1. INTENDED USE

BD Tritest™ CD4 fluorescein isothiocyanate (FITC)/CD8 phycoerythrin (PE)/CD3 peridinin chlorophyll protein (PerCP) is a three-color direct immunofluorescence reagent. It is used with BD Trucount™ absolute count tubes for identifying and determining absolute counts in cells/µL of mature human T lymphocytes (CD3+), helper/inducer (CD3+CD4+) T lymphocytes, and suppressor/cytotoxic (CD3+CD8+) T lymphocytes in erythrocyte-lysed whole blood.

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.

Clinical Applications

Helper/inducer lymphocytes are a subset of T lymphocytes (CD3+) that are CD4+. CD3+CD4+ counts are used to characterize and monitor some forms of immunodeficiency and autoimmune diseases. Determining counts of helper/inducer T lymphocytes can be useful in monitoring human immunodeficiency virus (HIV)-infected individuals. Individuals with HIV typically exhibit a steady decrease of helper/inducer T lymphocyte counts as the infection progresses.

Suppressor/cytotoxic lymphocytes are a subset of T lymphocytes (CD3+) that are CD8+. CD3+CD8+ counts are used to characterize and monitor some forms of immunodeficiency and autoimmune diseases.Suppressor/cytotoxic lymphocyte values lie outside the normal reference...
range in some autoimmune diseases, in certain immune reactions such as acute graft-versus-host disease (GVHD) and transplant rejection. The CD8+ subset is elevated in many patients with either congenital or acquired immune deficiencies, such as severe combined immunodeficiency (SCID) or acquired immune deficiency syndrome (AIDS). The CD8+ cell population is often decreased in active systemic lupus erythematosus (SLE), but can also be increased in SLE patients undergoing steroid therapy.

The Centers for Disease Control (CDC) recommends using reagent combinations containing CD3 antibodies for determining T-lymphocyte subsets in HIV-infected subjects. The BD Tritest CD4/CD8/CD3 reagent allows helper/inducer and suppressor/cytotoxic T lymphocytes to be identified and enumerated separately from contaminating CD3-CD4+ monocytes and CD3-CD8+ NK lymphocytes.

3. PRINCIPLES OF THE PROCEDURE

When whole blood is added to the reagent, the fluorochrome-labeled antibodies in the reagent bind specifically to leucocyte surface antigens. During acquisition, the cells travel past the laser beam and scatter the laser light. The stained cells fluoresce. These scatter and fluorescence signals, detected by the instrument, provide information about the cell’s size, internal complexity, and relative fluorescence intensity. BD Tritest reagents employ fluorescence triggering, allowing direct fluorescence gating of the lymphocyte population to reduce contamination of unlysed or nucleated red blood cells in the gate.

A known volume of sample is stained directly in a BD Trucount tube. The lyophilized pellet in the tube dissolves, releasing a known number of fluorescent beads. During analysis, the absolute number (cells/μL) of positive cells in the sample can be determined by comparing cellular events to bead events. If appropriate software such as BD Multiset™ is used, absolute counts will be determined by the software. If manually performing data analysis using software such as BD CellQuest™, simply divide the number of positive cellular events by the number of bead events, then multiply by the BD Trucount bead concentration.

4. REAGENT

Reagent Provided, Sufficient for 50 Tests

BD Tritest CD4/CD8/CD3 reagent is provided in 1 mL of buffered saline with 0.1% sodium azide. It contains FITC-labeled CD4, clone SK3; PE-labeled CD8, clone SK1; and PerCP-labeled CD3, clone SK7.

CD4 identifies helper/inducer T lymphocytes and recognizes the CD4 antigen, 59 kilodaltons (kDa), which interacts with class II molecules of the major histocompatibility complex (MHC) and is the primary receptor for HIV. The cytoplasmic portion of the antigen is associated with the protein tyrosine kinase p56Lck.

CD8 identifies suppressor/cytotoxic T lymphocytes and recognizes an antigen expressed as a disulfide-linked homodimeric complex with a 32-kDa α subunit. The cytoplasmic domain of the α subunit of the CD8 antigen is associated with the protein tyrosine kinase p56Lck. The CD8 molecule interacts
with class I MHC molecules resulting in increased adhesion between the CD8+ T lymphocytes and the target cells. Binding of the CD8 molecule to class I MHC molecules enhances the activation of resting T lymphocytes.

CD3 identifies T lymphocytes and recognizes the epsilon chain of the CD3 antigen/T-cell antigen receptor (TCR) complex. This complex is composed of at least 6 proteins that range in molecular weight from 20–30 kilodaltons (kDa). The antigen recognized by CD3 antibodies is noncovalently associated with either α/β or γδ TCR (70–90 kDa).

CD4, CD8, and CD3 antibodies are composed of mouse γ1 heavy chains and kappa light chains. BD Trucount tubes contain a freeze-dried pellet of fluorescent beads in a single-use tube. Each BD Trucount pouch contains 25 tubes, sufficient for 25 tests.

Precautions
- For In Vitro Diagnostic Use.
- Do not use the reagent if you observe any change in appearance. Precipitation or discoloration indicates instability or deterioration.

The antibody reagent contains sodium azide as a preservative; however, take care 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. Fixation has been reported to inactivate HIV.

- BD FACS™ lysing solution is required and contains diethylene glycol and formaldehyde. See the BD FACS lysing solution instructions for use (IFU) for warnings.
- The addition of a precise volume of blood is critical to achieving the result. Pipettes must be calibrated to deliver exactly 50 µL of sample. An electronic pipette that operates in the reverse pipetting mode is available through BD (see Reagents and Materials Required But Not Provided in Section 7, Procedure). If this or a similar pipette is not used, perform the reverse pipetting technique (see Reverse Pipetting in Section 7, Procedure, for a brief description). See the pipette manufacturer’s instructions for more information.
- Bead count varies by lot of BD Trucount tubes. It is critical to use the bead count shown on the current lot of BD Trucount tubes when entering this value in the software or when manually calculating absolute counts. Do not mix multiple lots of tubes in the same assay.
- BD Trucount tubes are designed for use with a specific lye/no-wash procedure. Do not attempt to threshold on forward scatter (FSC) for data collection.

Storage and Handling
- Store the reagent at 2°C–8°C. Do not use after the expiration date shown on the label.
- Do not freeze the reagent or expose it to direct light during storage or incubation with cells. Keep the reagent vial dry.
Store BD Trucount tubes in their original foil pouch at 2°C–25°C. To avoid potential condensation, open the pouch only after it has reached room temperature and carefully reseal the pouch immediately after removing a tube. Examine the desiccant each time you open the pouch. If the desiccant has turned from blue to lavender, discard the remaining tubes. Use tubes within 1 hour after removal from the foil pouch and do not use beyond the expiration date indicated on the packaging.

Concentration values are listed in the following table:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 FITC</td>
<td>1.0</td>
</tr>
<tr>
<td>CD8 PE</td>
<td>1.5</td>
</tr>
<tr>
<td>CD3 PerCP</td>
<td>6.25</td>
</tr>
</tbody>
</table>

5. INSTRUMENT

The BD Tritest CD4/CD8/CD3 reagent and BD Trucount tubes are designed for use on a flow cytometer equipped with appropriate computer hardware and software. The flow cytometer must be equipped with a 488-nm laser capable of detecting light scatter (forward and side) and three-color fluorescence with emission detectable in three ranges: 515–545 nm, 562–607 nm, and >650 nm. The instrument must be able to threshold or discriminate using the >650-nm channel. The BD FACS Loader can also be used with this product.

We recommend using BD Calibrite™ beads and BD FACSComp™ software, version 2.0 or later, for setting the photomultiplier tube (PMT) voltages, setting the fluorescence compensation, and checking instrument sensitivity before use. For users of flow cytometers manufactured by companies other than BD, see the manufacturer’s instructions for setting up three-color immunophenotyping.

BD has developed applications such as BD Multiset software, which automatically calculate absolute counts when BD Trucount tubes are used. However, other software packages can be used for data acquisition and analysis and the absolute counts can be calculated manually.

6. SPECIMEN COLLECTION AND PREPARATION

Collect blood aseptically by venipuncture into a sterile BD Vacutainer® EDTA blood collection tube. BD Tritest CD4/CD8/CD3 reagent has been validated with both liquid and dry formulations of EDTA. A minimum of 100 µL of whole blood is required for this procedure. Follow the collection tube manufacturer’s guidelines for the minimum volume of blood to be collected, to ensure proper use.

Obtain a white blood cell (WBC) count and a differential white-cell count from the same whole blood sample before staining to ensure that the WBC count is within the linear range (see Linearity in Section 11, Performance Characteristics). Anticoagulated blood stored at room temperature (20°C–25°C) must be stained within 48 hours of draw and then analyzed within 6 hours of staining. If samples are stained within 24 hours of draw, they can be analyzed within 24 hours of staining.
Interfering Conditions
Do not use previously fixed and stored patient specimens. Whole blood samples refrigerated before staining can give aberrant results. Samples obtained from patients taking immunosuppressive drugs can yield poor resolution. Blast cells can interfere with test results. Hemolyzed samples should be rejected. BD Tritest CD4/CD8/CD3 reagent has not been validated for use with heparin or acid citrate dextrose (ACD) liquid anticoagulants in determining absolute counts with BD Trucount tubes.

7. PROCEDURE
Reagent Provided
BD Tritest CD4 FITC/CD8 PE/CD3

Reagents and Materials Required But Not Provided
- BD Trucount Absolute Counting Tubes (Catalog No. 340334). (To order BD Tritest CD4/CD8/CD3 with BD Trucount tubes use Catalog No. 340401)
- BD Calibrite 3 beads (Catalog No. 340486)
- BD FACS lysing solution (10X), 100 mL (Catalog No. 349202). See the BD FACS Lysing Solution IFU for dilution instructions and warnings.
- Reagent-grade (distilled or deionized) water
- BD Vacutainer EDTA blood collection tubes or equivalent
- Vortex mixer
- Micropipettor with tips
- Bulk dispenser or pipettor (450 µL) for dispensing BD FACS lysing solution
- BD FACSFlow™ sheath fluid (Catalog No. 342003) or equivalent

CAUTION Use only BD FACSFlow sheath fluid diluent to dilute BD Calibrite beads.
- BD Trucount Controls (Catalog No. 340335)
- Lysable whole blood control (available commercially)

Staining the Cells
Lyse red blood cells following staining using diluted (1X) BD FACS lysing solution. Use care to protect the tubes from direct light. Perform the procedure at room temperature (20°C–25°C). See Precautions in Section 4, Reagent, and Interfering Conditions in Section 6, Specimen Collection and Preparation.

Reverse Pipetting
The addition of a precise volume of blood is critical to achieving the result. If a BD electronic pipette or a similar pipette that delivers a precise volume of blood is not used, perform reverse pipetting. This technique takes advantage of two stops in a pipette.
1. Depress the button to the second stop. Upon release, excess sample is drawn up into the tip.
2. Press the button to the first stop. A precise volume of sample is expelled, leaving excess sample in the tip.

Staining
1. For each patient sample, label a BD Trucount tube with the sample identification number.

NOTE Before use, verify that the BD Trucount bead pellet is intact and within the metal retainer at the
bottom of the tube. If this is not the case, discard the BD Trucount tube and replace it with another.

2. Pipette 20 µL of BD Tritest CD4/CD8/CD3 reagent into the bottom of the BD Trucount tube. Pipette just above the stainless steel retainer. Do not touch the pellet.

3. Pipette 50 µL of well-mixed, anticoagulated whole blood into the bottom of the tube.

   **NOTE** Avoid smearing blood down the side of the tube. If whole blood remains on the side of the tube, it will not be stained with the reagent. When using BD Trucount tubes, accuracy is critical. Use the reverse pipetting technique (see Reverse Pipetting section) to pipette sample onto the side of the tube just above the retainer.

4. Cap the tube and vortex gently to mix. Incubate for 15 minutes in the dark at room temperature (20°C–25°C).

5. Add 450 µL of 1X BD FACS lysing solution to the tube.

6. Cap the tube and vortex gently to mix. Incubate for 15 minutes in the dark at room temperature (20°C–25°C). The sample is now ready to be analyzed on the flow cytometer.

**Flow Cytometry**

- If using the BD FACS Loader, vortex tubes immediately before placing them into the loader racks.
- Acquire and analyze list-mode data using the appropriate application such as BD CellQuest or BD Multiset software.
- Before acquiring samples, adjust the threshold to minimize debris and ensure that populations of interest are included.

**Quality Control**

- Run a control sample daily from a normal adult subject or a commercially available whole blood control to optimize instrument settings and as a quality control check of the system.37
- Use commercial controls providing established values for percent positive and absolute counts with each run to assess system performance.
- Visually inspect the CD3 vs SSC dot plot. The T-lymphocyte population should appear as a compact, CD3+ cluster with low SSC. Do not proceed with analysis if the T-lymphocyte population is diffuse and if there is little or no separation between it and other clusters.

See Figure 1, Figure 2, and Figure 3 for representative data from a hematologically normal adult sample stained with CD4/CD8/CD3 in a BD Trucount tube.
8. RESULTS

Results are reported as the number of positive cells per microliter of blood (absolute count).

Calculating Absolute Counts

During analysis, the absolute number (cells/µL) of positive cells in the sample can be determined by comparing cellular events to bead events. If BD Multiset software is used, absolute counts will be determined by the software.

For manual data analysis using BD CellQuest or other software, simply divide the number of positive cellular events by the number of bead events. Then multiply by the BD Trucount bead concentration using the following equation:

\[ A = \frac{B}{C \times \frac{D}{E}} \]

Where:

- \( A \) = absolute count of cell population
- \( B \) = number of events in region containing cells
- \( C \) = BD Trucount bead concentration
- \( D \) = number of events in bead event
- \( E \) = number of bead events
C = number of events in absolute counts bead region
D = number of beads per test
E = test volume

9. LIMITATIONS

- Laboratories must establish their own normal reference ranges for the BD Tritest CD4/CD8/CD3 reagent parameters, which can be affected by sex of patient, age of patient, and preparative technique. Race of patient and individual variations of epitope expression can also have an effect, although sufficient data is not available to establish this. Age, sex, clinical characteristics, and race of patients should be known when a reference range is determined. Reference ranges provided are for information only.
- BD Tritest CD4/CD8/CD3 reagent has not been validated for use with heparin or acid citrate dextrose (ACD) liquid anticoagulants in determining absolute counts with BD Trucount tubes.
- BD Tritest CD4/CD8/CD3 reagent is not intended for screening samples for the presence of leukemic cells or for use in phenotyping samples from leukemia patients.
- Absolute counts are not comparable between laboratories using different manufacturers’ equipment.

10. EXPECTED VALUES

Reference Ranges

The reference ranges for CD4+, CD8+, and CD3+ T lymphocytes shown in Table 1 were determined internally at BD Biosciences in San Jose, CA, and at four external centers: Cleveland Clinic Foundation, Cleveland, OH; Johns Hopkins Hospital, Baltimore, MD; Institute of Tropical Medicine, Antwerp, Belgium; and University of North Carolina Hospital, Chapel Hill, NC. Subjects were hematologically normal adults between the ages of 18–65 years.

Table 1 Representative reference ranges in hematologically normal adults

<table>
<thead>
<tr>
<th>Subset</th>
<th>n</th>
<th>Mean</th>
<th>Lower 2.5 Percentile</th>
<th>Upper 97.5 Percentile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helper/inducer T lymphocytes (cells/μL)</td>
<td>523</td>
<td>880</td>
<td>410</td>
<td>1,590</td>
</tr>
<tr>
<td>Suppressor/cytotoxic T lymphocytes (cells/μL)</td>
<td>523</td>
<td>490</td>
<td>190</td>
<td>1,140</td>
</tr>
<tr>
<td>Total T lymphocytes (cells/μL)</td>
<td>516</td>
<td>1410</td>
<td>690</td>
<td>2,540</td>
</tr>
</tbody>
</table>

a. Absolute counts are rounded to the nearest 10 cells/μL.

These reference ranges are pooled ranges. See Section 9, Limitations, for more information about reference ranges.

11. PERFORMANCE CHARACTERISTICS

Performance of the reagents was established by testing at BD Biosciences laboratories in San Jose, CA, at an external clinical center in the US or Europe, or at a combination of sites.

* This value is found on the BD Trucount Absolute Count tube foil pouch label and might vary from lot to lot.
Accuracy

Absolute counts for CD3⁺CD4⁺ and CD3⁺CD8⁺ lymphocytes were compared to results from the BD FACSCount™ system.

Aliquots of the same blood sample from normal and abnormal donors were analyzed. Regression statistics reported in Table 2 indicate that the results are substantially equivalent.

Table 2 Regression analysis

<table>
<thead>
<tr>
<th>Subset</th>
<th>n</th>
<th>Slope</th>
<th>Intercept</th>
<th>r</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helper/inducer T lymphocytes (cells/µL)</td>
<td>201</td>
<td>1.02</td>
<td>4 cells/µL</td>
<td>0.99</td>
<td>0–1,880a</td>
</tr>
<tr>
<td>Suppressor/cytotoxic T lymphocytes (cells/µL)</td>
<td>196</td>
<td>1.00</td>
<td>29 cells/µL</td>
<td>0.99</td>
<td>70–1,980a</td>
</tr>
<tr>
<td>Total T lymphocytes (cells/µL)</td>
<td>199</td>
<td>1.02</td>
<td>8 cells/µL</td>
<td>0.99</td>
<td>70–2,860a</td>
</tr>
</tbody>
</table>

a. Absolute counts rounded to the nearest 10 cells/µL.

Within-Specimen Reproducibility

Ten aliquots of specimens from three samples representing high, medium, and low CD4 counts were assessed. Results are shown in Table 3.

Table 3 Within-specimen reproducibility for BD Tritest CD4/CD8/CD3 reagent

<table>
<thead>
<tr>
<th>Subset</th>
<th>Level</th>
<th>Mean</th>
<th>SDa</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helper/inducer T lymphocytes (cells/µL)</td>
<td>High</td>
<td>2,048</td>
<td>86</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>Med</td>
<td>1,311</td>
<td>37</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>355</td>
<td>10</td>
<td>2.7</td>
</tr>
<tr>
<td>Suppressor/cytotoxic T lymphocytes (cells/µL)</td>
<td>High</td>
<td>729</td>
<td>25</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>Med</td>
<td>483</td>
<td>19</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>334</td>
<td>12</td>
<td>3.7</td>
</tr>
</tbody>
</table>

a. SD = standard deviation

b. CV = coefficient of variation

Stability

A stability study was conducted to assess the effect of time with respect to BD Tritest reagent performance specifications. The study measured:
1) changes associated with the storage of whole blood before staining,
2) changes as a result of time between staining and data acquisition, and
3) the combined effect of the two.

Based on the results of this study, we recommend staining samples within 48 hours of draw and analyzing samples within 6 hours of staining; or staining samples within 24 hours of draw and analyzing within 24 hours of staining.

Cross-Reactivity

The CD4 antibody reacts with monocytes as well as with helper/inducer T lymphocytes. The CD8 antibody reacts with NK lymphocytes as well as with suppressor/cytotoxic T lymphocytes.

Linearity

The linearity was assessed by testing within a WBC concentration of 2.5 x 10³ to 31.0 x 10³ WBC/µL and a lymphocyte concentration of 2.0 x 10² to 16.7 x 10³ lymphocytes/µL. Results were observed to be linear within the CD3⁺CD4⁺ range (68 to 7.2 x 10³ cells/µL), the CD3⁺CD8⁺ range (43 to 3.9 x 10³ cells/µL), and the CD3⁺ range (123 to 9.1 x 10³ cells/µL).
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 herewith 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.

REFERENCES
