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.

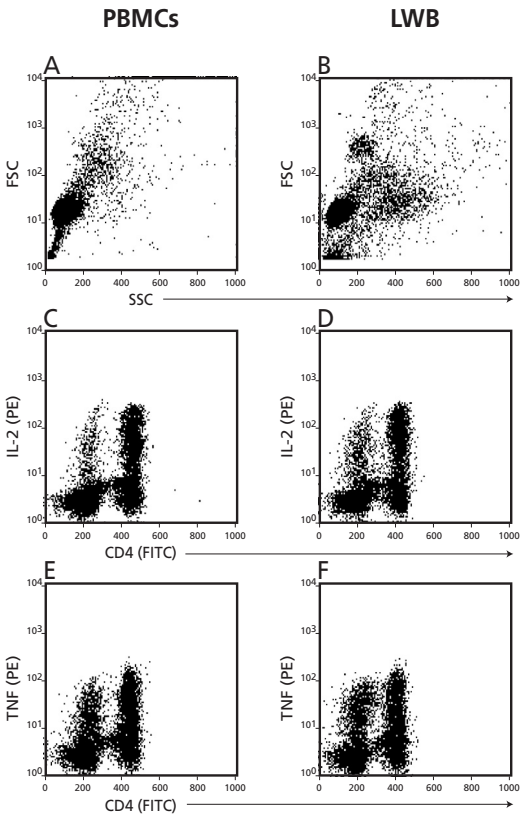


Figure 1. Analysis of IL-2 and TNF production in activated human PBMCs and lysed whole blood cells. Heparinized blood was either passed over Ficoll-Paque™ to isolate human PBMCs or treated with ammonium chloride buffer to lyse erythrocytes and obtain "lysed whole blood cells" (LWB). Both blood cell preparations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

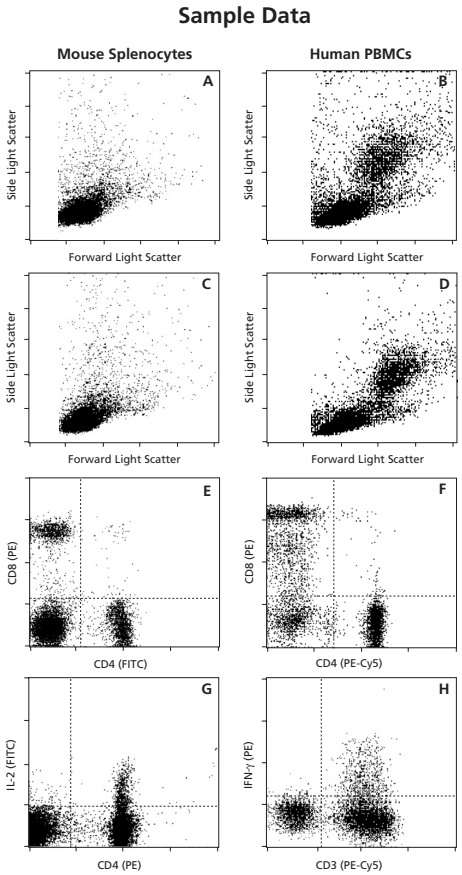


Figure 2. Effects of the BD Cytofix/Cytoperm Solution on cell light scattering properties, cell surface antigen staining, and intracellular cytokine staining. Panels A and B show the forward light scatter and side light scatter profiles for freshly-prepared, untreated mouse splenocytes and Ficoll-Hypaque-isolated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

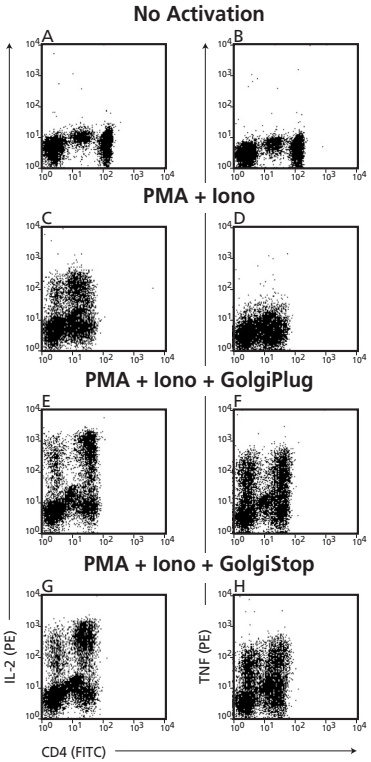


Figure 3. The effect of protein transport inhibitors on intracellular cytokine staining. Human PBMCs were isolated from heparinized whole blood by density centrifugation on Ficoll Paque™ and were either cultured for 4 hrs with no activators and no protein transport inhibitors (A, B) or were stimulated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

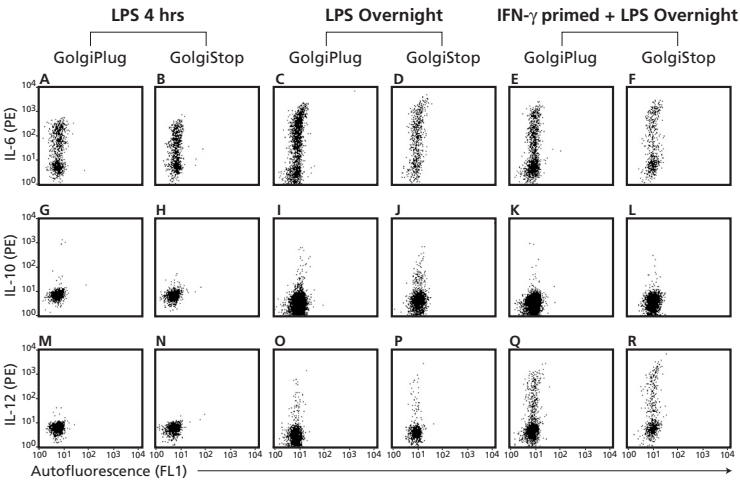


Figure 4. The effect of various activation conditions and various protein transport inhibitors on intracellular cytokine staining. Human PBMCs were isolated from heparinized whole blood by density gradient centrifugation (Ficoll-Paque™). The cells were stimulated with either LPS (1 µg/ml, Sigma, Cat.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

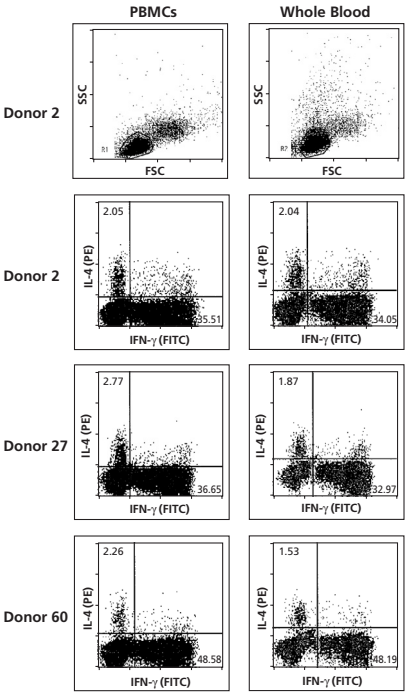
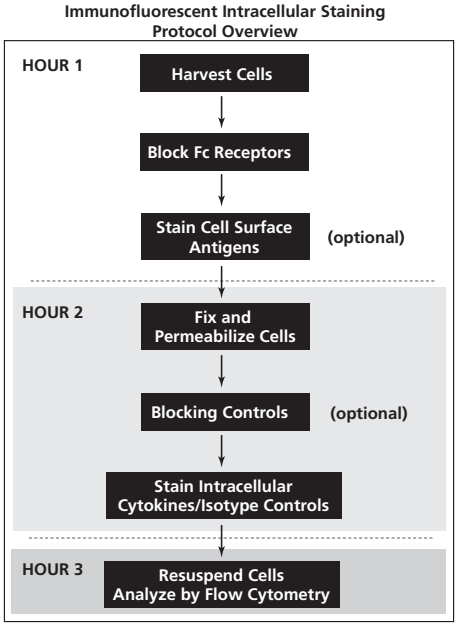


Figure 5. Frequencies of detectable cytokine-producing cells are comparable when staining activated PBMCs or activated whole blood from the same donor. Ficoll-Hypaque™ purified PBMCs (left panels) and whole blood (right panels) from each of the three donors were activated with PMA (50 ng/ml) and ionophore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

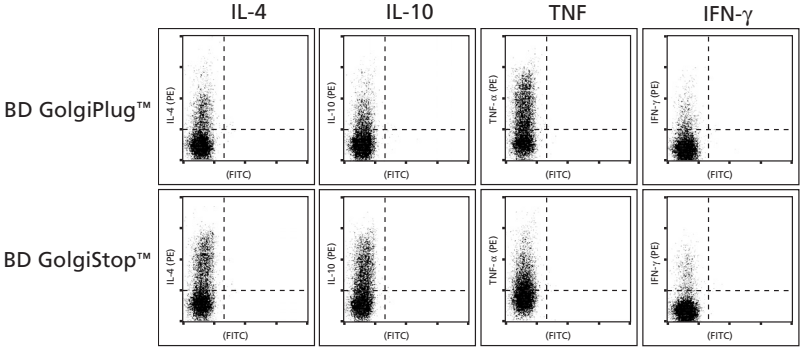
1. Thoroughly resuspend fixed/permeabilized cells in 50 µl of BD Perm/Wash Buffer (100 µl for staining in tubes) containing a pre-determined optimal concentration of a fluorescent anti-cytokine antibody or appropriate negative control. Incubate at 4°C for 15 – 30 min in the dark.
2. Wash cells 2 times 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).

References

1. Carter, L. L., and S. L. Swain. 1997. Single cell analyses of cytokine production. *Curr. Opin. Immunol.* 9:177.
2. Parks, D. R., L. A. Herzenberg, and L. A. Herzenberg. 1989. Flow cytometry and fluorescence-activated cell sorting. *In Fundamental Immunology, 2nd Edition.* W. E. Paul, ed. Raven Press Ltd., New York, p. 781-802.
3. Jung, T., U. Schauer, C. Heusser, C. Neumann and C. Rieger. 1993. Detection of intracellular cytokines by flow cytometry. *J. Immunol. Meth.* 159:197.
4. Vikingson, A., K. Pederson and D. Muller. 1994. Enumeration of IFN-γ producing lymphocytes by flow cytometry and correlation with quantitative measurement of IFN-γ. *J. Immunol. Meth.* 173:219.
5. Prussin, C. and D. Metcalfe. 1995. Detection of intracytoplasmic cytokine using flow cytometry and directly conjugated anti-cytokine antibodies. *J. Immunol. Meth*. 188: 117.
6. Elson, L. H., T. B. Nutman, D. D. Metcalfe and C. Prussin. 1995. Flow cytometric analysis for cytokine production identifies Th1, Th2, and Th0 cells within the human $CD4^+CD27^-$ lymphocyte subpopulation. *J. Immunol.* 154:4294.
7. Assenmacher, M., J. Schmitz and A. Radbruch. 1994. Flow cytometric determination of cytokines in activated murine T helper lymphocytes: Expression of interleukin-10 in interferon-γ and in interleukin-4-expressing cells. *Eur. J. Immunol.* 24:1097.
8. Picker, L. J., M. K. Singh, Z. Zdraveski, J. R. Treer, S. L. Waldrop, P. R. Bergstresser, and V. C. Maino. 1995. Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. *Blood* 86:1408.
9. Sallusto, F., C. R. Mackay, and A. Lanzavecchia. 1997. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. *Science* 277:2005.
10. Austrup, F., D. Vestweber, E. Borges, M. Löhning, R. Bräuer, U. Herz, H. Renz, R. Hallmann, A. Scheffold, A. Radbruch, and A. Hamann. 1997. P- and E-selectin mediate recruitment of T-helper-1 but not T-helper-2 cells into inflamed tissues. *Nature* 385:81.
11. Sander, B., J. Andersson and U. Andersson. 1991. Assessment of cytokines by immunofluorescence and the paraformaldehyde-saponin procedure. *Immunol. Rev.* 119:65.
12. Sander, B., I. Hoiden, U. Andersson, E. Moller, and J. Abrams. 1993. Similar frequencies and kinetics of cytokine producing cells in murine peripheral blood and spleen. *J. Immunol. Meth.* 166: 201.
13. Anderson, U. and J. Andersson. 1994. Immunolabelling of cytokine producing cells in tissues and suspension. In *Cytokine Producing Cells*, eds. D. Fradelizie and D. Emelie. INSERM, Paris. p. 32-49.
14. Ferrick, D. A., M. D. Schrenzel, T. Mulvania, B. Hsieh, W. G. Ferlin and H. Lepper. 1995. Differential production of interferon-γ and interleukin-4 in response to Th1- and Th2-stimulating pathogens by γδ T cells *in vivo*. *Nature* 373:255.
15. Sornasse, T., P. V. Larenas, K. A. Davis, J. E. de Vries, and H. Yssel. 1996. Differentiation and stability of T helper 1 and 2 cells derived from naive human neonatal $CD4^+$ T cells, analyzed at the single cell levels. *J. Exp. Med.* 184:473.
16. Andersson, S. J. and C. Coleclough. 1993. Regulation of CD4 and CD8 expression on mouse T cells. *J. Immunol.* 151: 5123.

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