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. Applications include studies of T-cell responses to antigens, such as herpes viruses, HIV, and tumor antigens.1-11

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. Extra BD FACS™ Lysing Solution and BD FACS™ Permeabilizing Solution 2 (Catalog No. 347692) 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. 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 cytokines 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 Solution
- BD FastImmune™ EDTA Solution
- BD FastImmune™ CD28/CD49d
- BD FACS Lysing Solution (10X)
- BD FACS Permeabilizing Solution 2 (10X)
- BD FastImmune™ γ2a FITC/γ1 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 (Catalog No. 340970), containing BD FastImmune™ Anti-Hu–IFN-γ FITC/CD69 PE/CD4 PerCP-Cy5.5
- Anti-Hu–IL-2 kit (Catalog No. 340971), containing BD FastImmune™ Anti-Hu–IL-2 FITC/CD69 PE/CD4 PerCP-Cy5.5
- Anti-Hu–TNF-α kit (Catalog No. 340972), containing BD FastImmune™ Anti-Hu–TNF-α FITC/CD69 PE/CD4 PerCP-Cy5.5

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.

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Anti-Hu–IFN-γ Catalog No. 340970
Anti-Hu–IL-2 Catalog No. 340971
Anti-Hu–TNF-α Catalog No. 340972

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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 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.

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.

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) or peptides, 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
- BD FACS™ brand flow cytometer. Refer to the appropriate instrument user’s guide for information.
- BD Calibrite™ beads (Catalog No. 349502) and BD Calibrite™ PerCP-Cy5.5–labeled beads (Catalog No. 345006, beads only; 345036, beads plus Bead Dilution Buffer). Refer to the beads product inserts 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. 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.
2. 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.

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

4. 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.

5. Add 30 μL of EDTA solution in PBS to each tube. Vortex vigorously, and incubate 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 1. 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)

   2. Aliquot 100 μL of each activated blood into the AIC tube and the AS tube.
   3. Aliquot 100 μL of each unstimulated blood into the UIC tube and the US tube.
   4. Proceed to step 7.

6b 1. 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.
2. Vortex and incubate at room temperature, and directly place the tubes in a freezer at –80°C.
3. 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 50 minutes at room temperature.
4. Decant the supernatant, and resuspend the pellet in 0.5 mL of wash buffer. 
5. 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 20 μL of the specific BD FastImmune anti-cytokine/CD69/CD4 to each of the AS and US tubes. Add 20 μL of the BD FastImmune γ2a/γ1/CD4 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 g for 5 minutes at room temperature.

11. Decant the supernatant, and add 20 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 from experiments performed on normal whole blood and analyzed on a BD FACS brand flow cytometer with laser excitation at 488 nm.

1. Analyze on a BD FACS brand flow cytometer.
2. Use BD Calibrite beads and appropriate software (BD FACSComp™, version 4.2 and later, or 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 inserts and software user's guide for flow cytometric setup, acquisition, and analysis.
3. Acquire data with BD CellQuest Pro 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 (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 within R1 and R2.
4. Display data as CD69 vs cytokine dot plots to determine cytokine expression (Figures 2A and 2B). Analyze data using BD CellQuest or BD Pain-A-Gate™ Pro software.
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.

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

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause</th>
<th>Solution</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor cell recovery</td>
<td>Inadequate centrifugation</td>
<td>Perform all spins at 500g for at least 5 minutes.</td>
<td>Fixed and permeabilized cells are more buoyant than live cells; therefore, they require higher centrifugal force to pellet.</td>
</tr>
<tr>
<td>Loss of pellet on aspiration</td>
<td>Cell pellets are loose and easily disturbed by aspiration.</td>
<td>Decant supernatants. Cell pellets are loose and easily disturbed by aspiration.</td>
<td></td>
</tr>
<tr>
<td>Low CD4 count</td>
<td>Inadequate activation, permeabilization, and/or staining</td>
<td>Stain 200–400 μL of blood per sample. Increase volume of BD FACS Lysing Solution accordingly; other reagent volumes need not be adjusted.</td>
<td></td>
</tr>
<tr>
<td>No cytokine-positive cells</td>
<td>Inadequate activation, permeabilization, and/or staining</td>
<td>Use a positive control, such as SEB activation, to assess the immune competence of the donor in question.</td>
<td></td>
</tr>
<tr>
<td>Lack of immune competence in the donor</td>
<td>Use a positive control, such as SEB activation, to assess the immune competence of the donor in question.</td>
<td>Perform SEB activation on a normal donor as a positive control for these steps.</td>
<td></td>
</tr>
<tr>
<td>Wrong anticoagulant used for blood collection</td>
<td>Only sodium heparin anticoagulant; Do not use lithium heparin, EDTA, or other calcium-chelating anticoagulants.</td>
<td>Calcium is required for lymphocyte activation; calcium-chelating anticoagulants prevent activation.</td>
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WARRANTY
Unless otherwise indicated in any applicable BD general conditions of sale for non-US customers, the following warranty applies to the purchase of these products.

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