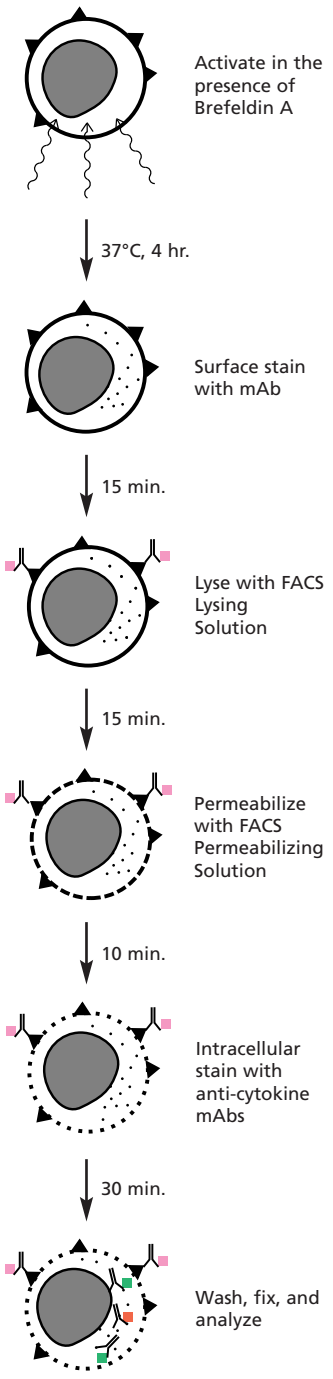
FastImmune Intracellular Cytokine Staining Procedures

BD has developed protocols for the detection of intracellular cytokines in activated lymphocytes and in activated monocytes. The procedures have been optimized for use with BD anti-cytokine monoclonal antibodies.

The FastImmune cytokine assay begins with activation of whole blood or peripheral blood mononuclear cells (PBMCs). Brefeldin A (BFA) is included during the last 4 hours of activation to inhibit intracellular transport. Thus antigens and cytokines produced during activation are retained inside the cell. The cells are stained with fluorochrome-conjugated monoclonal antibodies to human cell surface markers. Cells are lysed, permeabilized, and stained with fluorochrome-conjugated monoclonal antibodies to cytokines. Samples are washed and analyzed by flow cytometry.

Quick reference protocols are included in this catalog. For detailed assay information, please refer to our immune function application notes: *Detection of Intracellular Cytokines in Activated Lymphocytes* and *Detecting Intracellular Cytokines in Activated Monocytes*. They are available on our website or from your local BD representative.



FastImmune intracellular cytokine procedure

Detection of Intracellular Cytokines in Activated Lymphocytes

Equipment

1. Disposable 12 x 75-mm capped polystyrene Falcon test tubes (BD Cat. No. **2058**), or equivalent.
2. 37°C incubator with 5% to 7% CO_2
3. Vortex mixer
4. Centrifuge
5. Pipetman, or equivalent pipettors
6. FACS brand flow cytometer

Cells

Whole Blood

Collect blood for whole blood activation assays into sodium heparin VACUTAINER tubes (BD VACUTAINER Cat. No. **367673**). FastImmune assays are incompatible with lithium heparin, EDTA, and ACD anticoagulants.

For best results, assay blood within 8 hours of collection. If blood cannot be used within 8 hours, store VACUTAINER tubes horizontally at room temperature.

Peripheral Blood Mononuclear Cells (PBMCs) in Autologous Plasma

Prepare PBMCs using BD VACUTAINER Cell Preparation Tubes (CPTs) (Cat. No. **362753**) containing sodium heparin. See the VACUTAINER CPT product insert for detailed information. By gently inverting the tube, the leucocytes can be resuspended in the plasma and activated like whole blood in this assay.

Before storage, centrifuge CPTs and resuspend PBMCs in the autologous plasma by gently inverting each tube several times. Store each CPT at room temperature on its side. Assay the blood no later than 24 hours after collection.

PBMCs in Tissue Culture Medium

PBMCs can also be separated via Ficoll-Paque density-gradient centrifugation. Use standard techniques and resuspend at 2 x 10^6 cells/mL in RPMI-1640 with 10% heat-inactivated fetal bovine serum (FBS) for activation.

Cell Lines and T-Lymphocyte Clones

For activation, resuspend cells at 2 x 10^6 cells/mL in the fresh culture medium typically used to grow the cells.

NOTE: Heat inactivate FBS to denature complement.

Frozen Activated Whole Blood and PBMCs

Lyse and fix activated whole blood or PBMCs using 1X FACS Lysing Solution; wash with phosphate-buffered saline (PBS) and freeze in PBS with 1% bovine serum albumin (BSA) and 10% DMSO at –70°C. After thawing, aliquot cells into staining tubes. Wash cells by adding 2 to 3 mL wash buffer and centrifuge for 5 minutes at 500 x *g*; then permeabilize with 1X FACS Permeabilizing Solution and stain.

Detection of Intracellular Cytokines in Activated Lymphocytes

Reagents

The following procedures and reagents have been successfully used by the research laboratories at BD.

Reagents Used in Activation (Not Provided by BD)

1. Phorbol 12-myristate 13-acetate (PMA) (Sigma Cat. No. **P-8139**)
   a. Reconstitute in DMSO at 0.1 mg/mL.
   b. Store small aliquots (*eg*, 20 µL) at –20°C; do not refreeze aliquots after thawing.
   c. Dilute stock 1:100 in sterile PBS (without sodium azide) for each experiment.
   d. Use PMA at a final concentration of 10 ng/mL of cell suspension.
2. Ionomycin (Sigma Cat. No. **I-0634**)
   a. Reconstitute in EtOH at 0.5 mg/mL.
   b. Store at –20°C.
   c. Dilute stock 1:10 in sterile PBS (without sodium azide) for each experiment.
   d. Use ionomycin at a final concentration of 1 µg/mL of cell suspension.
3. Staphylococcal enterotoxin B (SEB) (Sigma Cat. No. **S-4881**)
   a. Reconstitute in sterile PBS (without sodium azide) at 0.5 mg/mL.
   b. Store at 4°C.
   c. Use SEB at a final concentration of 1 µg/mL of cell suspension.
4. Brefeldin A (BFA) (Sigma Cat. No. **B-7651**)
   a. Reconstitute in DMSO at 5 mg/mL.
   b. Store small aliquots (*eg*, 20 µL) at –20°C; do not refreeze aliquots after thawing.
   c. Dilute stock 1:10 in sterile PBS (without sodium azide) for each assay.
   d. Use BFA at 10 µg/mL of cell suspension for the last 4 to 5 hours of activation.

NOTE: Extensive incubation with BFA will reduce cell viability.

5. RPMI-1640 (BioWhittaker Cat. No. **12-167F**)
6. PBS without sodium azide (NaN_3), sterile filtered
7. DMSO (Sigma Cat. No. **D-8779**)
8. EtOH (Gold Shield Ethyl Alcohol), 200 proof
9. Wash buffer, PBS with 0.5% BSA and 0.1% NaN_3. Store at 4°C.
10. 1% paraformaldehyde in PBS; store at 4°C.

Reagents for Immunophenotypic Staining (BD)

1. Monoclonal antibody conjugates for surface staining.
2. FACS Lysing Solution

   FACS Lysing Solution is supplied as a 10X concentrate. Before use, dilute 1:10 in deionized water; refer to the product insert for instructions. Do not dilute in PBS or other buffers.
3. FACS Permeabilizing Solution

   FACS Permeabilizing Solution is supplied as a 10X concentrate. Before use, dilute 1:10 in deionized water; refer to the product insert for instructions. Do not dilute in PBS or other buffers.
4. BD monoclonal antibody conjugates for intracellular staining.

Detection of Intracellular Cytokines in Activated Lymphocytes

Activation

Activation is performed in the presence of BFA which inhibits intracellular transport of proteins,[1,2,3] so antigens and cytokines produced during activation will be retained inside the cell. The unstimulated control sample should also contain BFA. See the Assay Control section in this procedure. All activation procedures outlined are performed in 12 x 75-mm capped polystyrene test tubes (Falcon Cat. No. **2058**). The reagent concentrations indicated are final concentrations in the activation mixture using reagent preparations described previously.

1. PMA + ionomycin (PMA + I)
   a. Dilute whole blood or PBMCs in plasma 1:1 with RPMI 1640 without serum. (This dilution procedure is required only for PMA + I activation. Cells that have already been resuspended at 2 x 10^6/mL in medium need not be further diluted with RPMI.)
   b. Stimulate with 10 ng/mL of PMA (10 µL of working solution described previously per mL of blood) and 1 µg/mL of ionomycin (20 µL of working solution per mL of blood) in the presence of 10 µg/mL of BFA (20 µL of working solution per mL of blood).
   c. Incubate for 4 hours at 37°C, 5% to 7% CO_2 with tube caps loosened to allow entry of CO_2-containing air. (While a CO_2 incubator is preferred to ensure proper control of pH, the incubation can also be carried out in a water bath with each tube tightly capped.)
2. SEB
   a. Activate undiluted blood with 1 µg/mL of SEB in the presence of 10 µg/mL of BFA.
   b. Incubate for 4 to 6 hours at 37°C.
3. CD2/CD2R (BD Cat. No. **340366**)
   a. Activate undiluted blood with 20 µL of CD2/CD2R per mL of blood in the presence of BFA.
   b. Incubate for 4 to 6 hours at 37°C.
4. CD3
   a. Activate undiluted blood with immobilized CD3[4] in the presence of BFA.
   b. Incubate for 4 to 6 hours at 37°C. CD5 PerCP (BD Custom Conjugate) or CD45 PerCP (BD Cat. No. **347464**) are recommended for FL3 fluorescence triggering because the CD3 antigen is modulated by crosslinking of CD3.

NOTE: High-concentration, low-azide CD3 is available through the BD Custom Conjugate Program. Contact your local BD representative for more information.

5. CD28

   Use CD28 (BD Cat. No. **340975**) at 10 µg/mL to enhance activation responses to various stimuli, including SEB, CD3, and CD2/CD2R.

Assay Controls

Unstimulated Control

The unstimulated control is used to assess the level of residual cytokine synthesis from in vivo activation. Run this control for all samples. As the name implies, the unstimulated control is prepared by incubating the blood during the activation step with 10 µg/mL of BFA, but without a stimulus.

Isotype Controls

Fluorescent-conjugated isotype control antibodies are used at matching concentrations to detect non-specific binding to cells due to the class of the mouse monoclonal antibody. The FastImmune Cytokine System uses standard anti-KLH isotype controls specially formulated for intracellular detection systems.

Detection of Intracellular Cytokines in Activated Lymphocytes

Activation Control

The activation control uses surface expression of CD69 to assess whether activation has been achieved. If the expected level of CD69 is not seen, there is a problem with the activation step of the assay. Specifically, one of the reagents used in the activation step can be inactive, expired, or improperly prepared; or a solvent can be contaminated. Make fresh preparations of the stimuli and try again.

1. Activate one aliquot of blood with PMA + I (as described previously) but omit the BFA.

NOTE: Anti-secretory agents like BFA prevent surface expression of CD69 and must be omitted to permit surface expression and detection.

2. Surface stain only with CD69 PE/CD3 PerCP (BD Cat. No. **340368**). Omit the permeabilization and intracellular staining steps of the procedure.
3. Analyze results by fluorescence triggering on FL3 and assessing CD69 staining in the CD3-gated events. Surface staining for CD69 should be greater than 90% positive (Figure 1).

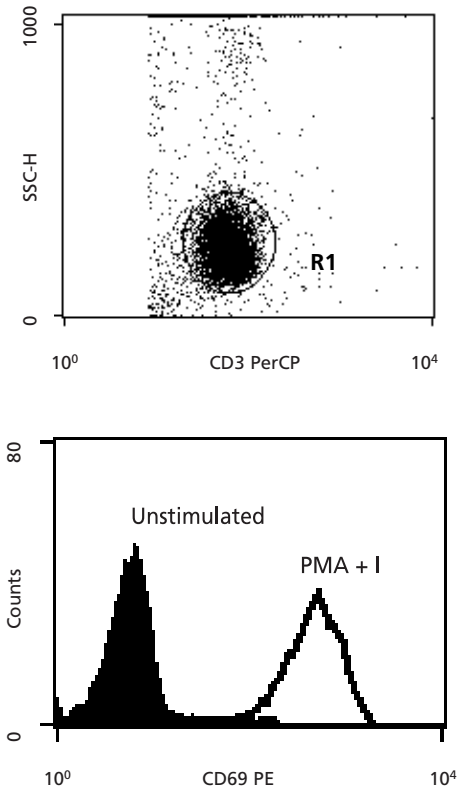


Figure 1 Activation control

Detection of Intracellular Cytokines in Activated Lymphocytes

Intracellular Staining Control

The intracellular staining control assesses intracellular staining of CD69 in conjunction with the results of the activation control to pinpoint whether permeabilization and intracellular staining are executed properly. If the activation control is greater than 90% positive, but a comparable level of CD69 is not detected by intracellular staining, there is a problem with the permeabilization or intracellular staining step of the assay. Make sure you have followed the procedure exactly as written, and try again.

1. Activate one aliquot of blood with PMA and ionomycin in the presence of BFA.
2. Omit the surface staining step. Stain intracellularly only with CD69 PE/CD3 PerCP (BD Cat. No. **340368**).
3. Analyze results by fluorescence triggering on FL3 and assessing CD69 staining in the CD3 gated events. Intracellular staining for CD69 should be greater than 90% positive (Figure 2).

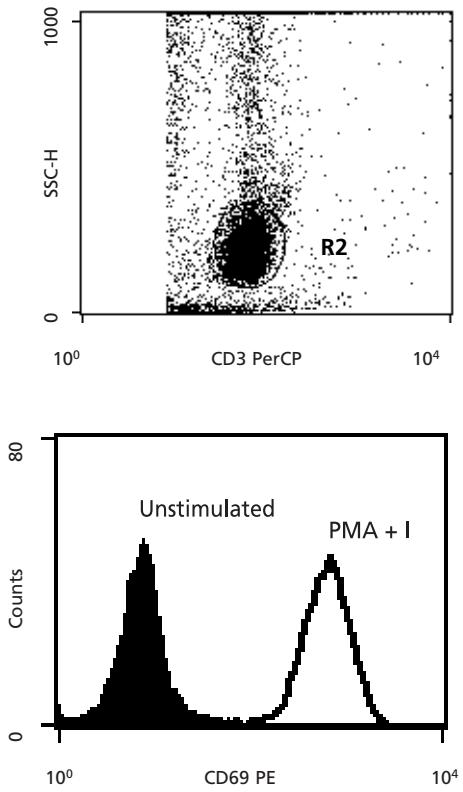


Figure 2 Intracellular staining control

Detection of Intracellular Cytokines in Activated Lymphocytes

Surface Staining

1. Add 20 µL of each BD surface staining reagent to 12 x 75-mm tubes.
2. Add 100 µL of diluted PMA + I activated blood or 50 µL of undiluted whole blood (activated by other stimuli) to the surface staining reagents. (Dilution of whole blood or PBMCs in plasma in RPMI is only required for PMA + I activation. Cells that have already been resuspended at 2 x 10^6 cells/mL in medium need not be further diluted with RPMI.)
3. Mix well and incubate for 15 minutes at room temperature in the dark.

Permeabilization and Intracellular Staining

1. Add 2 mL of 1X FACS Lysing Solution prepared according to the package insert. Incubate for 10 minutes at room temperature. When staining PBMCs or cultured cells, add FACS Lysing Solution to fix the surface epitopes and optimize the permeabilization process.

NOTE: PMA-activated whole blood does not always lyse completely.

2. Centrifuge for 5 minutes at 500 x *g* and remove the supernatant. Avoid disturbing the pellet. Add 500 µL of 1X FACS Permeabilizing Solution prepared according to the package insert and mix well. Incubate for 10 minutes at room temperature in the dark.
3. Add 2 to 3 mL of wash buffer and centrifuge for 5 minutes at 500 x *g*. Remove the supernatant.
4. Add fluorescent-conjugated anti-cytokine mAbs. Mix well and incubate for 30 minutes at room temperature in the dark.
5. Add 2 to 3 mL wash buffer and centrifuge for 5 minutes at 500 x *g*. Remove supernatant and add 500 µL 1% paraformaldehyde.

NOTE: Samples can be stored for up to 24 hours at 4°C in the dark prior to analysis.

Analysis

1. Analyze on a FACS brand flow cytometer.
2. Use CaliBRITE beads and appropriate software (FACSComp, version 1.1 or later, or AutoCOMP, version 3.0.2) for setting photomultiplier tube (PMT) voltages and fluorescence compensation and for checking instrument sensitivity prior to use. Refer to the appropriate TriTEST three-color application note for flow cytometric setup, acquisition, and analysis.

NOTE: Proper instrument setup with the correct version of FACSComp or AutoCOMP is important for obtaining accurate results with the FastImmune assay. Contact your BD representative if you have an older version of either FACSComp or AutoCOMP.

3. Acquire data with CellQuest or LYSYS II software, using a fluorescence or forward scatter (FSC) threshold. Typically, 10,000 gated events is sufficient.
4. Gate on $FL3^+$ cells. Display data as two-color dot plots to determine cytokine expression. Data can be analyzed using CellQuest, LYSYS II, PAINT-A-GATE, or Attractors software. With PMA activation, platelets can move into the $FL3^+$ gate. In this case, gate on FSC/SSC. In assays with a CD4 trigger, gate on FSC/SSC to exclude monocytes.

Calculate the Specific Response

As illustrated by the following formula, the specific response of cells to any stimulus is obtained by subtracting % positive events in the isotype control sample from % positive events in the anti-cytokine antibody-stained sample. Then subtract the isotype-corrected response of the unstimulated sample from that of the stimulated sample.

Formula: (AS – AIC) – (US – UIC)

where
AS = activated sample
AIC = activated isotype control
US = unstimulated sample
UIC = unstimulated isotype control

Detection of Intracellular Cytokines in Activated Lymphocytes

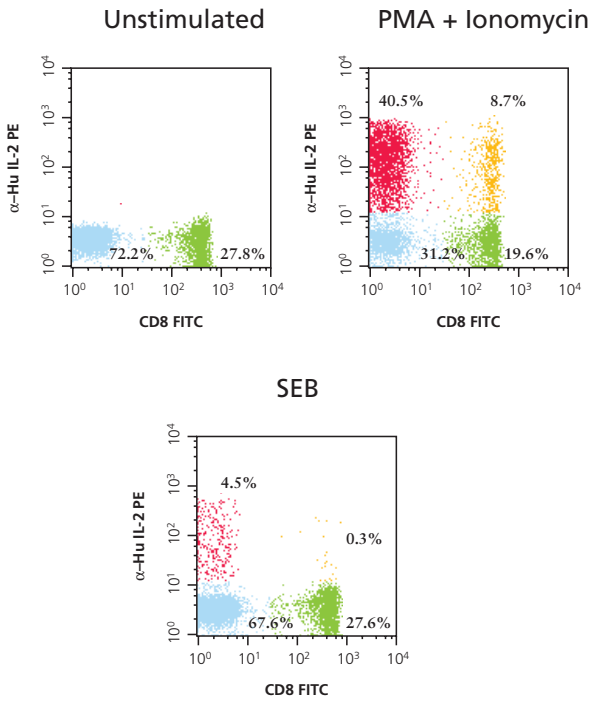


Figure 3 Functional subsets of CD8⁺ T cells

The FastImmune Cytokine System can be used to delineate functionally distinct subsets of CD8⁺ T cells. The two-color dot plots in Figure 3 compare the results of very general activation with PMA and ionomycin (PMA + I) versus more specific activation with superantigen staphylococcal enterotoxin B (SEB). Activation with PMA + I clearly induces IL-2 expression in the CD8⁺ and CD8⁻ (nominally CD4⁺) populations. In contrast, SEB stimulates IL-2 production in a less frequent population that is more strongly biased toward CD8⁻ cells. These subset-specific results cannot be observed in a bulk assay.

References

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3. Ferrick DA, Schrenzel MD, Mulvania T, Hsieh B, Ferlin WG, Lepper H. Differential production of interferon-γ and interleukin-4 in response to T_H1- and T_H2-stimulating pathogens by γ/δ T cells in vivo. *Nature*. 1995;373:255-257.
4. Cherwinski HM, Semenuk GT, Ransom JT. Stimulation of a T helper cell class 2 clone with immobilized anti-T cell receptor antibody activates a Ca^{2+} and protein kinase C-independent lethal signal pathway. *J Immunol*. 1992;148:2996-3003.