1. INTENDED USE

BD Multitest™ CD8 FITC/CD38 PE/CD3 PerCP/Anti–HLA-DR APC is intended for in vitro flow cytometric immunophenotyping of peripheral blood lymphocytes. CD8/CD38/CD3/Anti–HLA-DR assays are used in the evaluation of activated CD8+ T cells.\(^1\)-\(^3\)

2. COMPOSITION

CD8, clone SK1, is derived from hybridization of mouse NS-1 myeloma cells with spleen cells from BALB/c mice immunized with human peripheral blood T lymphocytes.

CD38, clone HB7, is derived from hybridization of mouse P3-X63-Ag8.653 myeloma cells with spleen cells from BALB/c mice immunized with the BJAB cell line.\(^4\)

CD3, clone SK7,\(^5\),\(^6\) is derived from hybridization of mouse NS-1 myeloma cells with spleen cells from BALB/c mice immunized with human thymocytes.

Anti–HLA-DR, clone L243, is derived from the hybridization of mouse NS-1/1-Ag4 myeloma cells with spleen cells from BALB/c mice immunized with the human lymphoblastoid B-cell line RPMI 8866.\(^7\)

CD8, CD38, and CD3 are each composed of mouse IgG1 heavy chains and kappa light chains.

Anti–HLA-DR is composed of mouse IgG2a heavy chains and kappa light chains.

This reagent is supplied as a combination of CD8 FITC, CD38 PE, CD3 PerCP, Anti–HLA-DR APC in 1 mL of phosphate-buffered saline (PBS) containing bovine serum albumin (BSA) and 0.1% sodium azide.
Antibody purity is as follows.

- FITC, PE, PerCP, APC: ≤20% free fluorophore at bottling, as measured by size-exclusion chromatography (SEC)

3. STORAGE AND HANDLING

The antibody reagent is stable until the expiration date shown on the label when stored at 2°C–8°C. Do not use after the expiration date. Do not freeze the reagent or expose it to direct light during storage or incubation with cells. Keep the outside of the reagent vial dry.

Do not use the reagent if you observe any change in appearance. Precipitation or discoloration indicates instability or deterioration.

4. REAGENTS OR MATERIALS REQUIRED BUT NOT PROVIDED

- Falcon® disposable 12 x 75-mm polystyrene test tubes or equivalent (if not using BD Trucount™ tubes)
- BD Vacutainer® EDTA blood collection tubes or equivalent
- Micropipettor with tips
- Bulk dispenser or pipettor (1,000 µL) for dispensing BD FACS™ lysing solution
- Vortex mixer
- BD FACS lysing solution (10X) (Catalog No. 349202). For dilution instructions and warnings, refer to the instructions for use (IFU).
- BD Calibrite™ 3 beads and APC beads (Catalog Nos. 340486 and 340487, respectively) or BD FACS™ 7-color setup beads (Catalog No. 335775)
- Reagent-grade (distilled or deionized) water
- BD FACSFlow™ sheath fluid (Catalog No. 342003) or equivalent.
- **CAUTION** Use only BD FACSFlow sheath fluid diluent to dilute BD Calibrite beads.
- BD Trucount™ Control Bead Kit (Catalog No. 340335), necessary if using BD Trucount™ tubes. BD Trucount Control Beads are recommended if using BD Multiset™ software.
- Properly equipped cytometer. Flow cytometers must have laser excitation set at 488 nm and 635 nm and must be equipped to detect light scatter and the appropriate fluorescence, and have the appropriate analysis software installed for data acquisition and analysis. Refer to the manual for details.
- Analysis software
  - For BD FACSCalibur™ flow cytometers, use BD Multiset™, or BD CellQuest™ Pro software.
  - For BD FACSCanto™ or BD FACSCanto™ II flow cytometers, use BD FACSCanto™ clinical software.

5. SPECIMEN(S)

BD Multitest CD8 FITC/CD38 PE/CD3 PerCP/Anti–HLA-DR APC can be used for immunophenotyping by flow cytometry with peripheral blood.

*Falcon is a registered trademark of Corning Incorporated.
WARNING All biological specimens and materials coming in contact with them are considered biohazards. Handle as if capable of transmitting infection\textsuperscript{8,9} 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.

6. PROCEDURE

When whole blood is added to the reagent, the fluorochrome-labeled antibodies in the reagent bind specifically to leukocyte 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 Multitest reagents employ fluorescence triggering, allowing direct fluorescence gating of the lymphocyte population\textsuperscript{10-12} to reduce contamination of unlysed or nucleated red blood cells in the gate.

When BD Trucount tubes are used, 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. Appropriate software such as BD Multiset software or BD FACSCanto clinical software can determine absolute counts. If manually performing data analysis using software such as BD CellQuest Pro, divide the number of positive cellular events by the number of bead events, then multiply by the BD Trucount bead concentration.

Staining the Cells

1. For each sample, label a tube with the sample identification.
   For absolute counts, use BD Trucount tubes. Refer to the BD Trucount Tubes IFU for more information.

2. Pipette 20 µL of BD Multitest CD8/CD38/CD3/Anti–HLA-DR reagent into the bottom of the tube.
   If using a BD Trucount tube, pipette just above the stainless steel retainer. Do not touch the pellet.

3. Pipette 30 µL of well-mixed, anticoagulated whole blood into the bottom of the tube.
   \textbf{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.

   If using a BD Trucount tube, accuracy is critical. Use the reverse pipetting technique to pipette sample onto the side of the tube just above the retainer. Refer to the BD Trucount Tubes IFU for more information.

4. Vortex gently and incubate for 15 to 20 minutes in the dark at room temperature (20°C–25°C).

5. Add 1 mL of 1X BD FACS lysing solution.
6. Vortex gently and incubate for 15 to 30 minutes in the dark at room temperature. The sample is now ready to analyze on the flow cytometer. If samples are not analyzed immediately, mix thoroughly before analysis.

Flow Cytometric Analysis

1. Set up the instrument as recommended by the manufacturer.
   Run a control sample daily from a normal adult subject to optimize instrument settings and as a quality control check of the system.

2. Vortex the cells thoroughly at low speed to reduce aggregation before running them on the flow cytometer.

3. Run the sample on the flow cytometer. Verify that all populations are on scale. Optimize instrument settings, if needed.

4. Acquire and analyze data.

5. Set a gate on the CD3+ lymphocyte population. If using either BD Multiset or BD FACSCanto clinical software, examine the gate set on the CD3+ lymphocyte population; adjust as necessary. Refer to the appropriate software guide for BD FACSCalibur flow cytometers. Refer to the BD Multitest CD8/CD38/CD3/Anti–HLA-DR Application Guide for BD FACSCanto and BD FACSCanto II flow cytometers.

6. Use the required combination of gates, regions, or quadrants to isolate the population of interest (Figure 1 and Figure 2).

7. Inspect all plots for expected patterns and verify that all gates include the populations of interest.

8. Determine antigen expression based on the sample negative population.

Figure 1 Gating strategy using BD Attractors™ or BD Multiset software

Figure 2 Gating strategy using BD FACSCanto clinical software
7. PERFORMANCE CHARACTERISTICS

Specificity
CD8 recognizes an antigen 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. The CD8 molecule interacts with class I major histocompatibility complex (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.

CD38 recognizes an integral membrane glycoprotein, 45 kDa, with a protein core of 35 kDa.

CD3 recognizes the epsilon chain of the CD3 antigen/T-cell antigen receptor (TCR) complex. This complex is composed of at least six proteins that range in molecular weight from 20 to 30 kDa. The antigen recognized by the CD3 antibody is noncovalently associated with either ζ/β or γ/δ TCR (70–90 kDa).

Anti–HLA-DR recognizes a human class II MHC antigen. The antigen is a transmembrane glycoprotein composed of ζ and β subunits that have molecular weights of 36 and 27 kDa, respectively.

Antigen Distribution
The CD8 antigen is expressed on the human suppressor/cytotoxic T-lymphocyte subset (CD3+CD8+), as well as on a subset of natural killer (NK) lymphocytes. The CD8 antigen is expressed on 19% to 48% of normal peripheral blood lymphocytes and the majority of normal thymocytes.

The CD38 antigen is expressed on essentially all pre-B lymphocytes, plasma cells, and thymocytes. It is also present on activated T lymphocytes, NK lymphocytes, myeloblasts, and erythroblasts. The antigen is expressed during the early stages of T- and B-lymphocyte differentiation, is lost during the intermediate stages of maturation, and then reappears during the final stages of maturation.

The CD3 antigen is expressed on 61% to 85% of normal peripheral blood lymphocytes and 65% to 85% of thymocytes.

HLA-DR is expressed on B lymphocytes, monocytes, macrophages, activated T lymphocytes, activated NK lymphocytes, and human progenitor cells.

8. PERFORMANCE DATA

Performance of the reagent was established by testing at BD Biosciences laboratories in San Jose, CA.

Accuracy
CD3+ T-lymphocyte absolute counts enumerated with BD Multitest CD8/CD38/CD3/Anti–HLA-DR reagent in BD Trucount tubes were compared with results from BD Multitest CD3/CD8/CD45/CD4 in BD Trucount tubes on BD FACSCanto and BD FACSCanto II systems using manual sample acquisition. Whole blood samples (n=60) were collected at random from normal donors at BD Biosciences and patient donors from two external clinical laboratories. CD3+ T-lymphocyte absolute counts ranged from 258 to 2,192 cells/µL on a
BD FACSCanto system and 258 to 2,159 cells/µL on a BD FACSCanto II system. Regression statistics are reported in Figure 3.

CD3+ T-lymphocyte absolute counts enumerated with BD Multitest CD8/CD38/CD3/Anti–HLA-DR reagent in BD Trucount tubes were compared with results from BD Multitest CD3/CD8/CD45/CD4 in BD Trucount tubes on BD FACSCanto and BD FACSCanto II systems using both manual and BD FACSTM Loader sample acquisition.

Whole blood samples (n=60) were collected at random from normal donors at BD Biosciences and patient donors from two external clinical laboratories. CD3+ T-lymphocyte absolute counts ranged from 281 to 3,005 cells/µL. Regression statistics are reported in Figure 4 and Figure 5.

The following table is a legend for the regression plots of Figure 3, Figure 4, and Figure 5:

<table>
<thead>
<tr>
<th>No.</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Regression Line</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Confidence Limit</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Ideal Line</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3** Regression plot of CD3+ T-lymphocyte absolute counts (cells/µL) on BD FACSCanto vs BD FACSCanto II systems

**Figure 4** Regression plot of CD3+ T-lymphocyte absolute counts (cells/µL) on a BD FACSCanto system
Precision
Estimates of assay precision were determined for two runs per day over 21 days from BD™ Multi-Check Control and BD™ Multi-Check CD4 Low Control stained in duplicate with BD Multitest CD8/CD38/CD3/Anti–HLA-DR reagent and run on the BD FACSCanto (Table 1) and BD FACSCanto II (Table 2) systems. Means and coefficients of variation (CVs) are provided for within-run and total precision in Table 1 and Table 2.

Table 1: Within-run and total precision of CD3+ T-lymphocyte absolute counts (cells/µL) on the BD FACSCanto system

<table>
<thead>
<tr>
<th>Control</th>
<th>Mean (cells/µL)</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD Multi-Check CD4 Low</td>
<td>310.15</td>
<td>5.82</td>
</tr>
<tr>
<td>Within-Run</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BD Multi-Check CD4 Low</td>
<td>310.15</td>
<td>6.27</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BD Multi-Check Normal</td>
<td>1,006</td>
<td>5.16</td>
</tr>
<tr>
<td>Within-Run</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BD Multi-Check Normal</td>
<td>1,006</td>
<td>7.24</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Within-run and total precision of CD3+ T-lymphocyte absolute counts (cells/µL) on the BD FACSCanto II system

<table>
<thead>
<tr>
<th>Control</th>
<th>Mean (cells/µL)</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD Multi-Check CD4 low</td>
<td>336.03</td>
<td>3.26</td>
</tr>
<tr>
<td>Within-Run</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BD Multi-Check CD4 low</td>
<td>336.03</td>
<td>6.50</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BD Multi-Check Normal</td>
<td>732.49</td>
<td>3.16</td>
</tr>
<tr>
<td>Within-Run</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BD Multi-Check Normal</td>
<td>732.49</td>
<td>7.11</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total Linearity
Linearity was assessed using triplicate measurements of nine concentrations of CD3+ T lymphocytes across a range of 100 to 3,000 cells/µL for CD3+ T-lymphocyte absolute counts on the BD FACSCanto and BD FACSCanto II systems. Results were observed to be linear within the CD3+ T-lymphocyte absolute count range.

9. LIMITATIONS
This reagent, BD Multitest CD8/CD38/CD3/Anti–HLA-DR, has not been validated by BD Biosciences for use with heparin or acid citrate dextrose (ACD) liquid anticoagulants in determining absolute counts with BD Trucount tubes. This reagent, BD Multitest CD8/CD38/CD3/Anti–HLA-DR, is not intended for screening samples for the presence of leukemic cells or for use in phenotyping samples from leukemia patients. Absolute counts calculated using hematology results are not comparable between laboratories using different manufacturers’ equipment.
Procedures using BD Multitest reagents must adhere to the instructions for use for the specific instrument, software, and quality control procedures used by your laboratory.

Samples with large numbers of nonviable cells can give erroneous results due to selective loss of populations and to increased nonspecific binding of antibodies to nonviable cells.

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 SOLELY HEREBY 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.

REFERENCES
