

## Application Note 3

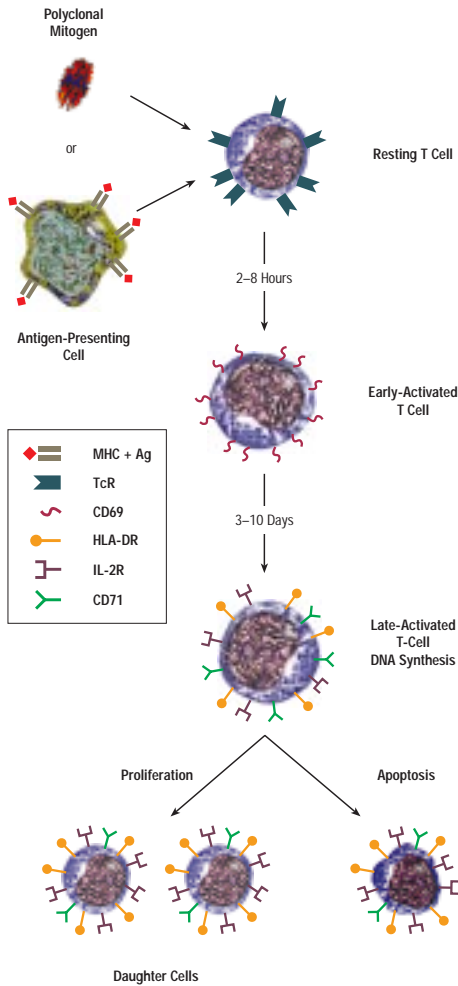
### Simultaneous Detection of Proliferation and Cytokine

#### Expression in Peripheral Blood Mononuclear Cells

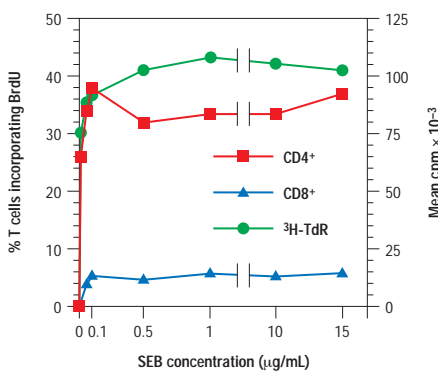
##### Scope

Activation and proliferation of appropriate immune-competent cells is one measure of a successful immune response to an infectious antigen. Cellular events that characterize lymphocyte responses to mitogenic and antigen-specific stimuli include expression of cell-surface activation antigens, production of cytokines, proliferation, and apoptosis (see Figure 1). Lymphocyte proliferation in response to mitogens and antigens is a conventional *in vitro* method to evaluate immune function that generally employs an isotopic readout such as measuring incorporation of tritiated [<sup>3</sup>H]thymidine into DNA.<sup>1-3</sup> This assay, however, has the disadvantage of being performed on bulk cultures, preventing the measurements of DNA synthesis in single cells. Also, traditional bulk assays do not provide information about multiple cell types recruited in the cellular responses to various stimuli and with distinct regulatory and effector functions.

Recently flow cytometric methods have been developed to allow multiparametric detection of cell-surface antigens and intracellular cytokine expression in response to polyclonal stimuli and antigen.<sup>4-7</sup> These methods have now been extended to simultaneously measure DNA synthesis, expression of cell surface activation antigens, and intracellular cytokines, enabling a more complete analysis of these functional activities at the single-cell level.<sup>8</sup> This method involves culturing activated peripheral blood mononuclear cells (PBMCs) in the presence of a thymidine analog bromodeoxyuridine (BrdU). After appropriate fixation and permeabilization, the uptake of the analog is detected with an Anti-BrdU antibody conjugated with a fluorescent dye.



**Figure 1** T-lymphocyte activation pathway



**Figure 2** Comparison of cell proliferation measured by [<sup>3</sup>H]thymidine ([<sup>3</sup>H]-TdR; green line) in PBMCs, and BrdU incorporation in CD4<sup>+</sup> T cells (red squares) and CD8<sup>+</sup> T cells (blue triangles) activated with increasing concentrations of SEB

The presence of an optimal amount of DNase denatures cellular DNA, enhancing the availability of incorporated BrdU to the antibody while preserving both the structure of intracellular proteins and the fluorescent properties of the fluorochrome.<sup>9-11</sup> This method obviates the use of DNA-denaturation conditions incompatible with simultaneous immunodetection of other cell surface and intracellular markers.<sup>12,13</sup> The assay also takes advantage of the unique chemistry and antibody selection protocols BD uses to ensure minimal background fluorescence from nonproliferating cells. Recent observations report that the percentage of BrdU<sup>+</sup>/CD4<sup>+</sup> proliferating T cells assessed by flow cytometry parallels the uptake of [<sup>3</sup>H]thymidine by PBMCs in response to a wide range of concentrations of the superantigen, staphylococcal enterotoxin B (SEB)<sup>14</sup> (see Figure 2). It is also clear that SEB predominantly stimulates CD4<sup>+</sup> T cells to incorporate BrdU and to express IL-2,<sup>14</sup> whereas both CD4<sup>+</sup> and CD8<sup>+</sup> subsets of T cells express CD69 and IFN- $\gamma$  in response to SEB stimulation.<sup>15,16</sup> This observation indicates that this flow cytometric proliferation assay can help delineate the subpopulations of cells with different activation phenotypes. Lymphoproliferative responses measured by detection of incorporated BrdU by flow cytometry have been demonstrated with a variety of mitogenic stimuli, including PHA, PMA + ionomycin (PMA + I), SEB, and specific antigen such as CMV.<sup>14</sup>

This assay\* promises to be a powerful research tool for answering basic biological questions in a broad range of applications.

- pathogenesis of AIDS or cancer
- T-cell subset responses to viral and bacterial antigens
- cell-mediated immune responses to opportunistic infection
- drug/vaccine efficacy assessment
- chemotherapy-immune status assessment
- immune regulation
- immune reconstitution
- transplantation
- toxicology

\* For research use only. Not for use in diagnostic or therapeutic procedures.

## Equipment

1. VACUTAINER® Cell Preparation Tubes (CPTs) (BD Catalog No. 362753) or equivalent. The VACUTAINER CPT is a blood draw tube that establishes a density-gradient plug upon centrifugation. Refer to the VACUTAINER CPT product insert for detailed information.
2. Optional: Ficoll-Paque® separation medium. Refer to the Ficoll-Paque package insert for materials and reagents required.
3. Falcon® 15-mL conical polypropylene tubes (BD Catalog No. 2096) or equivalent
4. 37°C incubator with 5% to 7% CO<sub>2</sub>
5. Vortex mixer
6. Falcon disposable 12 × 75-mm capped polystyrene test tubes (BD Catalog No. 2058) or equivalent
7. Centrifuge
8. Micropipettor with tips (BD Electronic Pipette, BD Catalog No. 34013290 or equivalent)
9. 5° slant rack
10. FACS™ brand flow cytometer. Refer to the appropriate instrument user's guide for information.
11. CaliBRITE™ beads (BD Catalog No. 349502). For detailed information on use, refer to the *CaliBRITE Beads* package insert.
12. FACSCComp™ software for instrument setup and CellQuest™ software for acquisition and analysis. Refer to appropriate software user's guide for detailed information.

## Reagents

### Reagents Not Provided by BD

1. Staphylococcal enterotoxin B (SEB) (Sigma Catalog No. S4881). Reconstitute at 0.5 mg/mL in sterile phosphate-buffered saline (PBS) containing no sodium azide. Store at 4°C. Use 10 µL of SEB per 1 mL of PBMCs (5 µg/mL of cell suspension final concentration).
2. Bromodeoxyuridine (BrdU) (Sigma Catalog No. B5002). Prepare at 60 mM in 1N NaOH. Store in small aliquots at -20°C. Store the thawed reagent at 4°C and use within one week. Dilute 1:10 in sterile PBS containing no sodium azide for each assay. Use 10 µL of BrdU per 1 mL of PBMCs (60 µM final concentration).

3. For intracellular cytokine staining only: Brefeldin A (BFA) (Sigma Catalog No. B7651). Reconstitute at 5 mg/mL in DMSO. Store in 20 µL aliquots at -20°C; do not refreeze aliquots after thawing. Dilute 1:10 in sterile PBS containing no sodium azide for each assay. Use 20 µL of BFA per 1 mL of PBMCs (10 µg/mL of cell suspension final concentration).
4. PBS containing no sodium azide, sterile filtered
5. Ethylene-diamine-tetra-acetic acid (EDTA). Prepare 20 mM stock solution in PBS containing no sodium azide. Sterilize by filtration and store at 4°C.
6. Wash buffer: PBS with 0.5% bovine serum albumin (BSA) and 0.1% sodium azide. Store at 4°C.
7. 1% paraformaldehyde solution prepared in PBS. Store at 4°C.

### BD Reagents for Immunophenotypic Staining

1. FACS™ Lysing Solution (10×) (BD Catalog No. 349202). For dilution instructions and warnings, refer to the *FACS Lysing Solution* package insert.
2. FACS™ Permeabilizing Solution (10×) (BD Catalog No. 340457). For dilution instructions and warnings, refer to the *FACS Permeabilizing Solution* package insert.
3. BD fluorochrome-conjugated monoclonal antibodies to human cell surface markers. The choice of specific surface markers depends on your particular application. In addition, the activation markers CD69, CD25, CD154 (PE\* conjugated, BD Catalog Nos. 347827, 347647, and 340477, respectively), and CD71 (PE conjugated, PharMingen Catalog No. 01595A) can be detected on proliferating lymphocytes. See the example protocol for staining conditions.
4. FastImmune™ Anti-BrdU FITC with DNase (BD Catalog No. 340649). The BrdU incorporated by the proliferating cells is detected by staining the cells with FITC-conjugated Anti-BrdU monoclonal antibody containing DNase. Use at 20 µL/test.
5. FastImmune fluorochrome-conjugated monoclonal antibodies to human intracellular cytokines.

**NOTE:** Most cytokines have passed their peak expression after the activation incubation (48 hours). Low frequencies of IFN-γ and IL-2 expressing cells can be detected using this protocol. Follow the FastImmune Cytokine System protocol for optimal detection of intracellular cytokines in activated lymphocytes and monocytes.

\* US Patent No. 4,520,110; European Patent No. 76,695; Canadian Patent No. 1,179,942

## Procedure

The following is an example of an assay to measure the frequency of CD4 T lymphocytes proliferating in response to SEB. Other antigens or mitogens also can be used, and additional lymphocyte subsets can be studied (see Figure 3).

### Preparation of Cells

**WARNING:** All biological specimens and materials with which they come into contact should be handled as if capable of transmitting infection and disposed of with proper precautions in accordance with federal, state, and local regulations. Never pipette by mouth. Avoid specimen contact with skin and mucous membranes.

Prepare PBMCs using VACUTAINER CPTs. The VACUTAINER CPT is a blood draw tube that establishes a density-gradient plug upon centrifugation. RBCs and granulocytes pass through the plug to the bottom of the tube while the lymphocytes and plasma remain above the plug. Gently invert each tube to resuspend the lymphocytes in autologous plasma.

PBMCs can also be separated using Ficoll-Paque density-gradient centrifugation. Use standard techniques and resuspend at  $2$  to  $3 \times 10^6$  cells/mL in RPMI-1640 with 10% heat-inactivated fetal bovine serum (FBS).

### Activation

Before you begin the activation procedure, set aside an unstimulated control tube that does not receive SEB. This tube is used to assess the residual proliferation from in vivo activation.

**NOTE:** A second control tube that does not contain BrdU can be used when there is residual proliferation from in vivo activation.

1. Add 1 mL of PBMCs to a 15-mL conical polypropylene tube. Cell density should be  $2$  to  $3 \times 10^6$  PBMCs/mL.
2. Add 10  $\mu$ L of SEB per 1 mL of PBMCs ( $5 \mu\text{g/mL}$  of cell suspension final concentration). Antibodies to co-receptors (for example, CD28 and CD49d [ $1 \mu\text{g/mL}$  each]) can also be added to enhance the response.<sup>6,7</sup>

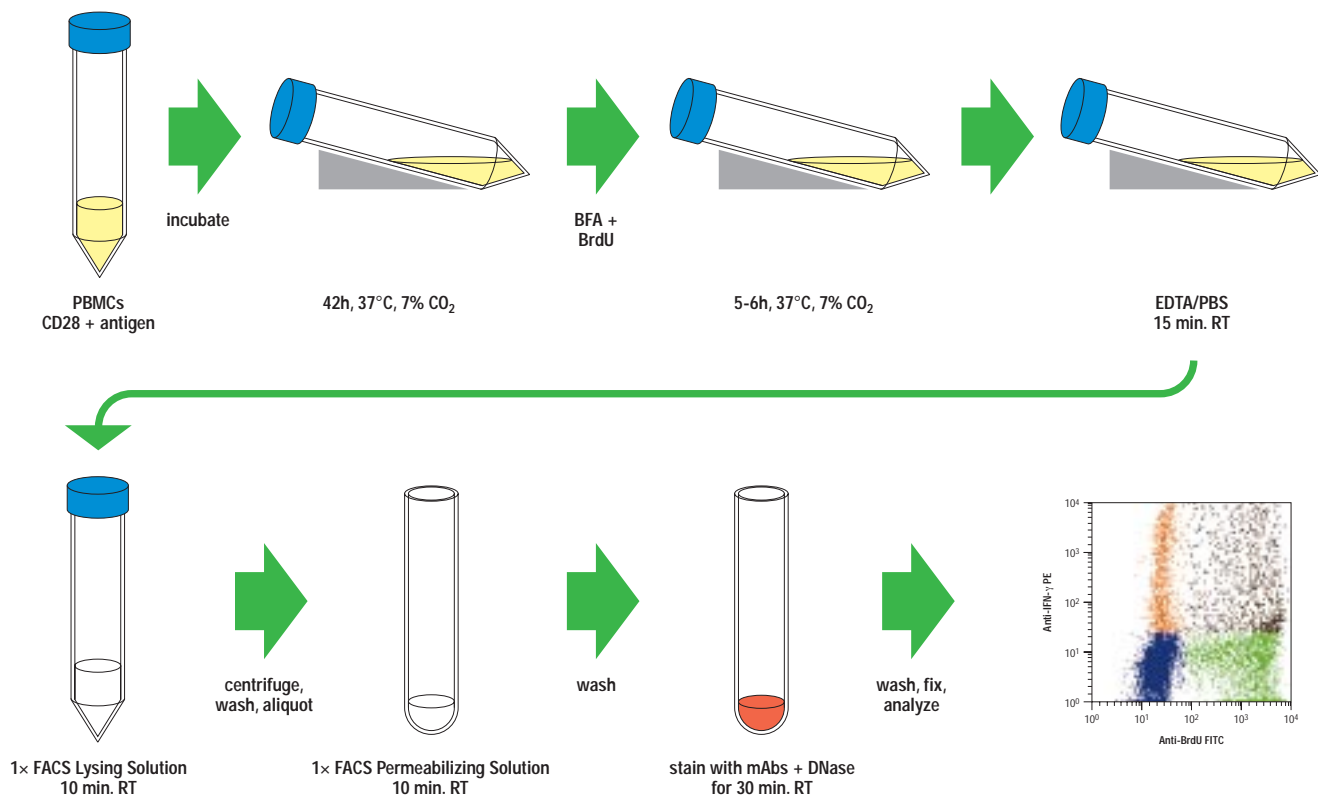


Figure 3 Method for assessing proliferation of PBMCs in response to antigen or mitogen stimulation

3. Incubate PBMCs in 15-mL conical polypropylene tubes positioned at a 5° slant from horizontal in a 5% to 7% CO<sub>2</sub> atmosphere for a total of 48 hours at 37°C.

**NOTE:** The use of a 5° slant rack is recommended but the assay performance may be compared by incubating the cultures either upright or in 24-well plates, depending on the antigen used for stimulation.

4. Add 10 µL of BrdU per 1 mL of PBMCs (60 µM final concentration) for the final 5 to 6 hours of activation.

**NOTE:** If cell concentration is greater than 3 × 10<sup>6</sup> cells/mL, add 20 µL of BrdU per 1 mL of PBMCs.

5. For intracellular cytokine detection: add 20 µL of BFA per 1 mL of PBMCs (10 µg/mL of cell suspension final concentration) for the last 5 to 6 hours of activation.

### *Harvesting the Cells*

1. Add 100 µL of 20 mM EDTA per 1 mL of PBMCs.
2. Vortex at high speed for 15 seconds.
3. Incubate the cell suspension in the same slant position for 15 minutes at room temperature (20° to 25°C).
4. Add 10 mL of cold PBS and mix vigorously.
5. Centrifuge at 500 × *g* for 10 minutes. Decant the supernatant leaving approximately 100 µL of fluid. Tap the tube to resuspend the pellet.

### *Fixation, Permeabilization, and Intracellular Staining*

1. Add 3 mL of 1× FACS Lysing Solution and mix well. Incubate for 10 to 15 minutes at room temperature. This treatment is necessary to lyse residual red blood cells (RBCs) in the PBMC preparation, fix the surface epitopes, and aid in permeabilization.
2. Centrifuge for 10 minutes at 500 × *g*. Remove the supernatant.
3. Resuspend the pellet in 1 mL of wash buffer and aliquot 100 µL into each labeled 12 × 75-mm tube.
4. Add 500 µL of 1× FACS Permeabilizing Solution and mix well. Incubate for 10 minutes at room temperature.
5. Add 2 to 3 mL of wash buffer and centrifuge for 10 minutes at 500 × *g*. Remove the supernatant.
6. Add the appropriate volume of fluorochrome-conjugated monoclonal antibodies to human cell surface markers (for example, CD4 PerCP<sup>\*</sup>), FastImmune Anti-BrdU FITC with DNase, and intracellular cytokines. Include appropriate isotype controls (for example, Mouse IgG<sub>1</sub> FITC) as negative controls. Mix well and incubate for 30 minutes in the dark at room temperature.

**NOTE:** Staining of CD3, CD4, and CD8 antigens can be performed either on the cell surface prior to permeabilization, or during the intracellular staining step after permeabilization. This does not apply to all antigens because epitopes vary in sensitivity to formaldehyde fixation.
7. Add 2 to 3 mL of wash buffer.
8. Centrifuge for 10 minutes at 500 × *g*. Remove the supernatant.
9. Add 200 µL of 1% paraformaldehyde solution. Samples can be stored in the dark at 4°C for up to 24 hours until analyzed.

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\* US Patent No. 4,876,190

## Analysis

1. Use CaliBRITE beads and appropriate software (FACSCComp, version 1.1 or later, or AutoCOMP™, version 3.0.2) for setting the photomultiplier tube (PMT) voltages and the fluorescence compensation, and for checking instrument sensitivity before use. Refer to the appropriate TriTEST™ three-color application note for flow cytometric setup, acquisition, and analysis.

**NOTE:** It has been observed that Anti-BrdU FITC drags up the BrdU-negative cell population approximately 10-fold. Therefore, cells from the unstimulated control tube should be used to set the quadrant marker during analysis.

2. Acquire data with CellQuest software, using a fluorescence or FSC threshold. Make sure to include larger cells (blasts) that fall into a larger FSC/SSC profile. Typically, acquisition of 10,000 gated events is sufficient. In cases with low frequencies of positive events (<1%), such as for antigen

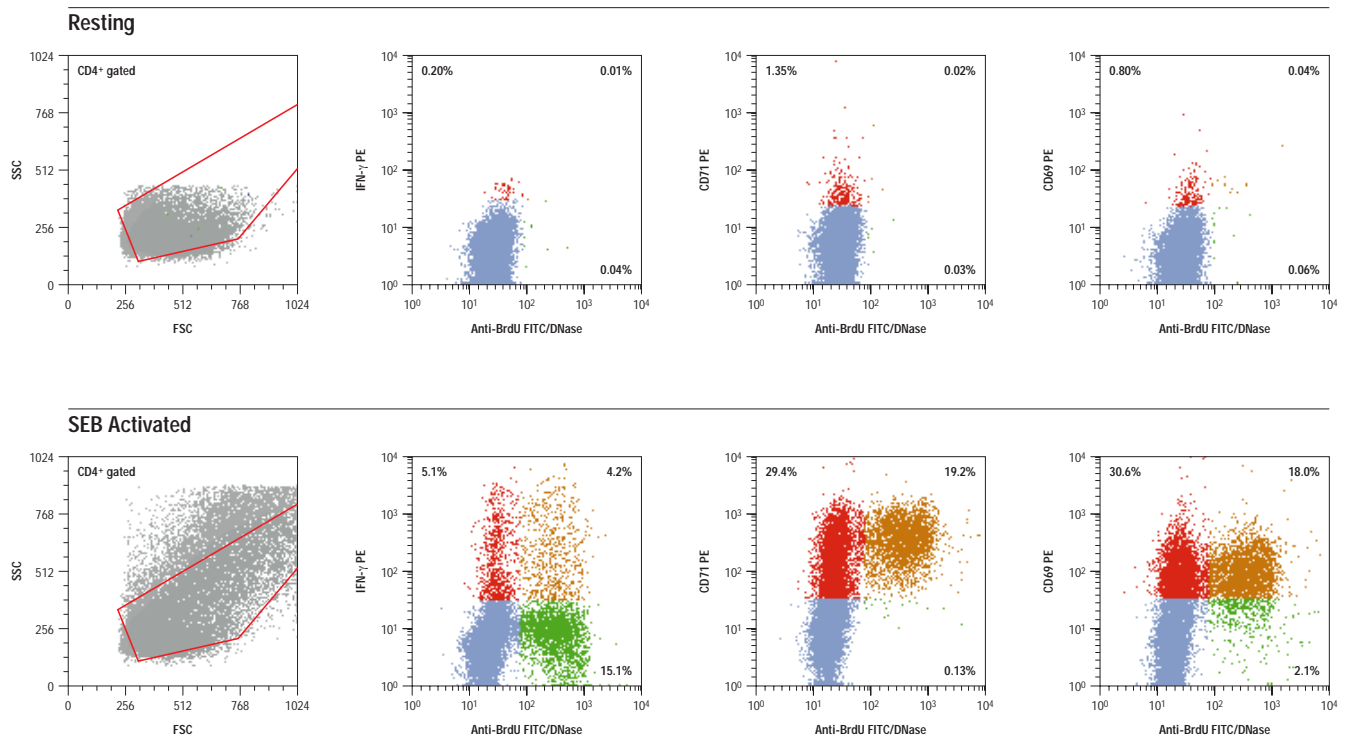
specific proliferation, acquisition of a minimum of 40,000 gated events is recommended (see Figure 4).

3. After gating the cell population of interest, display data as two-color plots to determine the phenotype and percentages of proliferating Anti-BrdU FITC-positive cells. Data can be analyzed using CellQuest or PAINT-A-GATE<sup>PRO</sup>™ software.

## Calculation of the Specific Response

Measuring the frequency of cells participating in a specific response to any stimulus is calculated by subtracting the percentage of positive events in the control samples from the percentage of positive events in the activated sample. The stimulus specific proliferation is calculated by the following formula:

$$\text{Frequency} = \% \text{ Activated Anti-BrdU FITC}^+ - \% \text{ Resting Anti-BrdU FITC}^+$$



**Figure 4** CD4<sup>+</sup> lymphocytes from a normal donor are gated on a FSC/SSC dot plot. Large cells (see activated example) are included in the CD4<sup>+</sup> gate. Data illustrates Anti-BrdU FITC staining versus IFN- $\gamma$  PE, CD71 PE, and CD69 PE. Since CD69 is an early activation marker, its expression at 48 hours is past its peak, and CD71 expression is higher. Intracellular BrdU staining and surface expression of activation markers can be investigated simultaneously in this assay.

## Troubleshooting

The following troubleshooting tips should help you pinpoint potential sources of problems in the assay. The most common sources of errors occur during the sample preparation and activation steps.

Problem	Possible Cause	Solution	Comments
No staining of BrdU or intracellular cytokines.  <b>NOTE:</b> Activating the cells for 48 hours might not be the optimal time for intracellular cytokine detection. BD has detected IFN- $\gamma$ and IL-2 in this assay.	Cells not activated	Activation reagents not prepared correctly. See Procedure section for stimulus preparation and storage.	>80% of BrdU <sup>+</sup> /CD4 <sup>+</sup> T lymphocytes should be CD71 <sup>+</sup> after 48-hour SEB stimulation.
	Proliferating blasts excluded from analysis	Gate should include large FSC/SSC events.	After 48 hours of activation, lymphocytes transform into lymphoblasts, which have large FSC/SSC characteristics.
	Cells not permeabilized	Treat cells with FACS Lysing Solution before treatment with FACS Permeabilizing Solution. FACS Lysing solution and FACS Permeabilizing Solution should be diluted in DI water. Do not dilute in PBS or other buffers.	FACS Lysing Solution conditions cells for permeabilization. Lysing and permeabilization should be performed at room temperature.
	BrdU or BFA inactive or prepared incorrectly	Prepare BrdU and BFA as directed. Aliquot BrdU and BFA stocks and store at -20°C.	BFA is necessary for accumulation of cytokines inside the cell enabling the intracellular cytokine staining.
	Reagent not stored properly	Store the Anti-BrdU FITC with DNase reagent at 4°C. The DNase enzyme can become inactive at room temperature or higher.	The Anti-BrdU FITC reagent contains DNase, which makes the BrdU (incorporated into the DNA of the proliferating cells) accessible for binding by Anti-BrdU antibody conjugate.
Unacceptable cell loss during procedure	Cells not recovered after 48-hour culture period	Treat the cells with EDTA (2 mM final concentration) for 15 minutes when harvesting the cells. Vortex vigorously after adding EDTA.	EDTA helps the detachment of cells cultured for 48 hours in polypropylene tubes.
	Cells not recovered during centrifuge washing steps	Centrifuge fixed and permeabilized cells at 500 $\times g$ for 10 minutes.	Activated blasts and fixed cells have lower density than resting, live cells and require higher centrifugal force and longer time to pellet.
	Cells not recovered during aspiration steps	Decant supernatant instead of aspirating with vacuum.	

*BD Biosciences publishes this*

*method as a service to*

*investigators. Detailed support*

*for non-flow cytometric aspects*

*of this procedure may not be*

*available from BD Biosciences.*

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