1. INTENDED USE

BD Simultest™ CD4/CD8 is a two-color direct immunofluorescence reagent for enumerating percentages of mature human helper/inducer (CD4+) and suppressor/cytotoxic (CD8+) lymphocytes in erythrocyte-lysed whole blood (LWB). The helper/suppressor ratio (CD4+/CD8+) can also be determined.

2. SUMMARY AND EXPLANATION

Human lymphocytes can be divided into three major populations based on their biologic function and cell-surface antigen expression: T lymphocytes, B lymphocytes, and natural killer (NK) lymphocytes. T lymphocytes participate in antigen-specific cell-mediated immunity and regulate the secretion of immunoglobulins by B lymphocytes. T lymphocytes can also be classified based on their functional properties as helper/inducer and suppressor/cytotoxic.

Clinical Applications*

Percentages of CD4+ and CD8+

T lymphocytes are used in monitoring the immune status of patients with immune deficiency diseases, autoimmune diseases, or immune reactions. The relative percentage of the CD4+ subset is depressed and the relative percentage of the CD8+ subset is elevated in many patients with congenital or acquired immune deficiencies such as severe combined immunodeficiency (SCID) and acquired immunodeficiency syndrome (AIDS).†

* Not all the studies in this section used BD reagents.
The percentage of suppressor/cytotoxic T lymphocytes can be outside the normal reference range in some autoimmune diseases and in certain immune reactions such as acute graft-versus-host disease (GVHD) and transplant rejection. The relative percentage of the CD8+ lymphocyte population can often be decreased in active systemic lupus erythematosus (SLE) but can also be increased in SLE patients undergoing steroid therapy.

The CD4+/CD8+ (helper/suppressor) T lymphocyte ratio, quantified as the ratio of CD4 FITC-positive lymphocytes to CD8 PE-positive lymphocytes, has been used to evaluate the immune status of patients with, or suspected of developing, autoimmune disorders or immune deficiencies. In many cases, the relative percentages of helper lymphocytes decline and suppressor lymphocytes increase in immune deficiency states. These states can also be marked by T-cell lymphopenia. In addition, the ratio has been used to monitor bone marrow transplant patients for onset of acute GVHD. While a useful indicator, the CD4+/CD8+ lymphocyte ratio has specific limitations.

3. **PRINCIPLES OF THE PROCEDURE**

When monoclonal antibody reagents are added to human whole blood, the fluorochrome-labeled antibodies bind specifically to antigens on the surface of leucocytes. Monoclonal antibodies can be used to identify lymphocyte subpopulations.

An aliquot of the stained patient sample is introduced into the flow cytometer and passed in a narrow stream through the path of a laser beam. The stained cells fluoresce when excited by the laser beam and the emitted light is collected and processed by the flow cytometer.

4. **REAGENT**

Reagent Provided, Sufficient for 50 Tests

The BD Simultest CD4/CD8 reagent, sufficient for 50 tests, is provided in 1 mL of buffered saline with gelatin and 0.1% sodium azide. It contains FITC-labeled CD4, clone SK3, to identify the helper/inducer lymphocyte population, and PE-labeled CD8, clone SK1, to identify the suppressor/cytotoxic lymphocyte population. The fluorescein-to-protein ratio (F:P) for BD IgG monoclonal antibody reagents is 2 to 5. The F:P ratio for CD4 FITC has been optimized for its intended use.

The CD4 antibody is composed of mouse IgG1 heavy chains and kappa light chains. The CD4 antibody recognizes the CD4 antigen, which interacts with class II molecules of the major histocompatibility complex (MHC) and is the primary receptor for HIV.
The CD8 antibody is composed of mouse IgG1 heavy chains and kappa light chains. The CD8 antigen is present on the human suppressor/cytotoxic T-lymphocyte subset as well as on a subset of NK lymphocytes. The CD8 antigen is expressed as a disulfide-linked bimolecular complex with a 32-kilodalton (kDa) α subunit. The cytoplasmic domain of the α-subunit of the CD8 antigen is associated with the protein tyrosine kinase p56lck.

**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 can cause erroneous results.

**WARNING** All biological specimens and materials coming into 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.

### 5. INSTRUMENT

BD Simultest CD4/CD8 reagent is designed for use on a BD FACS™ brand flow cytometer equipped with appropriate computer hardware, software, and gating electronics. The flow cytometer must be equipped to detect two-color fluorescence, forward scatter (FSC), and side scatter (SSC). We recommend using BD Simulset™ software, version 2.5 or later, for data acquisition and analysis.

All performance characteristics were obtained using a BD FACS™ flow cytometer. Other systems can have different characteristics and should be verified by the user.

### 6. SPECIMEN COLLECTION AND PREPARATION

Collect blood aseptically by venipuncture into a sterile (lavender top) BD Vacutainer® EDTA blood collection tube or equivalent. A minimum of 1 mL of whole blood is required for this procedure. Blood should be stained within 6 hours of venipuncture for optimal results. Anticoagulated blood can be stored at room temperature (20°C–25°C) for up to 6 hours until ready for staining. Blood samples refrigerated prior to staining can 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. An acceptable WBC concentration range is from 3.5 x 10^3 to 9.8 x 10^3 WBC/µL. Samples with counts greater than 9.8 x 10^3 WBC/µL must be diluted with 1X phosphate-buffered saline (PBS) containing 0.1% sodium azide. For samples with counts less than 3.5 x 10^3 WBC/µL, more blood might be needed and a separation
procedure can be required to concentrate the cells.

**Interfering Conditions**

Previously fixed and stored patient specimens should not be used. Whole blood samples refrigerated prior to staining can give aberrant results. For optimal results, blood samples should be stained within 6 hours of venipuncture. Samples obtained from patients taking immunosuppressive drugs can yield poor resolution. The presence of blast cells or unlysed or nucleated red blood cells (RBCs) can interfere with test results. Hemolyzed samples should be rejected. Follow the collection tube manufacturer’s guidelines for the minimum volume of blood to be collected.

**CAUTION** Use standard precautions when obtaining, handling, and disposing of all human blood samples and potentially carcinogenic reagents.

### 7. PROCEDURE

**Reagent Provided**

See Reagent Provided and Precautions in Section 4, Reagent.

**Reagents and Materials Required But Not Provided**

- BD Simultest™ Leucogate™ (CD45/CD14) reagent, 1 mL (Catalog No. 342408). For determining a lymphocyte analysis gate, see the **BD Simultest Leucogate instructions for use (IFU)** and the **BD Simulset Software User’s Guide**. Store at 2°C–8°C.
- BD Simultest™ Isotype Control γ/γ2a (IgG1/IgG2a) (both keyhole limpet hemocyanin-specific), 1 mL (Catalog No. 342409). Store at 2°C–8°C.
- BD FACS™ lysing solution (10X) (Catalog No. 349202). For dilution instructions and warnings, see the IFU.
- BD Calibrite™ beads (Catalog No. 349520). For detailed information on use, see the **BD Calibrite Beads IFU**.
- BD Vacutainer EDTA blood collection tubes or equivalent.
- Falcon® disposable 12 x 75-mm polystyrene test tubes or equivalent.
- Vortex mixer.
- Low-speed centrifuge (minimum speed 200g) with swinging-bucket rotor and 12 x 75-mm tube carriers.
- Vacuum aspirator with trap.
- Micropipettor with tips.
- BD CellWASH™ (Catalog No. 349524) or a wash buffer of PBS with 0.1% sodium azide.
- BD CellFIX™ (Catalog No. 340181) or 1% paraformaldehyde solution in PBS with 0.1% sodium azide. Store at 2°C–8°C in amber glass for up to 1 week.
- BD FACSVia™ sheath fluid (Catalog No. 342003) or equivalent.

**CAUTION** Use only BD FACSVia sheath fluid diluent to dilute BD Calibrite beads.

- Reagent-grade (both distilled and deionized) water.

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† Falcon is a registered trademark of Corning Incorporated.
Staining and Fixing the Cells

Whole blood samples are first stained with BD Simultest Leucogate (tube A), BD Simultest Control (tube B), and BD Simultest CD4/CD8 (tube C) reagents. Diluted (1X) BD FACS lysing solution is used to lyse RBCs following staining. Use care to protect the tubes from direct light. Perform the procedure at room temperature (20°C–25°C) using room temperature reagents. See Precautions in Section 4, Reagent.

1. For each patient sample, label three 12 x 75-mm tubes A, B, and C. Also label each tube with the sample identification number.

2. Place 20 µL of BD Simultest Leucogate reagent into tube A, 20 µL of BD Simultest Control reagent into tube B, and 20 µL of BD Simultest CD4/CD8 reagent into tube C.

3. For each sample tube, use a fresh micropipettor tip and carefully add 100 µL of well-mixed, anticoagulated whole blood into the bottom of each of the three labeled tubes. The recommended WBC concentration is 3.5 x 10^3 to 9.8 x 10^3 WBC/µL. Vortex thoroughly at low speed for 3 seconds and incubate for 15 to 30 minutes at room temperature (20°C–25°C).

   NOTE Protect samples from direct light during this incubation procedure and use care to prevent blood from running down the side of the tube. If whole blood remains on the side of the tube, it will not be stained with the reagent.

4. Add 2 mL of room temperature (20°C–25°C) 1X BD FACS lysing solution to each tube. Immediately vortex thoroughly at low speed for 3 seconds and incubate for 10 to 12 minutes at room temperature (20°C–25°C) in the dark. Do not exceed 12 minutes.

   NOTE Avoid prolonged exposure of the cells to lytic reagents, which can cause white cell destruction.

5. Immediately after incubation, centrifuge tubes at 300g for 5 minutes at room temperature (20°C–25°C).

6. Aspirate the supernatant leaving approximately 50 µL of residual fluid in the tube to avoid disturbing the pellet.

7. Vortex thoroughly at low speed to resuspend the cell pellet in the residual fluid, and then add 2 mL of BD CellWASH solution or PBS with 0.1% sodium azide to each tube. Vortex thoroughly at low speed for 3 seconds. Centrifuge at 200g for 5 minutes at room temperature (20°C–25°C).

8. Aspirate the supernatant leaving approximately 50 µL of residual fluid in the tube to avoid disturbing the pellet.

9. Vortex thoroughly at low speed to resuspend the cell pellet in the residual fluid, and then add 0.5 mL of BD CellFIX solution or 1% paraformaldehyde to each tube. Vortex thoroughly at low speed for 3 seconds. Make sure the cells are well mixed with the fixing solution.

10. The cells are now ready to be analyzed on the flow cytometer. Cap or cover the prepared tubes and store at 2°C–8°C in the dark until flow cytometric analysis. Analyze the fixed cells within 24 hours after staining. Vortex the cells thoroughly (at low speed) to reduce aggregation before running them on the flow cytometer.

**Flow Cytometry**
Follow the BD instructions for two-color flow cytometric analysis.

**Quality Control**
For optimal results, we recommend using BD Calibrite beads and BD FACSComp software for setting the photomultiplier tube (PMT) voltages, setting the fluorescence compensation, and checking instrument sensitivity prior to use of BD Simultest CD4/CD8 reagent on a BD FACScan flow cytometer.

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. Correct results for a hematologically normal patient are shown in Figure 1.

BD Simulset Control reagent is run with each patient sample to set fluorescence 1 (FL1) and fluorescence 2 (FL2) markers between negative and positively stained lymphocyte clusters and to detect the presence of nonspecific staining that would indicate erroneous patient results.

Visually inspect the dot plot for BD Simultest Control reagent tube B. If the negative cluster is diffuse and smeared over the FL2 intensity range, the marker might not be set correctly and results can be suspect.

BD Simulset software will automatically inspect the data and alert the operator with a number of possible error messages. See the **BD Simulset Software User’s Guide** for a list of possible messages. Use the following criteria for inspection of the dot plots obtained for each sample to evaluate the quality of the data obtained.

- The operator should reject the results if one or more of the following error messages is received for the normal control: no separation between cellular populations; too few lymphocytes (less than 500); excessive RBC or nucleated RBC contamination and debris (greater than 10%); or excessive monocyte (greater than 3%) or granulocyte (greater than 6%) contamination of the lymphocyte gate.
Figure 1  BD FACScan LWB sample, from a hematologically normal patient, stained with BD Simultest Leucogate (tube A), BD Simultest Control (tube B), and BD Simultest CD4/CD8 (tube C) reagents. BD Simultest Leucogate reagent was used to reduce debris, monocytes, and granulocytes in the gate shown under tube A. Dot plot displays of FL1 (x-axis) versus FL2 (y-axis) are shown for tubes B and C. Plots A, B and C show the data from tubes A, B, and C respectively.

- If there is no obvious reason for the normal control to fail, a sample from another normal control should be stained and run and the entire staining procedure repeated on all subsequent samples.

- Samples with nucleated RBCs can contain too much debris because of incomplete lysis of nucleated erythrocytes with BD FACS lysing solution. Too much debris can also occur when assaying blood samples from patients with certain hematologic disorders where red cells are difficult to lyse, as in myelofibrosis and spherocytosis. Nucleated erythrocytes will be counted as debris and, if debris exceeds 10%, the software will flag the sample as “too many nonlymphs in gate” and the sample results should be rejected.

8. RESULTS

Percent Lymphocyte Conversion
When the Percent Lymphocyte Conversion computation is performed, the CD4+ and CD8+ lymphocyte subsets are reported as a percentage of lymphocytes in the lymphocyte analysis gate. If the computation is not performed, results will be reported as a percentage of the gated events.

Three-Part Differential
For lysed whole blood, it is possible to estimate monocytes, lymphocytes, and granulocytes as a percentage of leucocytes using the BD Simultest Leucogate reagent (tube A). BD Simulset software automatically calculates a three-part differential. See the BD Simulset Software User's Guide for representative data printouts.
NOTE  The differential provided by BD Simulset software should be used only for comparison with an independent differential white cell count for quality control purposes and should not be used in place of an independent laboratory differential white cell count in patient charts or entered into BD Simulset software to obtain absolute counts.

Absolute Counts
An absolute cell count can be computed if a WBC and the lymphocyte percentage from an independent differential white cell count are obtained using standard laboratory procedures. For instructions on how to calculate absolute counts, see the BD Simulset Software User’s Guide.

9. LIMITATIONS

• Laboratories must establish their own normal reference ranges for the BD Simultest CD4/CD8 reagent parameters that can be affected by sex of patient, age of patient, and preparative technique. Race of patient can also have an effect, although sufficient data is not available to establish this. Age, sex, clinical status, and race of subjects should be known when a reference range is determined.

• Results from BD Simultest CD4/CD8 reagent must be used in conjunction with other information available from the clinical evaluation and additional independent diagnostic procedures.

• BD Simultest CD4/CD8 reagent is not intended for screening samples for the presence of leukemic cells or for use in phenotyping samples from leukemia patients. The presence of blast cells might not allow BD Simultest Leucogate reagent to set an adequate lymphocyte analysis gate. The software will flag the sample and results will not be printed.

• Absolute count values of lymphocyte subsets might not be comparable across laboratories due to variations in methods for determining white blood cell counts and/or white cell differential values.

10. EXPECTED VALUES

Leucocyte Subsets
The helper/inducer lymphocyte population present in quadrant 4 (Q4) and the suppressor/cytotoxic lymphocyte population present in quadrant 1 (Q1) of tube C are shown in Figure 1 and in the BD Simulset Software User’s Guide. The clinically significant results are found in the Physician Report.

BD has investigated the normal reference ranges for BD Simultest CD4/CD8 reagent parameters in normal male and female subjects using the BD FACScan flow cytometer at six sites (five European clinical sites and one US site). The expected normal reference ranges of helper/inducer and suppressor/cytotoxic lymphocytes and the helper/suppressor ratio for LWB are shown in Table 1.26 Adult reference ranges should not be used with pediatric blood samples.

Race can be a variable in the establishment of normal reference ranges, although insufficient data was collected by BD to determine this.
NOTE  Expected normal values can vary depending upon age, sex, or race of patient. Because of these differences, each laboratory should establish its own normal reference range for each parameter.

Table 1  Representative reference ranges for BD Simultest CD4/CD8 reagent parameters in hematologically normal adults as percentages of total gated lymphocytes (converted) (data pooled from six clinical sites)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sex</th>
<th>Age</th>
<th>n</th>
<th>Mean (%)</th>
<th>95% Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helper/inducer T lymphocytes (%)</td>
<td>Male</td>
<td>18–40</td>
<td>77</td>
<td>39.9</td>
<td>27.3–52.5</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>41–70</td>
<td>84</td>
<td>43.9</td>
<td>28.5–59.2</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>18–40</td>
<td>85</td>
<td>44.0</td>
<td>31.4–56.7</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>41–70</td>
<td>85</td>
<td>48.9</td>
<td>34.0–63.8</td>
</tr>
<tr>
<td>Suppressor/cytotoxic T lymphocytes (%)</td>
<td>Both</td>
<td>18–70</td>
<td>304</td>
<td>33.4</td>
<td>18.9–47.9</td>
</tr>
<tr>
<td>Helper/suppressor ratio</td>
<td>Male</td>
<td>18–40</td>
<td>77</td>
<td>1.2</td>
<td>0.6–2.2</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>41–70</td>
<td>84</td>
<td>1.5</td>
<td>0.6–3.0</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>18–40</td>
<td>85</td>
<td>1.3</td>
<td>0.7–2.8</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>41–70</td>
<td>58</td>
<td>1.7</td>
<td>0.8–3.1</td>
</tr>
</tbody>
</table>

Table 2  Within-sample reproducibility for BD Simultest CD4/CD8 reagent parameters (five normal subjects and five abnormal subjects) as percentages of lymphocytes (converted)

<table>
<thead>
<tr>
<th>Subjects</th>
<th>n</th>
<th>Parameter</th>
<th>Mean (%)</th>
<th>SD</th>
<th>CV</th>
<th>df</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>5</td>
<td>Helper/inducer T lymphocytes</td>
<td>46.3</td>
<td>1.5</td>
<td>3.2</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Suppressor/cytotoxic T lymphocytes</td>
<td>34.8</td>
<td>1.3</td>
<td>3.6</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Helper/suppressor ratio</td>
<td>1.4</td>
<td>0.1</td>
<td>5.2</td>
<td>15</td>
</tr>
<tr>
<td>Abnormal</td>
<td>5</td>
<td>Helper/inducer T lymphocytes</td>
<td>18.8</td>
<td>2.1</td>
<td>12.2</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Suppressor/cytotoxic T lymphocytes</td>
<td>63.5</td>
<td>2.6</td>
<td>3.7</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Helper/suppressor ratio</td>
<td>0.3</td>
<td>0.04</td>
<td>13.4</td>
<td>15</td>
</tr>
</tbody>
</table>

a. SD = standard deviation
b. CV = coefficient of variation
c. df = degrees of freedom

11. PERFORMANCE CHARACTERISTICS
Performance of the BD Simultest CD4/CD8 reagent was established by testing at one US clinical site and at BD laboratories in San Jose, California.

Within-Sample Reproducibility
Table 2 shows the average within-sample reproducibility obtained for both normal and abnormal subjects.

Between-Instrument Reproducibility
Table 3 shows the between-instrument reproducibility results.

Between-Laboratory Reproducibility
Between-laboratory reproducibility is indicated by the ability to pool the normal reference ranges for BD Simultest CD4/CD8 reagent parameters (Table 1).

BD Simultest CD4/CD8 versus Comparative Method
A summary of the results is presented in Table 4.
Cross-Reactivity

CD4 antibody reacts with monocytes as well as helper/inducer T lymphocytes.28 CD8 antibody reacts with suppressor/cytotoxic T lymphocytes as well as a subset of NK lymphocytes.16

Stability of Stained Cell Preparations

We recommend analyzing samples within 24 hours of staining.

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.

Table 3 Between-instrument reproducibility for BD Simultest CD4/CD8 reagent parameters (ten normal subjects and three instruments) as percentages of lymphocytes (converted)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean (%)</th>
<th>SDa</th>
<th>CVb</th>
<th>dfc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helper/inducer T lymphocytes</td>
<td>45.8</td>
<td>1.26</td>
<td>2.47</td>
<td>20</td>
</tr>
<tr>
<td>Suppressor/cytotoxic T lymphocytes</td>
<td>39.0</td>
<td>1.37</td>
<td>3.28</td>
<td>20</td>
</tr>
<tr>
<td>Helper/suppressor ratio</td>
<td>1.2</td>
<td>0.06</td>
<td>4.80</td>
<td>20</td>
</tr>
</tbody>
</table>

a. SD = standard deviation
b. CV = coefficient of variation
c. df = degrees of freedom

Table 4 BD Simultest CD4/CD8 reagent versus comparative method (BD Simultest IMK [CD4/CD8]) Using LWB and the BD FACScan Flow Cytometer

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Slope</th>
<th>Intercept</th>
<th>r</th>
<th>na</th>
<th>Range of Data (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helper/inducer T lymphocytes</td>
<td>1.16</td>
<td>-1.81</td>
<td>0.96</td>
<td>31</td>
<td>10–60</td>
</tr>
<tr>
<td>Suppressor/cytotoxic T lymphocytes</td>
<td>0.95</td>
<td>7.05</td>
<td>0.93</td>
<td>31</td>
<td>25–80</td>
</tr>
<tr>
<td>Helper/suppressor ratio</td>
<td>1.02</td>
<td>-0.02</td>
<td>0.98</td>
<td>31</td>
<td>0.125–2.0</td>
</tr>
</tbody>
</table>

a. Composed of normal and abnormal samples

REFERENCES


