1. INTENDED USE

BD CD3 fluorescein isothiocyanate (FITC) reagent is a single-color direct immunofluorescence reagent for identifying and enumerating T lymphocytes using fluorescence microscopy or flow cytometers such as the BD FACSCalibur™, BD FACSort™, BD FACScan™, or BD FACS™ Analyzer.

2. SUMMARY AND EXPLANATION

Human lymphocytes can be divided into three major subpopulations: T, B, and natural killer (NK) lymphocytes, based on biological function and cell surface antigens. T lymphocytes are responsible for antigen-specific cell-mediated immunity and regulate the secretion of immunoglobulin by B lymphocytes. T lymphocytes also express cell surface antigens that are indicators of cell differentiation.

Traditionally, T lymphocytes have been enumerated using the property of these cells to form rosettes with sheep red blood cells (SRBCs). The development of hybridoma technology has made it possible to develop immunologically pure antibodies highly specific for individual cell types, such as T lymphocytes.

Clinical Applications

Quantitation of T lymphocytes in peripheral blood has been found useful in categorizing some autoimmune and immunodeficiency diseases. The percentages of circulating T lymphocytes and T-lymphocyte subsets have been shown to vary among patients with systemic lupus erythematosus (SLE) and...
to correlate with clinical features of the disease. Patients with other autoimmune diseases such as rheumatoid arthritis may have reduced numbers of T lymphocytes. Therapy for certain immunodeficiency diseases can be monitored by following the relative percentage of T lymphocytes in peripheral blood. For example, severe combined immunodeficiency disease (SCID) is characterized by reduced numbers of T lymphocytes. In addition, patients with acquired immune deficiency syndrome (AIDS) complicated by opportunistic infections or Kaposi's sarcoma typically have significantly reduced numbers of peripheral blood T lymphocytes, as well as altered T-lymphocyte subset ratios, as compared to healthy controls.

Following successful bone marrow engraftment, the percentage of T lymphocytes rises. However, phenotypic analysis of the peripheral blood lymphocytes of bone marrow transplant patients has shown that the absolute number of circulating T lymphocytes can be reduced for up to 1 year following transplant, and that T-lymphocyte subset imbalances and abnormal immune function can persist beyond this time.

3. PRINCIPLES OF THE PROCEDURE

BD CD3 FITC reagent specifically binds to T lymphocytes, which can be detected when the fluorochrome is excited by light from a flow cytometer or fluorescence microscope. Lymphocytes that have bound antibody display green fluorescence.

Efficient detection of T lymphocytes in peripheral blood using a FITC-conjugated monoclonal antibody depends on the elimination of interfering cells. Cell separation methods isolate mononuclear cells (monocytes and lymphocytes) from the other blood components such as red blood cells (RBCs), granulocytes, platelets, and plasma.

Viability of the mononuclear cells should be determined using a stain such as ethidium bromide/acridine orange or trypan blue. With the ethidium bromide/acridine orange method, viable cells fluoresce green and nonviable cells fluoresce orange. Viable cells may then be quantitated as a percent of the total cells. Cell viability should be greater than 90% to ensure valid monoclonal antibody staining.

The T-lymphocyte count is usually reported as percent lymphocytes rather than percent mononuclear cells. If test results are analyzed with a fluorescence microscope, only lymphocytes should be counted. If the stained sample is analyzed by a flow cytometer, percent-positive lymphocytes can often be determined directly by excluding monocytes on the basis of forward scatter (FSC) and side scatter (SSC). Since each type of flow cytometer has different operating characteristics, refer to the instrument operating manual for further technical details.

4. REAGENTS

Reagent Provided, Sufficient for 50 or 250 Tests

BD CD3 FITC reagent is a monoclonal antibody provided in buffered saline with gelatin and 0.1% sodium azide and is available in two sizes: 50 or 250 tests. It contains FITC-labeled CD3, clone SK7. The CD3 antibody is composed
of mouse IgG1 heavy chains and kappa light chains.

CD3 identifies T lymphocytes and recognizes the epsilon chain of the CD3/T-cell antigen receptor (TCR) complex.\textsuperscript{13} This complex is composed of at least six proteins with a range in molecular weight of 20–30 kDa.\textsuperscript{14} The antigen recognized by CD3 antibodies is noncovalently associated with either α/β or γ/δ TCR (70–90 kDa).\textsuperscript{15}

Precautions

- For in vitro diagnostic use.
- When stored at 2°C–8°C, the antibody reagent is stable until the expiration date shown on the label. Do not use after the expiration date.
- The antibody reagent should not be frozen or exposed to direct light during storage or during incubation with cells. Keep the reagent vial dry.
- 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.
- The antibody reagent contains sodium azide as a preservative; however, care should be taken to avoid microbial contamination, which may cause erroneous results.

\textbf{WARNING} All biological specimens and materials coming into contact with them are considered biohazards. Handle as if capable of transmitting infection\textsuperscript{16,17} 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.

Concentration values are listed in the following table:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3 FITC</td>
<td>100</td>
</tr>
</tbody>
</table>

5. INSTRUMENT

The BD CD3 FITC reagent is designed for use on a flow cytometer equipped with appropriate computer hardware and software or on an epifluorescence microscope set up to excite fluorescein. The flow cytometer must be equipped to detect light scatter properties. We recommend using software such as BD CellQuest\textsuperscript{™} for data acquisition and analysis and a BD flow cytometer such as the BD FACSCalibur, BD FACSort, BD FACScan, or BD FACS Analyzer.

6. SPECIMEN AND COLLECTION PREPARATION

Collect blood aseptically by venipuncture into a sterile BD Vacutainer® EDTA blood collection tube. A minimum of 2 mL of whole blood is required for peripheral blood mononuclear cell (PBMC) preparation. Follow the collection tube manufacturer’s guidelines for the minimum volume of blood to be collected. Blood should be stained within 6 hours of venipuncture for optimal results. Anticoagulated blood may be stored at room temperature (20°C–25°C) for up to 6 hours until ready for staining. Blood samples refrigerated prior to staining may give aberrant results. A white blood cell (WBC) count and a differential white-cell count should be obtained from the same sample of whole blood before staining.
Interfering Conditions

Previously fixed and stored patient specimens should not be used. Whole blood samples refrigerated prior to staining may give aberrant results. Samples obtained from patients taking immunosuppressive drugs may yield poor resolution. The presence of blast cells may interfere with test results. Hemolyzed samples should be rejected.

7. PROCEDURE

Reagent Provided

CD3 FITC

Reagents and Materials Required But Not Provided

- BD Vacutainer EDTA blood collection tubes or equivalent.
- Phosphate-buffered saline (PBS) (1X) (Dulbecco’s modified, pH 7.2 ±0.2; 0.01 mol/L PO4; and 0.15 mol/L NaCl). This reagent does not contain calcium, magnesium, phenol red, or sodium azide. Filter PBS through a 0.2-µm filter before use. Store at 2°C–8°C.
- PBS with 0.1% sodium azide.
- Hank’s Balanced Salt Solution (BSS) containing 0.1% sodium azide. To obtain 10 mL of 10X BSS without calcium and magnesium (Gibco), add 90 mL of deionized water. Mix well, then add 100 mg sodium azide. Stir to dissolve. Store at 2°C–8°C.
- Ficoll-Paque separation medium for preparing a suspension of mononuclear cells.
- 3-mL disposable test tubes.
- 15-mL disposable test tubes.
- Refrigerated centrifuge capable of 400g with swinging-bucket rotor.
- 5- and 10-mL serological pipettes; Pasteur pipettes and bulbs.
- BD Calibrate beads (Catalog No. 349502). Refer to the BD Calibrate Beads instructions for use (IFU).
- Vortex mixer.
- Hemacytometer chamber and coverslip.
- Micropipettes with tips.
- Ice bath with cover.
- Paraformaldehyde, 1% weight/volume in PBS without sodium azide. Refer to Paraformaldehyde Fixation of Hematopoietic Cells Stained with Fluorochromes, Monoclonal Antibodies Source Book, Section 2.10.
- BD FACSFLOW sheath fluid (Catalog No. 342003) or equivalent.

CAUTION Use only BD FACSFLOW sheath fluid diluent to dilute BD Calibrate beads.

- 95-nm primary filter and 515-nm secondary filter for fluorescence microscope.
- Microscope slides and coverslips.
- Cell viability testing reagents and materials for the ethidium bromide/acridine orange method (refer to Viability Staining using Ethidium Bromide and Acridine Orange, Monoclonal Antibodies Source Book, Section 2.3) or the trypan blue exclusion method.

Staining the Cells

The staining procedure for PBMCs is described below. Use care to protect the

* Ficoll-Paque is a registered trademark of GE Healthcare.
tubes from direct light. Perform the procedure at room temperature (20°C–25°C), using reagents at 2°C–25°C. See Precautions in Section 4, Reagents.

**PBMC Preparation**

Peripheral blood samples are first separated using the Ficoll-Paque method. Refer to the Ficoll-Paque separation medium IFU for instructions on performing mononuclear cell separation.

1. After the cells have been separated, determine the viability of the cells by staining with ethidium bromide and acridine orange or by using trypan blue exclusion. Greater than 90% of the cells should be viable for the sample to be acceptable for use.

2. The cell suspension must be adjusted to a concentration of 1.5 to 2.5 x 10^7 cells/mL with PBS containing sodium azide. Count cells using a microscope and hemacytometer or a hematology cell counter.

3. For each sample, add 20 µL of CD3 FITC to a tube labeled Reagent. Add 50 µL PBMCs at 2 x 10^7 cells/mL to the tube.

4. Vortex for 2 seconds, and then incubate the tube for 30 minutes in an ice bath, covered to prevent light exposure.

5. Add 3 mL of cold Hank’s BSS to the tube, and vortex for 2 seconds.

6. Centrifuge the tube at 400g for 3 minutes at 2°C–8°C.

7. Aspirate the supernatant, leaving a small amount of fluid (approximately 50 µL) in the tube. Avoid disturbing the pellet.

8. Add 0.5 mL of 1% paraformaldehyde to the tube and immediately vortex for 2 seconds to resuspend cells.

9. Store the prepared tubes at 2°C–8°C in the dark until flow cytometric analysis is performed. Analyze the cells within 24 hours of staining.

**Flow Cytometry**

Analyze the stained samples within 24 hours after preparation. Vortex the cells thoroughly at low speed to reduce aggregation before running them on the flow cytometer.

**Fluorescence Microscopy**

Follow the PBMC procedure through step 8, and vortex to resuspend the cells. Prepare a slide (wet mount) using 10 µL of cell suspension. Cover with a coverslip and protect the slide from light. If the slide will not be counted immediately, seal the coverslip and store at 2°C–8°C. Slide should be read within 24 hours.

**Quality Control**

We recommend using BD Calibrite beads and appropriate software for setting the photomultiplier tube (PMT) voltages and checking instrument sensitivity prior to using BD FACSTM brand instruments. Refer to the BD Calibrite Beads IFU for alignment procedure.

We recommend that a control sample from a normal adult subject be run daily to optimize instrument settings and as a quality control check of the system.

**8. RESULTS**

Lymphocytes that bear the CD3 antigen will display apple-green ring fluorescence when examined by fluorescence microscopy or will appear as a fluorescent
cell peak when analyzed by flow cytometry.

**Flow Cytometry Using a BD FACSCalibur, BD FACSort, BD FACSscan, or BD FACS Analyzer Flow Cytometer**

Refer to the operator's manual for instrument setup. Figure 1 shows representative flow cytometry data obtained on density gradient–separated cells stained with CD3 FITC and analyzed with a BD FACS brand flow cytometer. Scatter gates were set to include only lymphocytes. Stained and unstained populations are clearly identifiable on this logarithmic display.

**Fluorescence Microscopy**

Count only lymphocytes with surface staining. Do not count monocytes or any cells with cytoplasmic staining. Count a total of 300 lymphocytes and record the number of positively stained and nonstained cells.

The percent T lymphocytes (A) can be obtained using the following equation:

$$A = \frac{X}{Y} \times 100$$

Where:

- $X$ = number of positively stained lymphocytes
- $Y$ = total number of lymphocytes

The absolute number of T lymphocytes/µL (B) can be obtained using the following equation:

$$B = \frac{C}{100} \times \frac{D}{100} \times \frac{WBC}{µL}$$

Where:

- $C$ = % T lymphocytes
- $D$ = % lymphocytes in the whole blood

![Figure 1](example.png)

**LIMITATIONS**

- Each laboratory should establish a normal range for T lymphocytes using its own test conditions.

<table>
<thead>
<tr>
<th>No</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Forward scatter vs side scatter dot plot with a gate around lymphocyte population</td>
</tr>
<tr>
<td>2</td>
<td>Control tube, FL1 vs counts</td>
</tr>
<tr>
<td>3</td>
<td>Reagent tube, FL1 vs counts</td>
</tr>
</tbody>
</table>
• The specimen should contain >90% viable cells. Nonviable cells may stain nonspecifically.

• The correlation (r values) between CD3 FITC and E-rosettes is 0.90 for flow cytometric assessment and 0.88 for microscopy. Known differences include E-rosette positive, CD3-negative NK lymphocytes. For more information, see Performance Characteristics, Section 11.

• If a total of 300 lymphocytes is not counted by microscopy, the precision of the test for T lymphocytes will be lower. If a WBC and differential count are available for the patient, initial sample volume or final sample dilution should be adjusted to ensure that 300 lymphocytes can be counted on the slide.

• Leucopenic patients or patients with low proportions of lymphocytes will sometimes not provide sufficient lymphocytes to count.

• Cells separated from whole blood by means of density gradients may not have the same relative concentrations of T and B lymphocytes as unseparated blood. This alteration is believed to be relatively insignificant for samples of blood from subjects with normal white blood counts. However, in leucopenic patients or patients with low proportions of lymphocytes, the selective loss of specific subsets may affect the accuracy of the determination.21,24

• Density gradient–separated PBMC samples from patients with lymphoma may have excessive debris, which may interfere with the flow cytometry analysis of lymphocytes.

• Prolonged contact of mononuclear cells with lymphocyte separation media may reduce cell viability. Remove cells within 5 minutes after centrifugation.

• If there is insufficient harvesting of mononuclear cells, results will not be valid.

• Incomplete density gradient separation may occur in diseases marked by changes in lymphocyte volume.

• Abnormal states of health are not always represented by abnormal percentages of T lymphocytes. That is, an individual who may be in an abnormal state of health may exhibit the same T-lymphocyte percentage as a healthy individual.

• Microscope results may be inaccurate unless a high-quality, properly aligned epifluorescence microscope is used.

10. EXPECTED VALUES

The normal range (central 95%) of T lymphocytes was determined on Ficoll-Paque–separated PBMCs from healthy subjects using the BD FACS Analyzer flow cytometer and a fluorescence microscope.

<table>
<thead>
<tr>
<th></th>
<th>BD FACS Analyzer</th>
<th>Fluorescence Microscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Range</td>
<td>63.0%–89.4%</td>
<td>48.2%–83.1%</td>
</tr>
<tr>
<td>Mean</td>
<td>77.0%</td>
<td>67.6%</td>
</tr>
<tr>
<td>Number of Samples</td>
<td>47</td>
<td>49</td>
</tr>
</tbody>
</table>

Expected values were also determined on 52 to 60 random hospital admissions. These values were combined with the values obtained from documented normal subjects to obtain the following values.

<table>
<thead>
<tr>
<th></th>
<th>BD FACS Analyzer</th>
<th>Fluorescence Microscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Range</td>
<td>59.6%–89.4%</td>
<td>42.0%–83.1%</td>
</tr>
</tbody>
</table>
11. PERFORMANCE CHARACTERISTICS†

Precision
The precision of this assay was determined on quadruplicate samples of Ficoll-Paque–separated PBMCs from healthy subjects.

<table>
<thead>
<tr>
<th></th>
<th>BD FACS Analyzer</th>
<th>Fluorescence Microscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>77.0%</td>
<td>66.4%</td>
</tr>
<tr>
<td>Number of Samples</td>
<td>47</td>
<td>109</td>
</tr>
</tbody>
</table>

Comparison with E-Rosette Technique
Comparison of test results with those obtained using a standard E-rosette technique showed that the CD3 FITC test gave good correlation. Ficoll-Paque–separated PBMCs from healthy subjects and immune-suppressed subjects were used.

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.

THE PRODUCTS SOLD HEREUNDER ARE WARRANTED ONLY TO CONFORM TO THE QUANTITY AND CONTENTS STATED ON THE LABEL OR IN THE PRODUCT LABELING AT THE TIME OF DELIVERY TO THE

CUSTOMER. BD DISCLAIMS HEREBY ALL OTHER WARRANTIES, EXPRESSED OR IMPLIED, INCLUDING WARRANTIES OF MERCHANTABILITY AND FITNESS FOR ANY PARTICULAR PURPOSE AND NONINFRINGEMENT. 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 OR ANY INCIDENTAL OR CONSEQUENTIAL DAMAGES, INCLUDING PERSONAL INJURY, OR ECONOMIC LOSS, CAUSED BY THE PRODUCT.

TROUBLESHOOTING

<table>
<thead>
<tr>
<th>Flow Cytometry Symptom</th>
<th>Possible Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor resolution between debris and lymphocytes</td>
<td>Combination of mononuclear cells with other cells and platelets</td>
<td>Prepare and stain more PBMCs.</td>
</tr>
<tr>
<td>Poor resolution between debris and lymphocytes</td>
<td>Rough handling of cell preparation</td>
<td>Check cell viability; centrifuge cells at lower speed.</td>
</tr>
<tr>
<td>Poor resolution between debris and lymphocytes</td>
<td>Cytometer not properly aligned</td>
<td>Troubleshoot instrument.</td>
</tr>
<tr>
<td>Staining is dim or fading</td>
<td>Cell concentration too high at staining step</td>
<td>Check and adjust cell concentration; repeat staining with fresh sample of PBMCs.</td>
</tr>
<tr>
<td>Staining is dim or fading</td>
<td>Insufficient reagent</td>
<td>Repeat staining with increased amount of CD3 FITC.</td>
</tr>
<tr>
<td>Staining is dim or fading</td>
<td>Cells not analyzed within 24 hours of staining</td>
<td>Repeat staining with fresh sample of PBMCs. Analyze promptly.</td>
</tr>
<tr>
<td>Staining is dim or fading</td>
<td>Improper medium preparation (sodium azide omitted)</td>
<td>Use sodium azide in staining medium and washing steps.</td>
</tr>
</tbody>
</table>
# REFERENCES


<table>
<thead>
<tr>
<th>Flow Cytometry Symptom</th>
<th>Possible Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Few or no cells</td>
<td>Cell concentration too low</td>
<td>Resuspend fresh sample of PBMCs at a higher concentration. Repeat staining and analysis.</td>
</tr>
<tr>
<td></td>
<td>Cytometer malfunctioning</td>
<td>Troubleshoot instrument.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Microscopy Symptom</th>
<th>Possible Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staining is dim or fading</td>
<td>Cell concentration too high at staining step</td>
<td>Check and adjust cell concentration with fresh sample of PBMCs.</td>
</tr>
<tr>
<td></td>
<td>Insufficient reagent</td>
<td>Check data sheet for correct amount of reagent.</td>
</tr>
<tr>
<td></td>
<td>Cells not analyzed within 24 hours of staining</td>
<td>Repeat staining with fresh sample of PBMCs. Analyze promptly.</td>
</tr>
<tr>
<td></td>
<td>Stained cells exposed to excessive light</td>
<td>Repeat staining with fresh sample of PBMCs. Avoid exposure of stained cells to light.</td>
</tr>
<tr>
<td></td>
<td>Improper medium preparation (sodium azide omitted)</td>
<td>Use sodium azide in staining medium and washing steps.</td>
</tr>
<tr>
<td>Not enough cells on slide</td>
<td>Cell concentration too low</td>
<td>Check and adjust cell concentration by resuspending cells in a smaller volume. Repeat staining and analysis.</td>
</tr>
<tr>
<td>Air bubbles in microscope field</td>
<td>Slide dried before analysis</td>
<td>Prepare new slide and seal correctly or read promptly.</td>
</tr>
<tr>
<td>Considerable cytoplasmic staining</td>
<td>Large numbers of dead cells in preparation</td>
<td>Check viability of cell preparation (should be &gt;90% viable). Obtain fresh sample; separate mononuclear cells; stain and analyze.</td>
</tr>
</tbody>
</table>


