-voltage vertical gel electrophoresis system
- Semi-dry electroblotter (eg, Integrated Separation Systems)
- Autoradiography film (Amersham Hyperfilm ECL) and film cassettes with intensifying screens
- Flat forceps (sterile)

Non-Rad RPA Protocol using Biotin-labeled Probes

Note: RNA is very sensitive to RNase contamination. Always use RNase-free supplies and reagents.

Probe Synthesis

1. Remove 5× Transcription Buffer, and 5× Nucleotide Mix, (from Non-Rad *In Vitro* Transcription Kit) and BD RiboQuant Multi-Probe Set from –20°C and thaw at room temperature. Mix each solution and collect solution by brief centrifugation. Store Nucleotide Mix on ice during reaction setup.

   Remove Enzyme Mix from –20°C, centrifuge briefly and store on ice during setup. (Store at –20°C immediately after use.)

2. **When first using the Non-Rad *In Vitro* Transcription Kit,** complete the 5× Nucleotide Mix by adding 3.5 µl 10 mM biotin-labeled UTP (eg, biotin-16-UTP, 10 mM, from Roche, Cat. No. 1388908) to the thawed vial of 5× Nucleotide Mix. Mix solution and centrifuge briefly. Store at –20°C.

3. For each probe synthesis, add the following in order to a 1.5 ml Eppendorf tube:

7 µl	RNase-free H_2O
4 µl	5× Transcription Buffer
4 µl	5× Complete Nucleotide Mix* (see Step 2, above)
2 µl	100 mM DTT
1 µl	Multi-Probe Template Set DNA (50 ng)
2 µl	Enzyme Mix
20 µl	

4. Mix by gently pipetting and centrifuge briefly. Incubate reaction at 37°C for 2 hr.

5. After *in vitro* transcription reaction is complete, add 2 µl DNase to degrade template DNA. Mix by gently pipetting and centrifuge briefly. Incubate at 37°C for 30 min.
6. To inactivate DNase, add 27 µl 20 mM EDTA to each tube and mix.
7. To precipitate probe, add:

   1 µl glycogen

   5 µl 4 M LiCl

   Mix.

   Add 150 µl cold 100% ethanol
8. Mix tubes by inverting several times and place on pulverized dry ice for 30 min or at –80°C for 1 hr.
9. Pellet RNA probe by centrifugation in a microfuge at full-speed at 4°C for >15 min.
10. Without disturbing the RNA precipitate, carefully remove the ethanol solution and add 100 µl of ice cold 70% ethanol. Vortex. Pellet RNA probe by centrifugation in microfuge at full-speed at 4°C for 5 min.
11. Without disturbing the RNA pellet, carefully remove all of the ethanol solution (if necessary centrifuge briefly and remove remaining ethanol). Allow the RNA probe to air dry for 5 to 10 min (do not dry in a vacuum evaporator centrifuge). Add 10 µl of hybridization buffer (Component No. 51-45015A, in RPA Kit, Cat. No. 556134) and solubilize the pellet by vortexing. Quick spin to collect the solubilized RNA probe.

Determining Probe Concentration

1. Dilute 1 µl of solubilized RNA probe into 99 µl RNase-free water and measure the probe concentration by determining absorbance at 260 nm. Calculate probe concentration using the following equation:

   40 µg/ml (RNA concentration per OD_{260}) × Abs at OD_{260} × 100 (dilution factor).

   Typically, a 20 µl IVT reaction will yield 5 – 10 µg of total RNA probe.
2. Once probe concentration has been determined, dilute remaining probe (9 µl) to 10 ng/µl with hybridization buffer.

RNA Preparation & Hybridization

1. Add the desired amount of target RNA (10 – 20 µg, optimizing for maximum signal and minimum background) into a labeled 1.5 ml Eppendorf tube. Set up two control hybridizations: into one tube add yeast tRNA as a background control, and into a second tube add 10 µl (10 µg) BD RiboQuant Control RNA (included with BD RiboQuant Template Set).

2. If RNA has been stored in water, freeze the RNA samples for 15 min at –70°C or on dry ice and place in a vacuum evaporator centrifuge (no heat) to dry.
3. Add 8 µl of hybridization buffer to each sample and solubilize the RNA by gently vortexing for 3 – 4 min. Centrifuge briefly to collect hybridization solution.
4. Add 2 µl (20 ng) biotin-labeled probe to each RNA sample and mix by pipetting repeatedly (final hybridization volume is 10 µl). Centrifuge briefly, then add two drops of mineral oil to each tube to prevent evaporation.
   We recommend titrating probe concentration in RPA to maximize signal and minimize background. Start with 20 ng per hybridization, then, if background occurs, optimize.
5. Incubate samples in a 90°C heat block (pre-warmed) for 5 min. Reduce heat block temperature to 56°C and hybridize for 12 – 16 hr (or overnight). Remove hybridization reaction from heat block and place at RT to allow temperature to ramp down slowly.

RNase Treatment

1. Prepare sufficient RNase cocktail for all hybridizations and controls (Number of samples + 2 controls + 1)

   100 µl RNase buffer / sample

   0.3 µl (100 U) Ribonuclease A + T1 / sample
2. Add 100 µl of the RNase cocktail directly into the aqueous phase of each sample and control hybridization reaction (beneath oil layer). Centrifuge samples for 10 seconds and incubate reactions for 45 min at room temperature.
3. Prepare sufficient Proteinase K cocktail for all hybridizations and controls (Number of samples + 2 controls + 1)

   15.6 µl Proteinase K buffer / sample

   1.2 µl Proteinase K / sample

   1.2 µl yeast tRNA / sample
4. Mix Proteinase K cocktail, centrifuge briefly and aliquot 18 µl to new, labeled Eppendorf tubes.
5. Using a pipetor, remove the RNase-digested probe from beneath the oil (do not carry over any residual oil) and transfer to the tubes containing the aliquoted Proteinase K cocktail. Mix briefly, centrifuge, and incubate samples for 15 min at 37°C.
6. After completion of Proteinase K digestion, add 12.8 µl 4 M LiCl (Non-Rad IVT Kit Component No. 51-46008Z), mix, then add 340 µl cold 100% ethanol. Mix samples by inverting the tubes several times and place on pulverized dry ice for 30 min. or at –70°C for 2 hr. Collect protected probe precipitate by full speed centrifugation in microfuge for >15 min at 4°C.

7. Carefully remove the ethanol solution and add 100 µl cold 90% ethanol (without disturbing the pellet). Spin in the microfuge for 5 min at 4°C.
8. Without disturbing the RNA pellet, carefully remove all of the ethanol solution (if necessary centrifuge briefly to remove remaining ethanol). Allow the protected RNA probe to air dry for 5 to 10 min (do not dry in a vacuum evaporator centrifuge). Add 5 µl of 1× loading buffer, mix for 2 – 3 min to solubilize RNA and centrifuge briefly. Biotin-labeled probe control is prepared by adding ~ 150 pg unhybridized probe to 5 µl of 1× loading buffer. Heat samples for 3 min at 90°C in a heat block to denature protected RNA probe and cool immediately on ice.

Gel Resolution of Protected Probes

1. Clean a set of gel plates (>40 cm in length, 0.5 mm spacers). Siliconize short plate. Assemble gel plates.
2. Combine the following to give a final concentration of 4.75% acrylamide (74.5 ml acrylamide solution at final ratio 19:1 acrylamide/bis):

Weigh:	35.82 g of urea
Add:	22.35 ml dH_2O 7.45 ml of 10× TBE
Heat:	~ 42°C to dissolve urea
Add:	8.85 ml of 40% acrylamide 9.31 ml of 2% bis acrylamide
Filter:	Using 0.4 – 0.8 µm filter
Add:	450 µl ammonium persulfate (10%) 60 µl TEMED

Note: Use recommended acrylamide concentration and acrylamide: bis ratio. It is critical for the correct resolution of unprotected and protected probe bands.

3. Mix acrylamide solution well, pour immediately into the gel cast removing any air bubbles that may form, and insert comb (we recommend using 0.5 cm width well; do not use sharkstooth comb).
4. After polymerization (about 1 h), place gel into a vertical gel rig that has a heat dispenser and add 0.5 × TBE running buffer into upper and lower reservoirs. Remove the gel comb and flush the wells thoroughly with 0.5 × TBE. Pre-run gel at 55 watts constant power for 45 min before loading samples.
5. Flush the wells with running buffer just prior to loading samples. Load samples and controls (including biotin-labeled probe, diluted with loading buffer to appropriate concentration based on titration of probe yield). Run the gel at 55 watts constant power until the leading edge of the Bromophenol Blue (BPB, front dye) reaches 30 cm from the bottom of the well.

Semi-dry Electrotransfer to Nylon Membrane

1. Disassemble gel mold and remove the short plate. Trim the gel, removing excess unused gel (see ***Figure 3***). Cut filter papers and transfer membrane to the same dimensions as the processed gel.

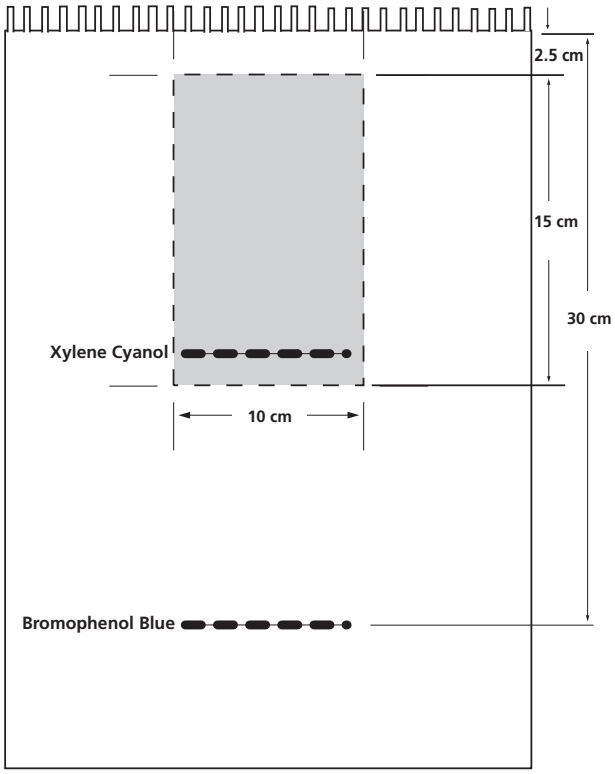

Figure 3. Trim Gel Prior to Transfer

2. Semi-dry electrotransfer is accomplished by creating a "sandwich" in which the gel and transfer membrane are enclosed by buffer-soaked filter papers as shown in ***Figure 4***. Absorb one sheet of filter paper onto the gel. On the semi-dry blotting apparatus, lay 3 pieces of 0.5× TBE saturated filter paper and place one sheet of buffer saturated positively- charged nylon membrane on top. Carefully lift filter-absorbed gel from the glass plate and place gel side down onto nylon membrane (gel is placed on top of nylon membrane). Add an additional 3 pieces of buffer saturated filter paper. Remove excess liquid and any air bubbles that may be trapped between filter papers, membrane, and gel using a pipette as a "rolling pin".

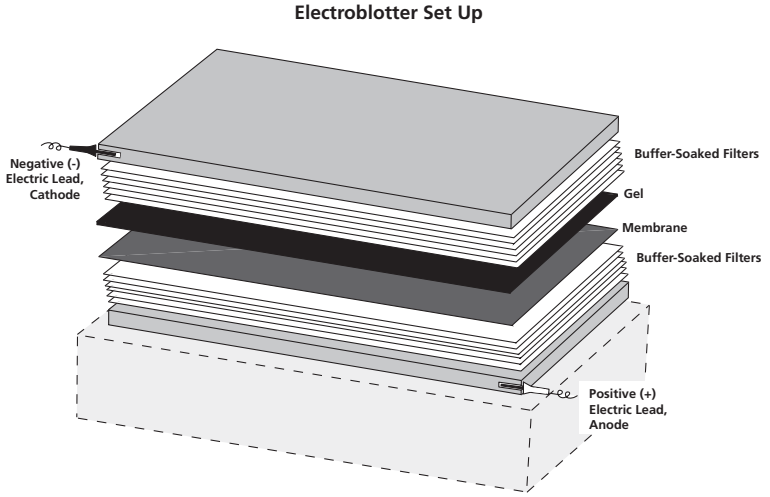

Figure 4. Electroblotter Set Up

3. Position the cathode electrode (black top) onto the completed stack. The red anode outlet will fit through the slot on one side of the black cover. After the negative and positive leads have been inserted, plug the leads into the power supply. Transfer protected labeled probe at 100 mA constant for 15–20 min.
4. After transfer is complete, carefully remove the black cover. Using smooth forceps, remove filter papers and gel slab and discard. Transfer the wet membrane to a clean, plastic container for UV crosslinking.

UV Crosslinking

1. Immobilized protected RNA probes are crosslinked to the wet membrane by exposing to UV light (UVP, mineralight lamp, model R-52G, 115V, 0.9 Amps) irradiated from 12 cm directly above the membrane for 5 min. Following UV-crosslinking, membrane should be completely air dried before developing. Membrane can be wrapped in plastic wrap for storage, but immediate signal development is recommended.

Chemiluminescent Probe Detection

Note: Use care in handling nylon membrane. Handle membrane with forceps cleaned with ethanol after each step. Wash containers should be changed before each new step. Avoid sodium azide in any solutions which may contact HRP as this may inactivate the HRP.

1. Add Membrane Blocking Buffer (Part No. 51-4562KC) to the membrane at >0.25 ml Blocking Buffer per cm^2 of membrane. Incubate for 15 min at RT with gentle agitation.
2. Add stabilized Streptavidin–HRP (Part No. 51-4528KC) into Membrane

Blocking Buffer at a 1:300 final dilution (100 µl Streptavidin-HRP to 30 ml Blocking buffer). Ensure conjugate does not directly contact membrane by tilting container and adding Sav-HRP to buffer at edge and mixing well. Incubate for 15 min at RT with gentle agitation.

3. Dilute Wash Buffer (4×) (Part No. 51-4563KC) to 1× with sterile dH_2O. Wash the membrane four times for 5 min per wash with ~35 ml 1× Wash Buffer at RT with gentle agitation. Discard wash buffer between washes. After final wash, briefly drain membrane and place into a clean container for the next step.
4. Add Substrate Equilibration Buffer (Part No. 51-4564KC) at 0.25 ml of buffer per cm^2 of membrane and incubate for 10 min at RT with gentle agitation.
5. Prepare the Substrate Working Solution by mixing equal volumes of the Stable Peroxide Solution (Part No. 51-4531KC) and Luminol/Enhancer (Part No. 51-4532KC). Prepare enough solution to completely cover the membrane (approximately 0.1 ml/cm^2, ~ 12 ml total). This working solution is stable for only 6 hr at RT after mixing.

   Note: Use a different pipette for each of the two solutions used to prepare the Working Solution.
6. Transfer the moist membrane onto clean plastic container and cover with Substrate Working Solution (0.1 ml/cm^2 membrane), making sure that the entire membrane is covered with the substrate. Incubate for 10 min at RT.

   Note: Once membrane is covered with working solution, never expose any part of the membrane to air as that may cause background.
7. Pour off excess substrate. Prior to exposing the film to the membrane, cover membrane by placing in a plastic sheet protector. Ensure there are no trapped air bubbles by gently lowering the top sheet of plastic protector from one edge to the opposite edge of the upward surface of the membrane to remove any bubbles present under the sheet and to create a liquid seal around the membrane.

   Note: Wiping the outside of the sheet protector with paper towel or wipes may cause static and therefore background.
8. Place the covered membrane into an X-ray film cassette and expose to film (we recommend Hyperfilm ECL, Amersham) for a period necessary to detect a clear signal with low background (30 sec–5 min).

Non-Rad Troubleshooting

(See also ***Troubleshooting for Radiolabeled Probe RPA, page 206***)

Probe Synthesis

1. Use new sterile disposable plasticware (pipette tips, Eppendorf tubes, etc.)
2. Use sterile DEPC-treated solutions.
3. Careless removal of ethanol from the precipitated probe can lead to significant losses. Glycogen is included as a carrier to facilitate probe precipitation.
4. Sometimes it is difficult to precipitate short, labeled probe quantitatively. Make sure that LiCl, glycogen and cold ethanol are mixed thoroughly with the reaction mix.
5. Wear powder-free gloves when setting up the reactions.
6. Using a beta box for probe precipitation in –80°C freezer will maintain temperature during transport to centrifuge. Otherwise, transport on dry ice.

Transfer To Nylon Membrane

1. Avoid touching the membrane with fingers (gloved or ungloved). Use ethanol-washed, blunt-ended forceps to pick up membrane. Handle membrane only at the edges.
2. The nylon membrane requires no pretreatment; simply wet in 0.5× TBE and put it into transfer "sandwich."
3. The transfer time can vary depending on current, gel thickness, percent acrylamide, and the molecular weight of the RNA. In this non-rad RPA protocol, 15 – 30 min. will be sufficient to transfer probes and protected probes (100 – 500 bases) to membrane.
4. Semi-dry electroblotting is more likely to allow the sample to move through and out of the membrane, therefore, longer transfer time is not recommended.
5. For best detection results, mark the side of the membrane exposed to the gel with a pen and always place this side up in tray.

Detection

1. To reduce the background, use freshly washed trays for each step and shake membrane throughout entire detection procedure.
2. The blocking and washing steps can be prolonged, but do not prolong the streptavidin reaction.
3. Spotty, mottled background may be caused by an unsuitable membrane, or a precipitate in the streptavidin preparation. Use the membrane supplied by BD Biosciences Pharmingen. Centrifuge the streptavidin for 1 min. before removing an aliquot for dilution.

4. The stabilized Streptavidin-HRP conjugate binds to biotin very quickly. Add stabilized Streptavidin-HRP conjugate to the corner of the tray and quickly mix with the whole blocking solution to avoid over-concentrated conjugate on some parts of the membrane; do not add it directly to the surface of the membrane.
5. When the signal is missing in a specific area, this may indicate that a bubble occurred between the membrane and the gel during the transfer. Remove any air bubbles between the gel and the membrane by rolling a clean pipette gently across the "sandwich" before electroblotting.
6. Irregular smears of background may be caused by non-uniform distribution of chemiluminescent substrate during chemiluminescent detection. This may occur if the membrane is not shaken during detection procedure or if certain parts of the membrane become dry.
7. When exposing the film to the membrane, place the membrane in a plastic sheet protector after substrate reaction. Gently lower the top sheet of plastic protector from one edge to the opposite edge of the upward surface of membrane to remove any bubbles present under the sheet and to create a liquid seal around the membrane.
8. Spots on the exposed ECL film, both outside and within the signal area, can be caused by electrostatic charge on the plastic sheet protector. Wear gloves and touch the membrane only at the edges with a clean forceps. Never press or wipe the plastic protector containing the membrane.
9. Inadequate sensitivity or high background may be caused by underexposure or overexposure. Adjust the exposure time.

Background Bands

1. Optimizing Proteinase K treatment of the completed RNase digestion can enhance the sensitivity and reduce background.
2. Titrate amount of probe used for hybridization to minimize background and maximize signal.

BD RiboScreen™ Human–1 Membranes

Through the application of large numbers of different DNA probes or complementary oligonucleotides on membranes or glass substrates, DNA arrays provide a powerful method enabling the expression analysis of multiple genes in parallel.

The BD RiboScreen™ Human-1 Membrane is a discovery tool which is a membrane array with 289 different human genes and allows the simultaneous monitoring of a wide spectrum of gene expression patterns from a single experimental sample. All of the genes represented on our BD RiboScreen membrane can be found in the BD RiboQuant RNase Protection Assay System. Thus, the BD RiboScreen membrane can be used as a stand alone system or as a prescreening tool in conjunction with BD RiboQuant RPA. Together these products provide gene expression screening and a highly specific quantitation system.

The BD RiboScreen membrane is a positively-charged nylon membrane with dimensions of 7.6 cm × 11.4 cm. Genes are deposited in a 3 × 3 double offset pattern that results in duplicates of 3 discrete genes and a reference ubiquitous housekeeping gene, L32. BD RiboScreen Human–1 Membranes are ready for use by hybridization with labeled first-strand cDNA probes synthesized from sample poly(A)$^+$ RNA. The signal strength corresponding to the mRNA gene expression patterns represented on the membrane can be visualized using autoradiography or phosphorimaging. Two membranes are supplied per package to allow for duplicate or parallel hybridizations, as required for comparative analysis of gene expression patterns in response to experimental conditions.

Use the BD Riboscreen membrane system to detect differential gene expression among various treatment conditions, tissue types, pathologies or genetic manipulations. BD Riboscreen membranes allow the screening of representative genes that participate in the following diverse cellular functions:

- Apoptosis-Related Molecules
- Cell Cycle Regulators and Origin Recognition Complex Molecules
- Clotting Factors and Angiogenesis-Regulating Molecules
- Cytokines, Cytokine Receptors, and Cell Surface Antigens
- Developmental Genes and Fibroblast Growth Factors
- Glucose Transporters
- G-Protein Coupled Receptors and Orphan Receptors

Comparative Analysis Using Human-1 Membranes

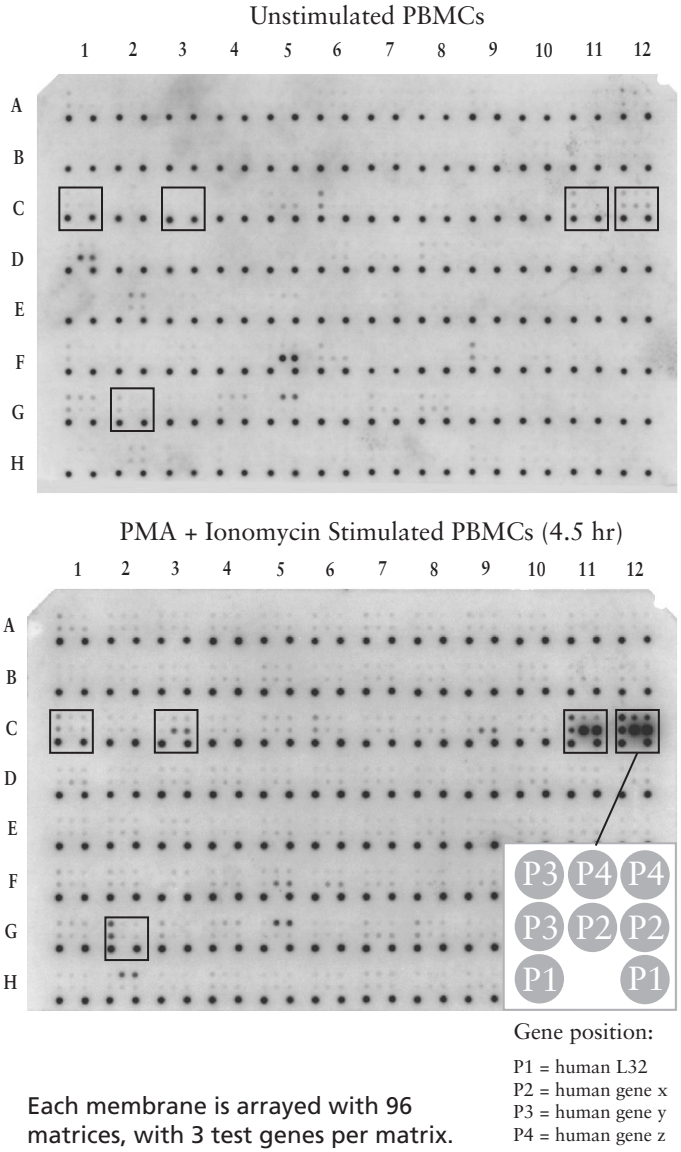

Figure 5. Gene expression analysis using BD RiboScreen Human–1 Membranes (Cat No. 559436). α-^{32}P–labeled cDNA probes were synthesized from 1.5 µg each of poly-(A)$^+$ RNA isolated from unstimulated peripheral blood mononuclear cells (PBMCs) and PBMCs stimulated with PMA/Ionomycin for 4.5 hr. PMA/Ionomycin treatment stimulates and represses the expression of various genes.

BD RiboQuant™ Multi-Probe Ribonuclease Protection Assay Template Sets

For a complete listing of available template sets and Control RNA's, please refer to the ***BD RiboQuant™ Multi-Probe RNase Protection Assay System Instruction Manual, 7th Ed.*** or visit our website at **www.bdbiosciences.com**

Description	Templates	Cat. No.
Human Cytokines/Chemokines		
hCK-1	IL-5, IL-4, IL-10, IL-14, IL-15, IL-9, IL-2, IL-13, IFN-g, L32, GAPDH	556151
hCK-2b	IL-12p35, IL-12p40, IL-10, IL-1α, IL-1β, IL-Rα, IL-6, IGIF, IFN-γ, L32, GAPDH	559242
hCK-3	LT-α, LTβ, TNF, IFN-γ, TGFβ3, TGFβ2, TGFβ1, L32, GAPDH	556153
hCK-4	IL-3, IL-7, GM-CSF, M-CSF, G-CSF, IL-6, LIF, SCF, OSM, L32, GAPDH	556154
hCK-5	Ltn, RANTES, IP-10, MIP-1β, MIP-1α, MCP-1, IL-8, I-309, L32, GAPDH	556155
hCK-8	Eotaxin, RANTES, MCP-4, MIP-1β, MCP-2, MIP-1α, MCP-1, MCP-3, I-309, HCC-1, L32, GAPDH	551787
hCK-9	TECK, MPIF-1, MDC, SLC, MIP-3α, MIP-3β, PARC, TARC, HCC-4, HCC-2, L32, GAPDH	551488
Mouse Cytokines/Chemokines		
mCK-1	IL-4, IL-5, IL-10, IL-13, IL-15, IL-9, IL-2, IL-6, IFN-γ, L32, GAPDH	556121
mCK-1b	IL-4, IL-5, IL-10, IL-13, IL-15, IL-9, IL-2, IL-3, IFN-γ, L32, GAPDH	556157
mCK-2b	IL-12p35, IL-12p40, IL-10, IL-1α, IL-1β, IL-1Rα, IL-18/IGIF, IL-6, IFN-γ, MIF, L32, GAPDH	556156
mCK-3b	LT-α, LT-β, TNF, IL-6, IFN-γ, IFN-β, TGF-β1, TGF-β2, TGF-β3, MIF, L32, GAPDH	556158
mCK-4	IL-3, IL-11, IL-7, GM-CSF, M-CSF, G-CSF, LIF, IL-6, SCF, L32, GAPDH	556145
mCK-5c	Ltn, RANTES, MIP-1β, MIP-1α, MIP-2, IP-10, MCP-1, TCA-3, Eotaxin, L32, GAPDH	551943
Rat and Pig Cytokines/Chemokines		
rCK-1	IL-1α, IL-1β, LT-α, IL-3, IL-4, IL-5, IL-6, IL-10, TNF, IL-2, IFN-γ, L32, GAPDH	556147
rCK-2	IL-1α, IL-1β, IL-1Rα, IL-12p40, IL-18/IGIF, IL-6, IL-10, MIF, IFN-γ, L32, GAPDH	556244
rCK-3	IFN-β, LT-α, GM-CSF, TGF-β1, TGF-β3, TGF-β2, LTβ, TNFα, MIF, IFN-γ, L32, GAPDH	556245
pCK-1	IL-4, IL-10, IL-15, IL-2, IL-6, IFN-γ, L32, GAPDH	557062
pCK-2	IL-12p35, IL-12p40, IL-10, IL-1α, IL-1β, IL-18, IL-6, IFN-γ, L32, GAPDH	557063
pCK-3	LT-α, TNF, IFN-β, TGF-β2, IL-6, IFN-γ, L32, GAPDH	557350
pCK-4	AMCF-11, GM-CSF, G-CSF, MCP-1, MCP-2, IL-8, RANTES, L32, GAPDH	557468

BD RiboQuant Multi-Probe Ribonuclease Protection Assay Template Sets

Description	Templates	Cat. No.
Human Cytokine Receptors		
hCR-1b	IL-13Rα1, IL-7Rα, IL-9Rα, IL-13Rα, IL-15Rα, IL-4Rα, γc, IL-2Rβ, IL-2Rα, L32, GAPDH	557197
hCR-2	IL-5Rα, IL-3Rα, GM-CSFRα, βc, IL-6Rα, gp130, LIFRα, G-CSFR, L32, GAPDH	556203
hCR-3b	IL-10R2, IL-10R, IL-11R, IL-12Rβ1, IL-12Rβ2, IL-6Rα, gp130, IFN-γRα, IFN-γRβ, L32, GAPDH	558833
hCR-4	IL-1RI, IL-1RII, TNFRp75, TNFRp55, IL-6Rα, gp130, TGF-βRI, TGF-βRII, L32, GAPDH	556205
hCR-5	CCR1, CCR3, CCR4, CCR5, CCR8, CCR2a+b, CCR2a, CCR2b, L32, GAPDH	556206
hCR-6	CXCR1, CXCR2, CXCR3, CXCR4, BLR-1, CCR7, V28, L32, GAPDH	556207
hCR-7	CCR5, L32, GAPDH	556208
hCR-8	STRL33, US28, CCR3, CCR5, CXCR4, CCR8, GPR15, GPR1, V28, CCR2b, L32, GAPDH	556210
Mouse Cytokine Receptors		
mCR-1	IL-7Rα, IL-9Rα, IL-13Rα, IL-15Rα, IL-4Rα, γc, IL-2Rβ, IL-2Rα, L32, GAPDH	556196
mCR-2	IL-5Rα, AIC2A, GM-CSFRα, IL-3Rα, IL-6Rα, gp130, AIC2B, LIFRα, G-CSFR, L32, GAPDH	556197
mCR-3	IL-10R, IL-11R, IL-12Rβ1, IL-12Rβ2, IL-6Rα, gp130, IFN-γRα, IFN-γRβ, L32, GAPDH	556198
mCR-4	IL-1RI, IL-1RII, TNFRp75, TNFRp55, IL-6Rα, gp130, IFN-γRα, IFN-γRβ, L32, GAPDH	556199
mCR-5	CCR1, CCR1b, CCR3, CCR4, CCR5, CCR2, L32, GAPDH	556200
mCR-6	CXCR2, CXCR4, BLR-1, L32, GAPDH	556201
Human and Mouse Cell Surface Antigens		
hCD-1	TCRδ, TCRα, CD3ε, CD4, CD8α, CD8β, CD19, CD14, CD45, L32, GAPDH	556230
mCD-1	TCRδ, TCRα, CD3ε, CD4, CD8α, CD8β, CD19, F4/80, CD45, L32, GAPDH	556228
Human Apoptosis		
hAPO-1	caspase-8, Granzyme B, caspase-3, caspase-6, caspase-5, caspase-2 (S), caspase-7, caspase-1, caspase-2 (L), caspase-9, L32, GAPDH	556209
hAPO1c	caspase-8, caspase-4, caspase-3, caspase-6, caspase-10a, caspase-5, caspase-2 (S), caspase-7, caspase-1, caspase-2 (L), caspase-9, L32, GAPDH	556233
hAPO-2b	bcl-w, bcl-x (L), bcl-x (S), bfl-1, BID, bik, bak, bax, bcl-2, mcl-1, L32, GAPDH	556240
hAPO-2c	bcl-w, bcl-x (L), bcl-x (S), bfl-1, bad, bik, bak, bax, bcl-2, mcl-1, L32, GAPDH	556163
hAPO-3	caspase-8, FasL, Fas, FADD, DR3, FAP, FAF, TRAIL, TNFRp55, TRADD, RIP, L32, GAPDH	556163
hAPO-3b	caspase-8, FasL, Fas, CLARP, FAP, CRADD, DAXX, MADD, RIP, L32, GAPDH	556237
hAPO-3d	caspase-8, FasL, Fas, DcR-1, DR3, DR5, DR4, TRAIL, DcR-2, TNFRp55, TRADD, RIP, L32, GAPDH	557278
hAPO-4	Granzyme A, Granzyme B, DAD1, FAST K, Granzyme H, RVP1, Dr-nm23, Granzyme 3, Requiem, CAS, Perforin, L32, GAPDH	556164
hAPO-5	XIAP, TRAF1, TRAF2, TRAF4, NAIP, c-IAP-2, c-IAP-1, TRPM-2, TRAF3, L32, GAPDH	556165
hAPO-5b	TRAF1, TRAF2, TRAF4, I-TRAF, TRAF5, TRAF6, TRAF3, TRIP, L32, GAPDH	556236
hAPO-5c	XIAP, Survivin, NAIP, c-IAP-2, c-IAP-1, TRPM-2, L32, GAPDH	556239
hAPO-6	IPL, ASK1, Harakiri, SIAH, DFF, Nip2, Nip3, Nip1, DAP-K, DAP, DRM, L32, GAPDH	556238

BD RiboQuant Multi-Probe Ribonuclease Protection Assay Template Sets

Description	Templates	Cat. No.
Mouse and Rat Apoptosis		
mAPO-1	caspase-8, caspase-3, caspase-6, caspase-11, caspase-12, caspase-2 (L), caspase-7, caspase-1, caspase-14, caspase-2 (S), L32, GAPDH	556195
mAPO-2	bcl-w, bfl1, bcl-x (L), bcl-x (S), bak, bax, bcl2, bad, L32, GAPDH	556191
mAPO-3	caspase-8, FasL, Fas, FADD, FAP, FAF, TRAIL, TNFRp55, TRADD, RIP, L32, GAPDH	556192
r-APO-1	FAS, bcl-x (L),bcl-x (S), FasL, caspase-1, caspase-3, caspase-2, bax, bcl-2, L32, GAPDH	556227
Human Cell Cycle Regulators		
hCC-1	Cdk1, Cdk2, Cdk3, Cdk4, p27, p21, PISSLRE, p16, L32, GAPDH	556159
hCC-2	p130, Rb, p107, p53, p57, p27, p21, p19, p18, p16, p14/15, L32, GAPDH	556160
hCYC-1	Cyclin A, Cyclin B, Cyclin C, Cyclin D1, Cyclin D2, Cyclin D3, Cyclin A1, L32, GAPDH	556189
hCYC-2	Cyclin E, Cyclin F, Cyclin G1, Cyclin G2, Cyclin I, Cyclin H, L32, GAPDH	556190
hStress-1	bcl-x, p53, GADD45, c-fos, p21, bax, bcl-2, mcl-1, L32, GAPDH	556188
hTS-1	p130, Rb, p107, DP1, DP2, E2F1, E2F2, E2F4, L32, GAPDH	556161
Mouse Cell Cycle Regulators		
mCC-1	Cdk1, Cdk2, Cdk4, Cdk5, Cdk7, Cdk8, KKLIARE, PCTAIRE3, PCTAIRE1, PITAIRE/CHED, PITALRE, PITSLRE, L32, GAPDH	559540
mCYC-1	Cyclin A2, Cyclin B1, Cyclin C, Cyclin D1, Cyclin D2, Cyclin D3, Cyclin A1, Cyclin B2, L32, GAPDH	556241
mCYC-2	Cyclin E, Cyclin F, Cyclin G1, Cyclin G2, Cyclin I, Cyclin H, L32, GAPDH	556242
Mouse Complement		
mComplement	C3aR, C5aR, C1qRp, Factor B, C1q-A chain, C3, Factor D, Crry, L32, GAPDH	551490
Human and Mouse Proto-Oncogenes		
hMyc	mad3, rox, N-myc, L-myc, mad4, max, mad, mixi1, sin3, c-myc, L32, GAPDH	551984
mMyc	Sin3, c-myc, N-myc, L-myc, b-myc, max, mad, mxi, mad3, mad4, mnt, L32, GAPDH	556193
mFos/Jun	c-jun, jun-B, jun-D, c-fos, fos B, fra-1, fra-2, L32, GAPDH	556194
Human Fibroblast Growth Factors		
hFGF-1	FGF-1, FGF-2, FGF-10, FGF-11, FHF-2, FHF-4, L32, GAPDH	556243
Human and Mouse Angiogenesis		
hAngio-1	flt1, flt4, TIE, Thrombin Rec., TIE2, CD31, Endoglin, Angiopoietin, VEGF, VEGF-C, L32, GAPDH	556232
hAngio-2	Endothelin RA, Endothelin RB, Endothelin RB-Like, Thrombin Rec., PAR2, PAR3, PAFR, H963-PAFR, PAFR-like, L32, GAPDH	556851
hAngio-3	EDG1, EDG2, EDG3, GPR41, KIAA0001, FEG1, L32, GAPDH	559900
mAngio-1	flt1, flt4, TIE, TIE2, Thrombin Rec., CD31, VEGF-C, Endoglin, VEGF, Angiopoietin-1, L32, GAPDH	551418

BD RiboQuant Multi-Probe Ribonuclease Protection Assay Template Sets

Description	Templates	Cat. No.
Rat Neutrophins		
rNT-1	bNGF, BDNF, GDNF, CNTF, NT3, NT4, L32, GAPDH	556148
Human Signal Transduction and Transcriptional Activators		
hMAPK	ERK1, ERK2, ERK3, ERK3 rel, ERK5/ERK4, ERK6, p38, p38b, p38d, L32, GAPDH	559471
hMKK	MEK1, MEK2, MEK3, MEK4 (JNKK1), MEK5, MKK6 (SAPKK3), MKK7 (JNKK2), L32, GAPDH	559953
hMKKK	MAPKAPK2, MAPKAPK3, MAPKAPK5, MEKK3, MTK1/MEKK4, MYK5/ASK, L32, GAPDH	559870
hSmad	Smad1, Smad2, Smad3, Smad4, Smad5, Smad6, Smad7, Smad8, L32, GAPDH	559646
hSOCS	CIS, SOCS7, SOCS6, SOCS5, SOCS3, SOCS2, SOCS1, L32, GAPDH	559927
hSTAT	Stat1, Stat2, Stat3, Stat4, Stat5A, Stat5B, Stat6, L32, GAPDH	558834
Human and Mouse Matrix Metalloproteinases		
hMMP-1	MMP8, MMP12, TIMP3, MMP1, MMP2, MMP7, MMP9, MMP13, TIMP2, L32, GAPDH	551274
hMMP-2	MMP8, MMP12, TIMP3, MMP1, MMP2, MMP3, MMP14, TIMP1, TIMP2, L32, GAPDH	551275
mMMP-1	MMP1, MMP2, MMP3, MMP9, MMP8, TIMP4, TIMP3, TIMP1, TIMP2, L32, GAPDH	551276
mMMP-2	MMP1, MMP12, MMP3, MMP9, MMP8, MMP7, TIMP3, TIMP1, TIMP2, L32, GAPDH	551277
Human Integrins		
hITG-1	ITGA1, ITGA2, ITGA3, ITGA4, ITGA5, ITGA6, ITGA7, ITGA8, ITGA9, ITGAv, ITGB1, L32, GAPDH	559928
hITG-2	ITGAe, ITGB4, ITGA4, ITGB5, ITGA6, ITGB6, ITGB7, ITGB8, ITGAv, L32, GAPDH	550355
mITG-3	ITGA2b, ITGAd, ITGAL, ITGAm, ITGAx, ITGB2, ITGB3, ITGB7, ITGB8, ITGAv, L32, GAPDH	550356
Human Toxicology		
hTox-1b	CYP2B6, NCPR, CYP2C8, CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP3A4, CYP2D6, L32, GAPDH	550793
hTox-2	FMO1, FMO2, FMO3, FMO4, FMO5, SULT2A1, SULT2B1, SULT1C1, SULT1A, L32, GAPDH	550794
hTox-3	UGT1A1, UGT1A7, UGT1A10, UGT1A9, UGT1A6, UGT1A8, UGT1A4, UGT2B4, UGT2B15, UGT2B7, L32, GAPDH	552134
hTox-4	GSTM3, GSTM2, GSTM1, GSTM1, GSTM4, GSTM5, GSTA4, GSTA2, GSTA3, L32, GAPDH	552135

BD RiboQuant Multi-Probe Ribonuclease Protection Assay Template Sets

Description	Templates	Cat. No.
Human Toll Like Receptors		
hTLR-1	TLR1, TLR2, TLR4, TLR5, TLR6, L32, GAPDH	551983
Human and Mouse T-cell Receptor		
hTCR Vβ-A	Vβ16 (14s1), Vβ18 (18s1), Vβ22 (2s1), Vβ5 (5s1), Vβ23 (13s1), Vβ6 (7s3), Vβ9 (3s1), C2	552583
hTCR Vβ-B	Vβ14 (27s1), Vβ21 (11s1), Vβ2 (20s1), Vβ13 (6s1), Vβ17 (19s1), Vβ8 (12s3), Vβ7 (4s1), C2	552586
hTCR Vβ-C	Vβ20 (30s1), Vβ3 (28s1), Vβ4 (29s1), Vβ11 (25s1), Vβ2 (10s1), Vβ15 (24s1), Vβ24 (15s1), Vβ1 (9s1), C2	552587
mTCR Vβ-A	Vβ2, Vβ11, Vβ16, Vβ12, Vβ1, Vβ4, C2, C1	552588
mTCR Vβ-B	Vβ5, Vβ14, Vβ3, Vβ20, Vβ6, Vβ9, Vβ7, C2, C1	552589
mTCR Vβ-C	Vβ18, Vβ5, Vβ13, Vβ8, Vβ10, C2, C1	552590

Notes

Chapter 12

Tools to Study the Complement System

Introduction

The Complement System is an integral part of the Innate and Adaptive Immune System.[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 ***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.[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.[1,2]

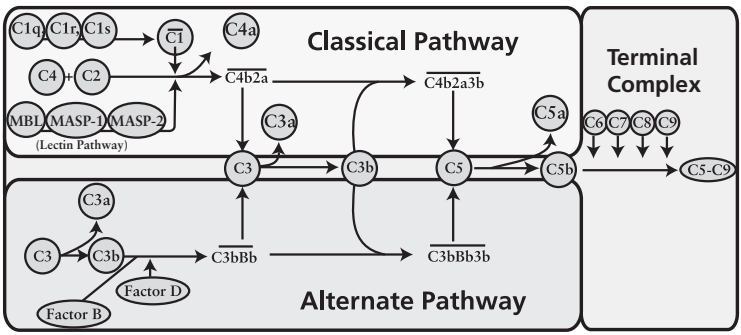

Figure 1. Overview of complement cascades.

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 Pharmingen recently-introduced C3a-desArg, C4a-desArg and C5a-desArg BD OptEIA™ Anaphylatoxin ELISA Kits (Cat. Nos. 550499, 550947 and 550500) and the Human Anaphylatoxin Cytometric Bead Array (CBA) 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. Using the BD OptEIA ELISA Kits allows the researcher a choice in assay development. The Human Anaphylatoxin CBA Kit provides a new platform to perform simultaneous, highly-sensitive measurements of human anaphylatoxins C3a-desArg, C4a-desArg and C5a-desArg from one small volume sample. For a general description of the OptEIA ELISA Kit protocol, please refer to ***Chapter 8*** 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.[9,10] 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 Pharmingen 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.[4,5,7] BD Biosciences Pharmingen 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, refer to the product listing at the end of this chapter. 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

1. 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]

2. Protocol

a. Reagents

1. Lyophilized samples of FUT-175 (Cat. No.552035). Each vial contains 5 mg FUT-175 (Futhan). Store lyophilized vials at 4ºC, protected from light and moisture. Prior to use, reconstitute FUT-175 (Futhan) with 1 ml dH_2O to get a 100× stock solution. After reconstitution with dH_2O, FUT-175 is stable for 4 weeks if stored at 4ºC and several months if it is stored at –80ºC.

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 ≤ –80ºC. 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 37ºC 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

Tube	Test Conditions	Typical C4a-desArg Values* (ng/ml)
A	No Futhan, 1 hour @ 0ºC	150–450
B	No Futhan, 1 hour @ 37ºC	8,000–12,000
C	50 µg/ml Futhan, 1 hour @ 0ºC	150–450
D	50 µg/ml Futhan, 1 hour @ 37ºC	150–450

*Based on donor to donor variation, and plasma collection/handling

3. Mix well and incubate samples A and C for 1 hour @ 0ºC, incubate samples B and D for 1 hour @ 37ºC.
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 –80ºC 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 8*** 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 7*** 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.

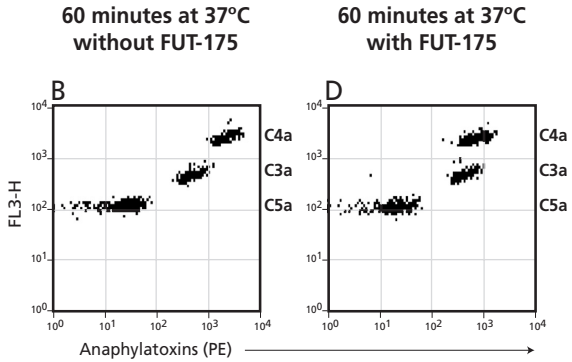

Figure 2. Anaphylatoxin levels measured by CBA analysis. Anaphylatoxin levels were measured using the BD CBA Human Anaphylatoxin Kit from samples B (left panel) and D (right panel) from the experimental setup described above (***Table 1, page 235***), using 1:1600 sample dilution. Bivariate dot plots (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:

Sample	C3a (ng/ml)	C4a (ng/ml)	C5a (ng/ml)
EDTA plasma @ 37°C for 60 min without FUT-175	448.6	>8,000.0	12.3
EDTA plasma @ 37°C for 60 min with FUT-175	339.5	1,446.0	13.3

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.

Helpful tips 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 dH_2O to get a 5 mg/ml = 100× stock solution. After reconstitution, FUT-175 is stable for 4 weeks if stored at 4°C and several months if stored at –80°C.

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.

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FUT-175 (Futhan)

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Complement System Related Products

Description	Other Names	Ligand	Clone	Isotype	Format	Cat. No.
Complement Receptors/membrane proteins						
Human						
C1qR(p)	CD93	C1q, SPA	R139	M IgG_{2b}	Purified	551087
					FITC	551531
					PE	551509
					NA/LE	552954
			R3	M IgM	Purified	551454
					Biotin	552117
C3aR	Anaphylatoxin C3a Receptor	C3a	8H1		Purified	557173
C5aR	CD88, Anaphylatoxin C5a Receptor	C5a, C5a-desArg	C85-4124	Rabbit IgG mAb	Purified	559159
			C85-2506.1	Rabbit IgG mAb	PE	552993
			D53-1473	M IgG_1	Purified	550733
					Purified	550493
					PE	550494
CR1	CD35, C3b/C4b receptor	C3b, C4b, iC3b	E11	M IgG_1	Purified	555451
					FITC	555452
					PE	559872
CR2	CD21, C3d receptor, Epstein-Barr virus receptor	iC3b, C3dg, C3d, EBV	1048	M IgG_1	Purified	552727
			B-ly4		Purified	555421
					PE	555422
					APC	559867
					PE-Cy5	551064
			B-ly4*		Purified	557327
CR3	CD11b/CD18, Mac-1, aMb2	iC3b, C3dg, C3d	ICRF44	M IgG_1	Purified	555386
					Biotin	555387
					PE	555388
					APC	550019
					PE-Cy5	555389
					NA/LE	555385
			ICRF44*		Purified	557320
					PE	557321
CR3/CR4	CD11b/CD18, & CD11c/CD18	iC3b, C3dg	L130	M IgG_1	Purified	556084
			6.7		Purified	555922
					FITC	555923
					PE	555924
					APC	551960
					PE-Cy5	557528
			6.7*		FITC	557156
					PE	557157
DAF	CD55, Decay Acceleration Factor huMACIF	Accelerates decay of C4b-C2a and C3b-Bb	IA10	M IgG_{2a}	Purified	555691
					Biotin	555692
					FITC	555693
					PE	555694
					APC	555696
					PE-Cy5	555695

* cross-reacts with non-human primates: clone Bly4 and clone ICRF44 cross-react with Baboon, Cynomolgus, and Rhesus, clone 6.7 cross-reacts with Cynomolgus and Rhesus.

Complement System Related Products

Description	Other Names	Ligand	Clone	Isotype	Format	Cat. No.
Human*(continued)*						
MCP	CD46, Membrane Cofactor Protein	Cofactor for Factor I-mediated cleavage of C3b & C4b	E4.3	M IgG_{2a}	Purified FITC	555948 555949
CD59	HRF20, MIRL protectin	Binds C8 & C9, blocking MAC assembly	P282(H19)	M IgG_{2a}	Purified Biotin FITC PE PE*	555761 555762 555763 555764 557141
Mouse						
C5aR	CD88 Anaphylatoxin C5a Receptor	C5a, C5a-desArg	Rabbit poly IgG		Purified	552837
ccry/p65			1F2	Rat IgG_{2a}	Purified Biotin	550058 550059
CR1	CD35/CD21b, Cr2-190	C3b, C4b	8C12	Rat IgG_{2a}	Purified Biotin	558768 553816
CR2/CR1	CD21/CD35		7G6	Rat IgG_{2b}	Purified FITC NA/LE	553817 553818 559831
CR3	CD11b/ CD18 Mac-1	iC3b, C3dg, C3d	M1/70	Rat IgG_{2b}	Purified Biotin FITC PE APC PerCP–Cy5.5 NA/LE	553308 553309 557396 557397 553312 550993 553307
CR3/CR4	CD11b/CD18 & CD11c/CD18	iC3b, C3dg, C3d	GAME-46	Rat IgG_1	Purified NA/LE	557440 555280
			C71/16	Rat IgG_{2a}	Biotin FITC PE	557439 553292 553293
CR4	CD11c/CD18, gp150,95	iC3b, C3dg	HL3	Ham IgG_1, λ	Purified Biotin FITC PE APC	553799 553800 553801 553802 550261
			M18/2	Rat IgG_{2a}	Purified NA/LE	557437 553341

* cross-reacts with non-human primates: clone Bly4 and clone ICRF44 cross-react with Baboon, Cynomolgus, and Rhesus, clone 6.7 cross-reacts with Cynomolgus and Rhesus.

Complement System Related Products

Description	Other Names	Ligand	Clone	Isotype	Format	Cat. No.
Rat						
C1qRp	CD93	C1q, MBL, SPA	LOV8	M IgG_1	Purified	552294
					PE	552295
ccry/p65	CD35/Crry/p65		512	M IgG_1	Purified	554991
CR3	CD11b/CD18, Mac-1, aMb2	iC3b, C3dg, C3d	OX-42	M IgG_{2a}	Purified	554859
					FITC	554861
					PE	554862
					NA/LE	554858
			WT.5	M IgA	Purified	554980
					Biotin	554981
					FITC	554982
CR3/CR4	CD11b/CD18 & CD11c/Cd18		WT.3	M IgG_1	Purified	554977
					FITC	554979
					NA/LE	554976
CD59	HRF20, MIRK, ratMACIF protectin	Binds C8 & C9, blocking MAC assembly	TH9	M IgG_1	Purified	special order*
					FITC	550976

Kits and Other Related Reagents

Description	Contains	Apps	Format	Size	Cat. No.
Cytometric Bead Arrays (CBA)					
Human					
Anaphylatoxin Kit	C3a, C4a, C5a	FCM	Kit	50 tests	552363

Description	Assay Range	Sensitivity	Apps	Size	Cat. No.
ELISA					
Human					
C3a	80-5000 pg/ml	7.3 pg/ml	ELISA	1 plate	550499
C4a	31.3-2000 pg/ml	6.2 pg/ml	ELISA	1 plate	550947
C5a	0.63-40 ng/ml	0.06 ng/ml	ELISA	1 plate	550500
FUT-175 (Futhan)		Sample Stabilizer		5 mg	552035

* cross-reacts with non-human primates: clone Bly4 and clone ICRF44 cross-react with Baboon, Cynomolgus, and Rhesus, clone 6.7 cross-reacts with Cynomolgus and Rhesus.

Notes

Chapter 13

Detection of *In Vivo* Cytokine Production with the *In Vivo* Capture Assay for Cytokines

Analysis of the types and levels of cytokines that are produced *in vivo* is often difficult. Many cytokines have short *in vivo* half-lives and do not accumulate to detectable levels as measured in serum samples. Cytokine mRNA accumulation by cells *in vivo* and cytokine protein secretion by cells restimulated *in vitro* are relatively easy to measure. However, mRNA levels and *in vitro* cytokine protein secretion may not fully reflect cytokine protein secretion *in vivo*.

The *In Vivo* Capture Assay for Cytokines, also known as Cincinnati Cytokine Capture Assay, more directly measures the *in vivo* production of cytokine proteins. In this assay, mice are injected with 10 µg of a no azide/low endotoxin (NA/LE), biotin-labeled antibody that binds to the targeted cytokine protein as it is secreted, forming a long-lived soluble complex that accumulates in the blood. Mice are bled and the complex is captured from serum and added to ELISA plate microwells coated with antibody that binds to a different epitope of the same cytokine. The complex is detected by streptavidin-horseradish peroxidase followed by the addition of a chromogenic substrate solution.

Compared to the standard ELISA detection of serum cytokines, the *In Vivo* Capture Assay for Cytokines often increases the sensitivity of detection 50 – 100-fold. The amount of cytokine detected is directly proportional to the amount secreted, regardless of the site of production. Because only a fraction of the secreted cytokine is captured by the injected NA/LE biotinylated antibody, the *In Vivo* Capture Assay for Cytokines does not inhibit cytokine-dependent processes. Thus, the *In Vivo* Capture Assay for Cytokines allows measurement of multiple cytokines secreted during the course of an immune response, without interfering with that response.

For more information please consult our Immune Function Homepage (**www.bdbiosciences.com/Immune_Function**) and the listed references.

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Notes

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