



BD FastImmune

CD4 Intracellular Cytokine Detection Kit

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

Anti-Hu-IFN- γ Catalog No. 340970

Anti-Hu-IL-2 Catalog No. 340971

Anti-Hu-TNF- α Catalog No. 340972

11/01

23-4767-01

INTENDED USE

The BD FastImmune™ CD4 Intracellular Cytokine Detection Kit is designed for the detection of intracellular cytokines and the activation marker CD69 in antigen-activated CD4⁺ T lymphocytes in whole blood.¹⁻⁷ Applications include studies of T-cell responses to antigens, such as herpes viruses,^{1,3,6,7} HIV,⁸⁻¹⁰ and tumor antigens.¹¹

SUMMARY AND EXPLANATION

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. Extra BD FACS™ Lysing Solution* and BD FACS Permeabilizing Solution 2 are provided for additional antibody staining.

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 CD4⁺ T cells that respond to specific soluble antigens in short term restimulation assays.¹⁻¹³ Whole blood is stimulated with antigen and co-stimulatory antibodies (CD28 and CD49d)¹³ 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. 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 CD4 Intracellular Cytokine Detection Kit includes:

- BD FastImmune Brefeldin A (BFA) Solution
- BD FastImmune EDTA Solution
- BD FastImmune CD28/CD49d
- BD FACS Lysing Solution (10X)
- BD FACS Permeabilizing Solution 2 (10X)
- BD FastImmune γ_{2a} FITC/ γ_1 PE[†]/CD4 PerCP[‡]-Cy5.5[§] isotype control
- BD FastImmune anti-cytokine FITC/CD69 PE/CD4 PerCP-Cy5.5 (kit-specific):
 - Anti-Hu-IFN- γ kit (BD Catalog No. 340970[¶]), containing BD FastImmune Anti-Hu-IFN- γ FITC/CD69 PE/CD4 PerCP-Cy5.5
 - Anti-Hu-IL-2 kit (BD Catalog No. 340971[¶]), containing BD FastImmune Anti-Hu-IL-2 FITC/CD69 PE/CD4 PerCP-Cy5.5
 - Anti-Hu-TNF- α kit (BD Catalog No. 340972[¶]), containing BD FastImmune Anti-Hu-TNF- α FITC/CD69 PE/CD4 PerCP-Cy5.5

Storage and Handling

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

Store each kit at 2° to 8°C.

BD FACS Lysing Solution (10X) and BD FACS Permeabilizing Solution 2 (10X) are each stable for the period shown on the appropriate bottle label when stored as directed. Do not use either reagent if discoloration occurs or a precipitate forms. Dilute 1:10 in deionized (DI) water. Use at room temperature.

When stored at 2° to 8°C, each antibody reagent is stable until the expiration date shown on the label. Do not use after the expiration date. Conjugated forms should **not** be frozen and should be protected from prolonged exposure to light. 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

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 risks 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.

WARNING: BD FastImmune Brefeldin A (BFA) Solution also contains dimethyl sulfoxide (DMSO). The toxicological properties of BFA and DMSO have not been determined. Keep out of reach of children (S2). Keep container in a well-ventilated place (S9). Keep away from food, drink, and animal feedingstuff (S13). Can be harmful by all routes of entry, including inhalation. Danger of serious damage to health by prolonged exposure (R48). Can irritate

* US Patent Nos. 4,654,312; 4,902,613; and 5,098,849

† US Patent No. 4,520,110, 4,859,582, and 5,055,556; European Patent No. 76,695; and Canadian Patent No. 1,179,942

‡ US Patent No. 4,876,190

§ US Patent Nos. 5,268,486; 5,486,616; 5,569,587; 5,569,766; and 5,627,027

¶ Use of this product 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.

eyes, respiratory system, and skin (R36/37/38). Prolonged or repeated skin contact can cause dermatitis. DMSO is readily absorbed through skin and can increase the tendency for other chemicals, such as BFA, to penetrate the skin. If swallowed, seek medical advice immediately and show the container or label (S46). DMSO and BFA are not listed (IARC, NTP, OSHA) as cancer causing agents.

All specimens and materials with which they come into contact are considered biohazards and should be handled as if capable of transmitting infection.^{14,15}

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.

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

WARNING: Sodium azide is harmful if swallowed (R22). Keep out of reach of children (S2). Keep away from food, drink, and animal feedingstuff (S13). Wear suitable protective clothing (S36). If swallowed, seek medical advice immediately and show this container or label (S46). Contact with acids liberates very toxic gas (R32). Azide compounds should be flushed with large volumes of water during disposal to avoid deposits in lead or copper plumbing where explosive conditions can develop.

SPECIMEN COLLECTION AND PREPARATION _____

Blood should be collected in sodium heparin as 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) or peptides, but it also works with superantigens such as SEB.
- wash buffer: First prepare stock solutions of 5% BSA in 1X phosphate-buffered saline (PBS) (filter sterilize) and 10% NaN₃ in 1X PBS. Then prepare 500 mL of wash buffer by adding 50 mL of 5% BSA stock solution and 5 mL of 10% NaN₃ stock solution to 445 mL of 1X sterile PBS. This represents final concentrations of 0.5% BSA and 0.1% NaN₃ in PBS. Store at 4°C.
- 1% paraformaldehyde solution prepared in PBS containing 0.1% sodium azide. Store at 2° to 8°C in amber glass for up to 1 week. Refer to the product insert for warnings.
- 15-mL polypropylene tubes (BD Catalog No. 352096)
- 5-mL polystyrene tubes (BD Catalog No. 352058)
- vortex mixer
- micropipettor with tips (BD Electronic Pipette, BD Biosciences 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.
- CaliBRITE™ beads (BD Biosciences Catalog No. 349502) and CaliBRITE PerCP-Cy5.5-labeled beads (BD Biosciences Catalog No. 345006, beads only; 345036, beads plus Bead Dilution Buffer). Refer to the CaliBRITE beads product inserts for instructions.
- FACSCOMP™ software, version 4.2, for instrument setup and CellQuest™ Pro or 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 found on our website (www.bdfacs.com) or ask your local BD representative for a copy.

1. Label a 15-mL polypropylene tube *Activated*; add 0.5 mL of heparinized whole blood, antigen at titer (or other activation agent), and 5 µL of CD28/CD49d monoclonal antibody cocktail.

Label a second 15-mL polypropylene tube *Unstimulated*; add 0.5 mL of heparinized whole blood and 5 µL of CD28/CD49d monoclonal antibody cocktail.

Vortex each tube gently and incubate 2 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.

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. 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.
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 μ L 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.
 7. 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.
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 μ L of the specific BD FastImmune anti-cytokine FITC/CD69 PE/CD4 PerCP-Cy5.5 to each of the AS and US tubes. Add 20 μ L of the BD FastImmune γ_{2a} FITC/ γ_1 PE/CD4 PerCP-Cy5.5 isotype control to each of the AIC and UIC tubes. 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 μ L 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

1. Analyze on a FACS brand flow cytometer. The figures that follow show representative data from experiments performed on normal whole blood and analyzed on a FACS brand flow cytometer with laser excitation at 488 nm.
2. Use CaliBRITE beads and appropriate software (FACSCComp, version 4.2 or later, or AutoCOMP™, version 3.0.2) for setting photomultiplier tube (PMT) voltages and fluorescence compensation and for checking instrument sensitivity before use. Refer to the CaliBRITE beads product inserts and software user's guide for flow cytometric setup, acquisition, and analysis.
3. Acquire data with CellQuest Pro or 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 (Figure 1). 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.

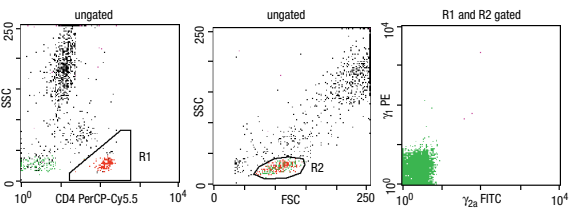


Figure 1 Gating strategy and isotype control

4. Display data as CD69 vs cytokine dot plots to determine cytokine expression (Figures 2A and 2B). Analyze data using CellQuest or PAINT-A-GATE PRO™ software.

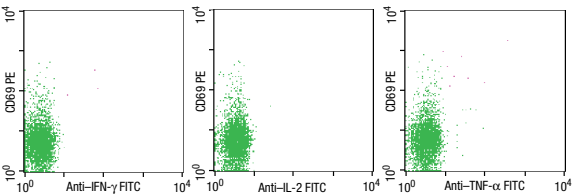


Figure 2A Unstimulated samples, R1 and R2 gated

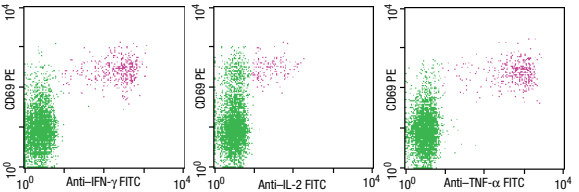


Figure 2B CMV-activated samples, R1 and R2 gated

5. To obtain statistics, draw a region around the CD69 and cytokine double-positive events in a positive control sample (Figure 3), 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.

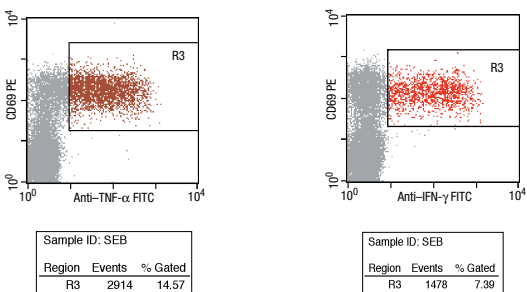


Figure 3 SEB-activated control, R1 and R2 gated

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 cytokine.

TROUBLESHOOTING

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 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 <i>Low numbers of cytokine-positive cells</i> and <i>Low intensity of cytokine staining</i> 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 <i>Low intensity of cytokine staining</i> in this table. The number of cytokine-producing cells will vary depending upon the antigen and cytokine, and the individual donor.
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. Minimize residual volume after each wash by shaking the tube once or twice after decanting supernatant. Use 500 µL/sample of BD FACS Permeabilizing Solution 2 for a full 10 minutes at room temperature. Vortex thoroughly to resuspend cells in BD FACS Permeabilizing Solution 2.	Do NOT dilute BD FACS Lysing Solution or BD FACS Permeabilizing Solution 2 in PBS or other buffers. A low residual volume of about 100 µL is needed to avoid excessive dilution of BD FACS Permeabilizing Solution 2 or staining mAb. BD FACS Lysing Solution and BD FACS Permeabilizing Solution 2 should be used at room temperature, and all incubations should be at room temperature.
High background in unstimulated samples	Poor compensation	Set up using 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 CD4 vs SSC to include CD4 dim lymphocytes, but exclude monocytes, platelets, and dead lymphocytes. Use an exclusion channel, such as CD33 APC + CD62P APC, to simplify exclusion of monocytes and activated platelets.	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 non-specific staining. 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 CD4 ⁺ 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 CD4 ⁺ cells in sample	See <i>Poor cell recovery</i> in this table.	

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WARRANTY

The product sold hereunder is warranted only to conform to the quantity and contents stated on the label at the time of delivery to the customer. There are no warranties, expressed or implied, that extend beyond the description on the label of the product. BD's sole liability is limited to either replacement of the products or refund of the purchase price. BD is not liable for property damage, personal injury, or economic loss caused by the product.

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