APPLICATION NOTE

Cytokine Detection in Antigen-Activated $CD8^+$ and $CD4^+$ T Cells

IMMUNE FUNCTION

FastImmune Cytokine System

Introduction

The quantitative and qualitative measurement of antigen-specific T cells is important to 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 37ºC. After a stimulation period of 6 hours, EDTA is added to the sample in order to arrest activation and to remove adherent cells from the activation vessel. This step is followed by the simultaneous lysis of erythrocytes and fixation of leucocytes using BD 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.

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.

BD FastImmune CD8 cytokine four-color kit:

Anti-Hu–IFN-γ Kit (BD Catalog 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 Catalog 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

Table 1 Antigens in this assay

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.

BD FastImmune CD4 cytokine three-color kits:

Anti-Hu–IL-2 Kit (BD Catalog 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 Catalog 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 (10X)
- BD FACS Permeabilizing Solution 2 (10X)

Kit Working Solutions

- BD FastImmune Brefeldin A (BFA) Solution
  Upon receipt, thaw BFA, dispense into 10-µL aliquotes, and store at -20°C.

- BD FACS Lysing Solution
  Dilute 10X stock to 1X with deionized (DI) water. Store and use 1X solution at room temperature.

- BD FACS Permeabilizing Solution 2
  Dilute 10X stock to 1X with deionized water. Store and use 1X solution at room temperature.

  WARNING: BD FACS Lysing Solution (10X) and BD FACS Permeabilizing Solution 2 (10X) 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 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 Catalog No. 349502; unlabeled, FITC, and PE beads); CaliBRITE PerCP-Cy5.5–labeled beads (BD Catalog No. 345036; beads plus Bead Dilution Buffer); CaliBRITE APC beads (BD Catalog No. 340487, to support the FastImmune CD8 Kit only).
  Refer to the CaliBRITE beads product inserts for instructions.

- Software
  BD FACSComp™ software, version 4.2, for instrument setup and BD CeIIQuest™ 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 1X PBS (Store at 4°C.)

- 1% parafolmaldehyde in 1X PBS (Store at 4°C.)
  Refer to the paraformaldehyde product insert for warnings.
- 15-mL polypropylene tubes
  (BD Catalog No. 352096)

- 5-mL polystyrene tubes (BD Catalog No. 352058)

- micropipettor with tips (BD Electronic Pipette, BD Catalog No. 343246 or equivalent)
- vortex mixer

- 37°C water bath or incubator

- centrifuge

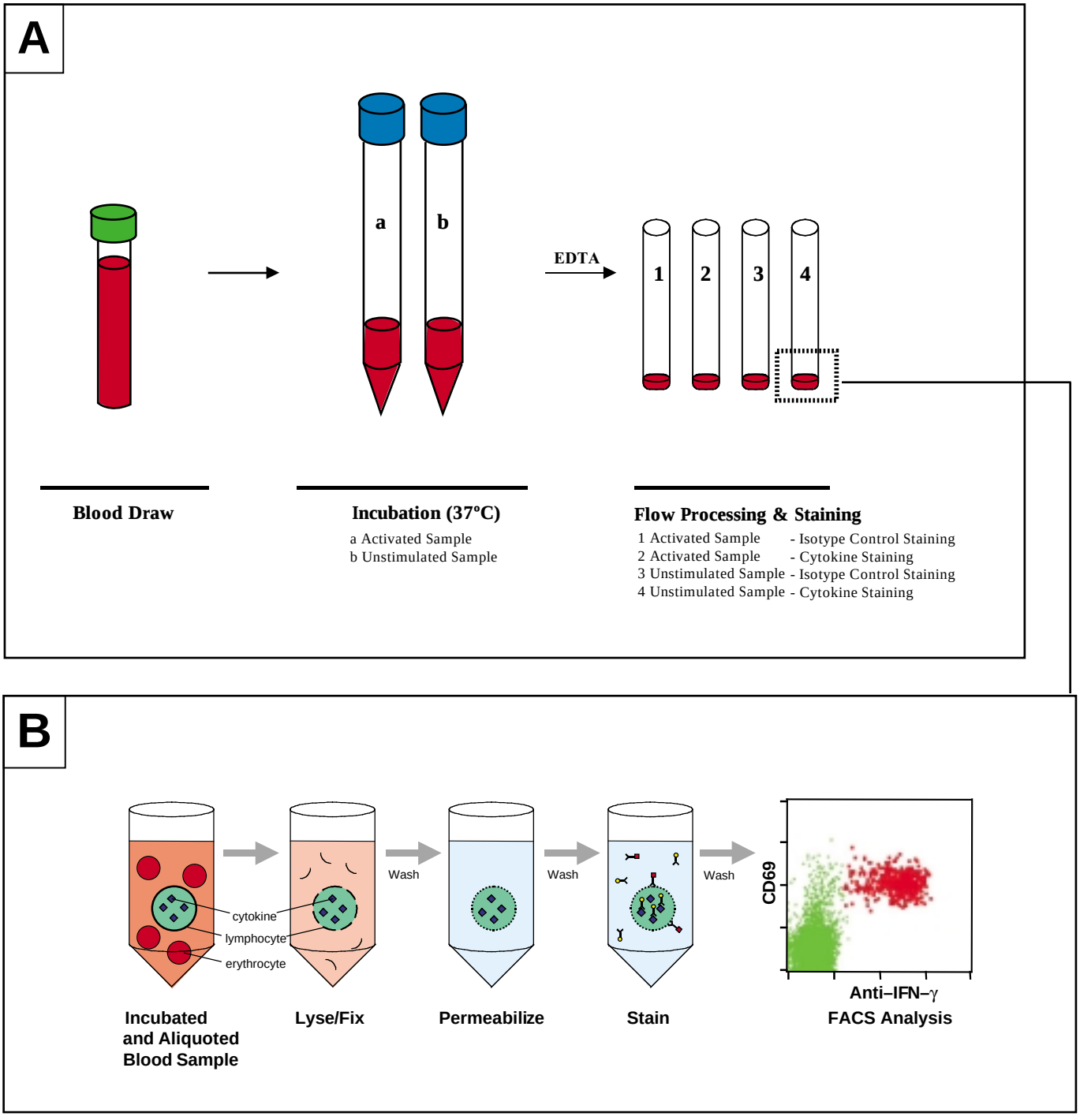


Figure 1 Schematic of whole blood FastImmune antigen-specific assay: Part A From blood draw to sample activation to flow cytometric sample processing; Part B Staining and processing of samples for flow cytometric analysis, applies to tubes 1 to 4 from Part A

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 1X BD FACS Lysing Solution (dilute 10X solution 1:10 with DI water before use) to each activated and unstimulated 0.5-mL whole blood sample.
- 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 x *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 1X BD FACS Lysing Solution (dilute 10X 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 x *g* for 5 minutes at room temperature. Decant the supernatant.

7. Add 0.5 mL of 1X BD FACS Permeabilizing Solution 2 (dilute 10X 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 x *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 x *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 18°C 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 x *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 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 $CD8^{dim}/CD3^{dim}$ cells for maximum detection of cytokine-positive events.

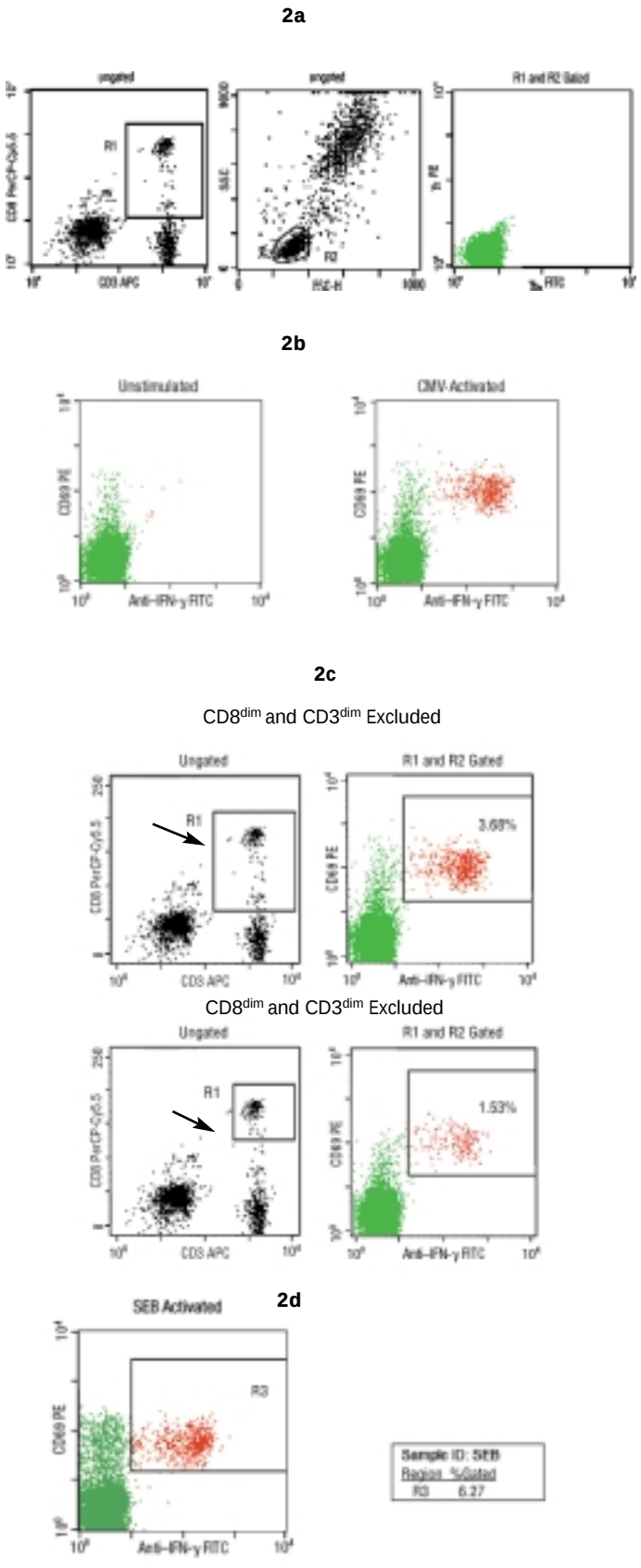


Figure 2

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

(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.

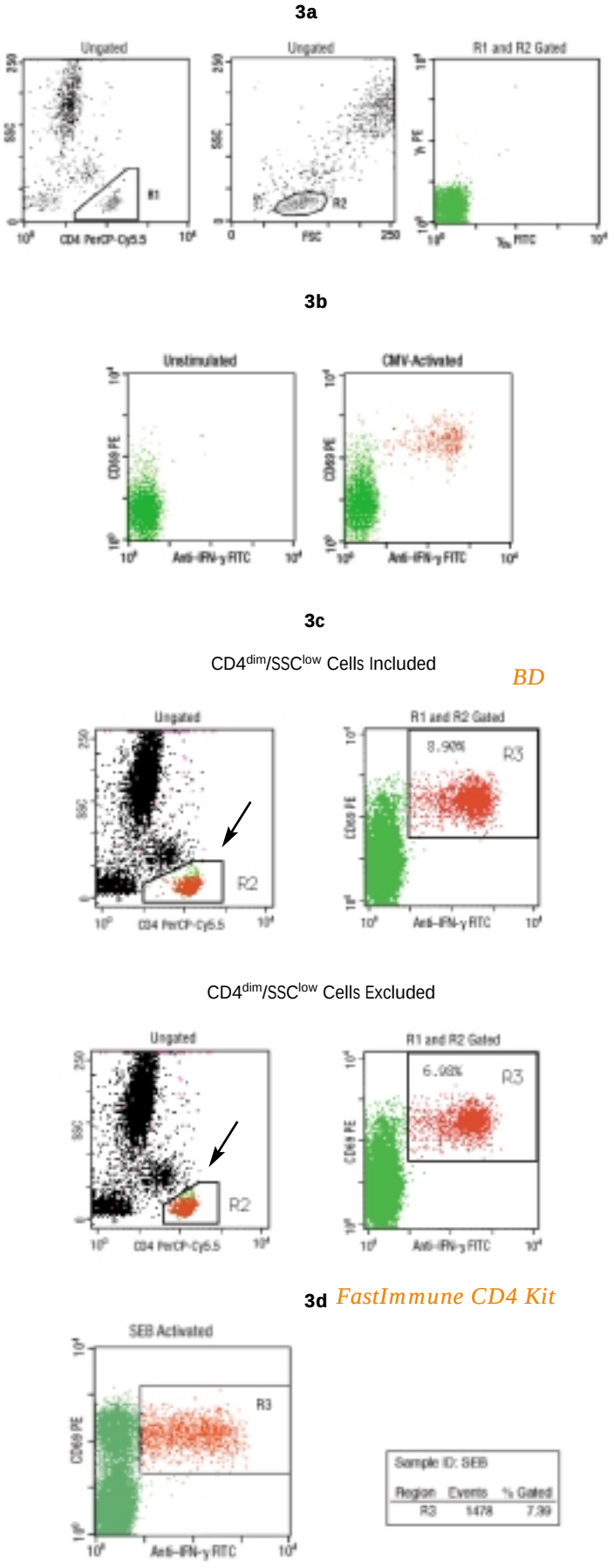


Figure 3

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 Catalog 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

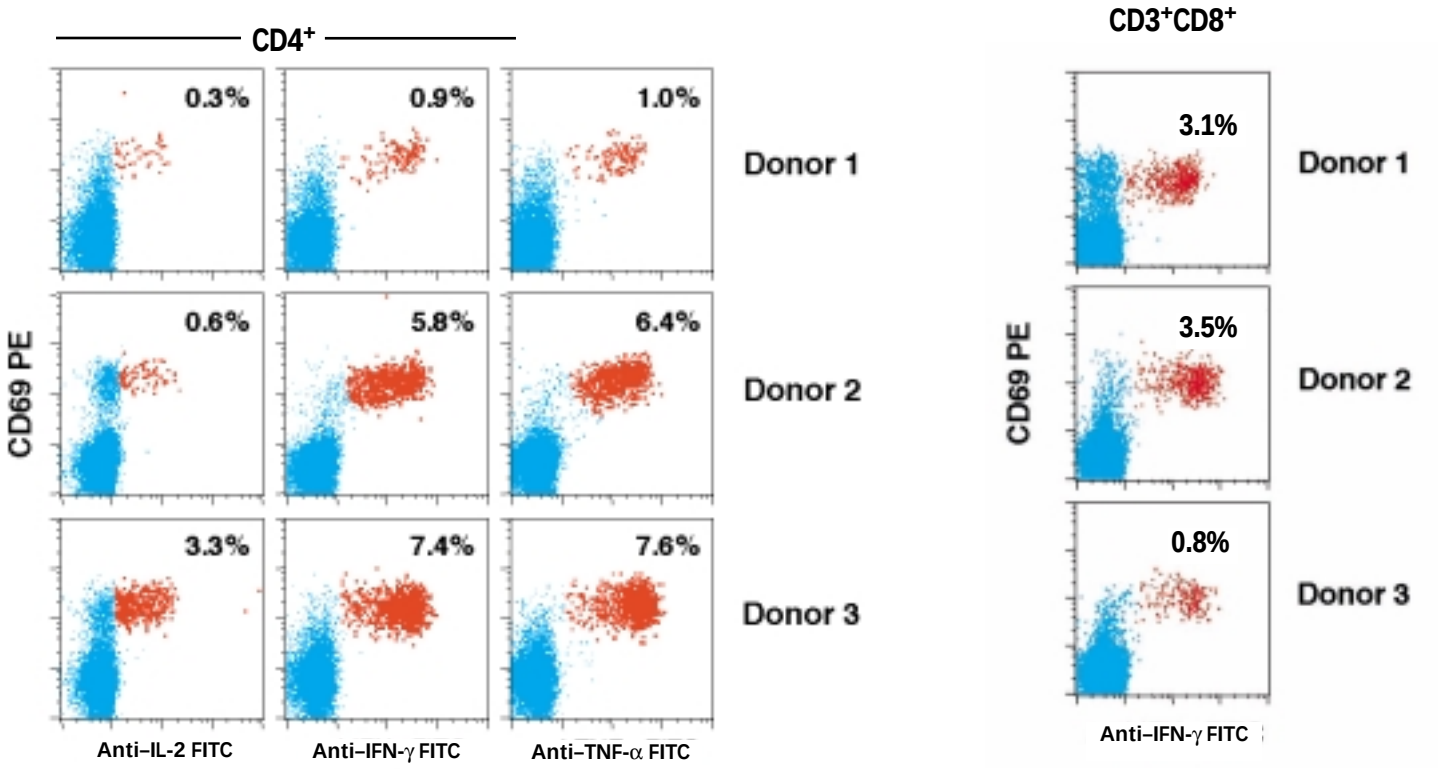
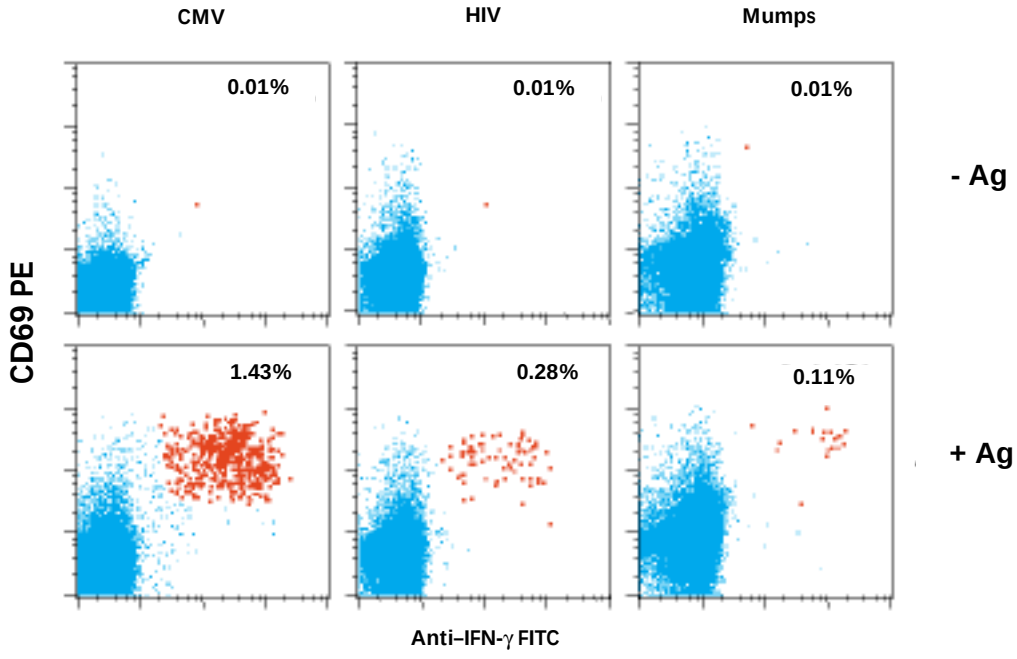


Figure 5 Typical CD4 IFN-γ responses to three different antigens



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.[22,23]
Low intensity of cytokine staining	Inadequate permeabilization or staining or both	Dilute BD FACS Lysing Solution and BD FACS Permeabilizing Solution 2 to 1X 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 $CD4^{dim}$ lymphocytes, but exclude monocytes, platelets, and dead lymphocytes. Gate carefully on CD8 vs CD3 to include $CD8^{dim}$ and $CD3^{dim}$ lymphocytes.	Activated lymphocytes can down-modulate CD4 to become $CD4^{dim}$. Monocytes are $CD4^{dim}$ 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 $CD8^{dim.}$
		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.	

Notes:

Notes:

BD Biosciences publishes this method as a service to investigators. 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 Catalog 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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