BD FastImmune™

CD8 Intracellular Cytokine Detection Kit

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

Anti-Hu–IFN-γ   Catalog No. 346049

5/2015   23-6124-01

RESEARCH APPLICATIONS

The BD FastImmune™ CD8 Intracellular Cytokine Detection Kit is designed for the detection of intracellular cytokines and the activation marker CD69 in antigen-activated $CD8^+$ T lymphocytes in whole blood.[1-6] Applications include studies of T-cell responses to antigens, such as herpes viruses,[7-12] HIV,[13-16] and tumor antigens.[17]

DESCRIPTION

Each kit supplies sufficient reagents for 25 activated whole blood and 25 unstimulated control samples. In performing the assay, 0.5 mL of whole blood is activated with antigen (activation agent not included in the kit) and 0.5 mL of blood remains as an unstimulated control. Both activated and unstimulated blood are then stained with an isotype control and an anti-cytokine antibody cocktail.

NOTE: If you are using a specific antigen as the activation agent, you should activate an additional 0.5 mL of blood with a superantigen such as staphylococcal enterotoxin B (SEB). This tube is used as a positive activation control and simplifies gating.

PRINCIPLES OF THE PROCEDURE

This technique allows the detection of functional populations of $CD8^+$ T cells that respond to specific soluble antigens in short term restimulation assays.[1-17] Whole blood is stimulated with antigen and costimulatory antibodies (CD28 and CD49d)[18] for an initial two-hour period. Brefeldin A (BFA) is then added to inhibit the secretion of newly synthesized cytokine and CD69, and the blood is incubated an additional four hours. Next, EDTA is added to remove adherent cells from the activation vessel, followed by the simultaneous lysis of erythrocytes and fixation of leucocytes using BD FACS™ Lysing Solution. Cells are then washed and permeabilized using BD FACS™ Permeabilizing Solution 2 (Catalog No. 347692). BD FACS Permeabilizing Solution 2 has been optimized for intracellular staining of antigen-activated whole blood. After an additional wash, surface and intracellular staining antibodies are added in a single staining step. Finally, the cells are washed and fixed for analysis on a flow cytometer.

REAGENTS

The BD FastImmune CD8 Intracellular Cytokine Detection Kit includes:

- BD FastImmune™ Anti-Hu–IFN-γ FITC/CD69 PE/CD8 PerCP-Cy™5.5*/CD3 APC
- BD FastImmune™ γ_{2a} FITC/γ_1 PE/CD8 PerCP-Cy5.5/CD3 APC isotype control
- BD FastImmune™ Brefeldin A Solution
- BD FastImmune™ EDTA Solution
- BD FastImmune™ CD28/CD49d
- BD FACS Lysing Solution (10X)
- BD FACS Permeabilizing Solution 2 (10X)

Handling and Storage

Upon receipt, thaw BFA, dispense into 10-µL aliquots, and store at –20°C.

Before use, dilute BD FACS Lysing Solution and BD FACS Permeabilizing Solution 2 1:10 in deionized (DI) water. Use at room temperature.

Store vials at 2°C–8°C. Conjugated forms should not be frozen. Protect from exposure to light. Each reagent is stable until the expiration date shown on the bottle label when stored as directed. Alteration in the appearance of the reagent, such as precipitation or discoloration, indicates instability or deterioration. In such cases, the reagent should not be used.

Precautions

Each antibody reagent contains sodium azide as a preservative; however, care should be taken to avoid microbial contamination, which can cause erroneous results.

WARNING: All biological specimens and materials coming in contact with them are considered biohazards. Handle as if capable of transmitting infection[19,20] and dispose of with proper precautions in accordance with federal, state, and local regulations. Never pipette by mouth. Wear suitable protective clothing, eyewear, and gloves.

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Reagent contains 30.0% diethylene glycol, CAS number 111-46-6, 9.99% formaldehyde, CAS number 50-00-0, and 3.51% methanol, CAS number 67-56-1.

Danger

H311 Toxic in contact with skin.
H331Toxic if inhaled.

H341 Suspected of causing genetic defects.
H350 May cause cancer. Route of exposure: Inhalative.
H371-H335 May cause damage to organs. May cause respiratory irritation.
H373 May cause damage to the kidneys through prolonged or repeated exposure. Route of exposure: Oral.

H318 Causes serious eye damage.

H302 Harmful if swallowed.
H315 Causes skin irritation.
H317 May cause an allergic skin reaction.

Wear protective clothing / eye protection. Wear protective gloves. Avoid breathing mist/vapours/spray. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing. IF SWALLOWED: Immediately call a doctor.

SPECIMEN COLLECTION AND PREPARATION

Blood should be collected in sodium heparin, since other anticoagulants severely compromise the functional capacity of lymphocytes. It should be stored at room temperature to avoid platelet activation prior to use but should be used within 8 hours of collection. Antigen-presenting cell function is compromised with longer storage times, and loss of function can be compounded by shipping.

REAGENTS AND MATERIALS REQUIRED BUT NOT PROVIDED

- Heparinized whole blood
- Activation agent: This kit is optimized for activation by specific antigens, such as cytomegalovirus (CMV) peptides or peptide mixes, but it also works with superantigens such as SEB.
- Wash buffer: First prepare stock solutions of 5% bovine serum albumin (BSA) in 1X phosphate-buffered saline (PBS) (filter sterilize) and 10% sodium azide in 1X PBS. Then prepare 500 mL of wash buffer by adding 50 mL of 5% BSA stock solution and 5 mL of 10% sodium azide stock solution to 445 mL of 1X sterile PBS. This represents final concentrations of 0.5% BSA and 0.1% sodium azide in PBS. Store at 4°C.
- 1% paraformaldehyde solution prepared in PBS containing 0.1% sodium azide. Store at 2°–8°C in amber glass for up to 1 week.
- 15-mL polypropylene tubes
- 5-mL polystyrene tubes
- Vortex mixer
- Micropipettor with tips (BD Electronic Pipette, Catalog No. 343246 or equivalent)
- 37°C water bath or incubator
- Centrifuge
- BD FACS™ brand flow cytometer. Refer to the appropriate instrument user's guide for information.
- BD Calibrite™ beads (Catalog No. 349502), BD Calibrite™ PerCP-Cy5.5–labeled beads (Catalog No. 345006, beads only; 345036, beads plus Bead Dilution Buffer), and BD Calibrite APC beads (Catalog No. 340487). Refer to the beads product insert for instructions.
- BD FACSComp™ software, version 4.2, for instrument setup and BD CellQuest™ Pro or BD CellQuest™ software for acquisition and analysis. Refer to the appropriate software user's guide for detailed information.

PROCEDURE

For more details and troubleshooting tips, refer to the appropriate application note on our website.

1. Remove an aliquot of BFA from the freezer and dilute 1:10 with sterile PBS.
2. Label a 15-mL polypropylene tube *Activated;* add 0.5 mL of heparinized whole blood, antigen at titer (or other activation agent), 5 µL of CD28/CD49d monoclonal antibody cocktail, and 10 µL of diluted BFA stock to each tube.
3. Label a second 15-mL polypropylene tube *Unstimulated;* add 0.5 mL of heparinized whole blood, 5 µL of CD28/CD49d monoclonal antibody cocktail, and 10 µL of diluted BFA stock to each tube.
4. Vortex each tube gently and incubate 6 hours at 37°C.
   NOTE: If you are using a specific antigen for the activation agent, you should activate an additional 0.5 mL of blood with a strong activation agent, such as SEB (final concentration of 1 µg/mL of blood), and stain with an isotype control and anti-cytokine antibody. This tube is used as a positive control and simplifies gating.
5. Add 50 µL of EDTA solution in PBS to each tube. Vortex vigorously and incubate 15 minutes at room temperature. Vortex again on high setting for 10 seconds.

6. **If cells are to be stained fresh, proceed with step 6a; if cells are to be frozen for later staining, proceed with step 6b.**

   6a

   - 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 7.

   6b

   - 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*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 6a, Tubes 1–4.
   - Proceed to step 9.
7. 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.
8. Add 2 mL of wash buffer to each tube, and centrifuge at 500*g* for 5 minutes at room temperature.
9. Decant the supernatant, and add 0.5 mL of 1X BD FACS Permeabilizing Solution 2 (dilute 10X solution 1:10 with DI water before use) to each tube.
10. Vortex to resuspend the pellet. Incubate for 10 minutes at room temperature.
11. Add 2 mL of wash buffer to each tube, and centrifuge at 500*g* for 5 minutes at room temperature.
12. Decant the supernatant, and add 20 µL of the specific BD FastImmune Anti-Hu–IFN-γ/CD69/CD8/CD3 to each of the AS and US tubes. Add 20 µL of the BD FastImmune γ_{2a}/γ_1/CD8/CD3 isotype control to each of the AIC and UIC tubes. Incubate at room temperature for 30 minutes in the dark.
13. Add 2 mL of wash buffer to each tube, and centrifuge at 500*g* for 5 minutes at room temperature.
14. 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. It is therefore recommended that decantation is used to remove the supernatant instead of the typical aspiration.

DATA ACQUISITION AND ANALYSIS

The figures that follow show representative data performed on whole blood and analyzed on a dual-laser BD FACS brand flow cytometer with laser excitation at 488 nm and 635 nm.

1. Analyze on a BD FACS brand flow cytometer.
2. Use BD Calibrite beads and appropriate software (BD FACSComp, version 4.2, and BD Autocomp™, version 3.0.2) for setting photomultiplier tube (PMT) voltages and fluorescence compensation and for checking instrument sensitivity before use. Refer to the beads product insert and the appropriate software user's guide for flow cytometric setup, acquisition, and analysis.
3. Acquire data with BD CellQuest Pro or BD CellQuest software, using a forward scatter (FSC) threshold. During acquisition, set up a CD3 vs CD8 dot plot (Figure 1). 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.

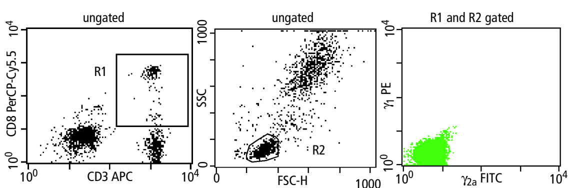

Figure 1 Gating strategy and isotype control

4. Analyze data using BD CellQuest Pro, BD CellQuest, or BD Paint-A-Gate™ Pro software. Display data as Anti-Hu–IFN-γ vs CD69 dot plots to determine cytokine expression (Figure 2). The dot plots are gated with the same requirements for G3 as determined during acquisition ($CD3^+/CD8^+$ and lymphocyte scatter characteristics). See Figure 2.

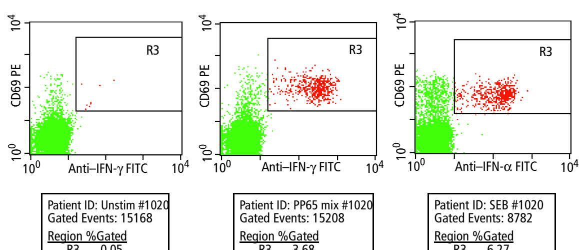

Figure 2 Unstimulated, CMV-pp65 peptide mix stimulated, and SEB-stimulated samples, R1 and R2 gated

5. To obtain statistics, draw a region around the CD69 and Anti-Hu–IFN-γ double-positive events in a positive control sample (for example, SEB), and apply this region to your sample files (Figure 2). The % gated statistic gives frequency of cytokine-producing $CD8^+$ cells.
   NOTE: It is important to include $CD8^{dim}/CD3^{dim}$ cells for maximum detection of cytokine-positive events. See Figure 3.

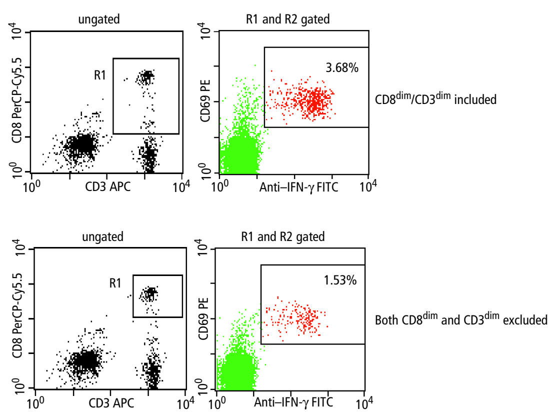

Figure 3 Precision of gating, inclusion of $CD8^{dim}/CD3^{dim}$ cytokine-positive events

Calculating the Specific Response

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.

LIMITATIONS

Specific responses will vary by donor and by antigen used. Processing of complex antigens and presentation of relevant peptide epitopes on host class I–MHC molecules are inefficient when antigen is used in soluble form. Optimal class I–restricted CD8 T-cell responses are obtrained by exogenous addition of peptide(s) or peptide mixes spanning the entire protein to whole blood or PBMCs.

TROUBLESHOOTING

Problem	Possible Cause	Solution	Comments
Poor cell recovery	Inadequate centrifugation	Perform all spins at 500g 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 CD8 count (for example, in HIV-infected samples)	Stain 200–400 µL of blood per sample.	Increase volume of BD FACS Lysing Solution accordingly; other reagent volumes need not be adjusted.
No cytokine-positive cells	Inadequate activation, permeabilization, and/or staining	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. Use a freshly diluted aliquot of BFA, and store aliquots of BFA at –20°C.	See reference number 6 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. Processing of complex antigens and presentation of relevant peptide epitopes on host class I–MHC molecules are inefficient when antigen is used in soluble form. Optimal class I–restricted CD8 T-cell responses are obtained by exogenous addition of peptide(s) or peptide mixes spanning the entire protein to whole blood or PBMCs.
Low intensity of cytokine staining	Inadequate permeabilization and/or staining	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 Lyse/No- Wash 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 CD8 vs CD3 to include CD3 dim and CD8 dim lymphocytes.	Activated lymphocytes can down-modulate CD8 to become CD8 dim. Activated platelets can bind to lymphocytes and therefore require an additional marker to distinguish. See reference number 6 for information on exclusion channel or refer to the appropriate BD application note.
Long run time needed to acquire adequate number of CD8⁺ events	Excessive dilution of samples in fixative prior to 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 return the cytometer to Run.
	Poor cell recovery or limited number of CD8⁺ cells in sample	See *Poor cell recovery* in this table.	

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