1. INTENDED USE
BD Simultest™ Control γ₁/γ₂a (IgG₁ FITC/IgG₂a PE) is a two-color direct immunofluorescence reagent for use as a negative control in flow cytometric immunophenotyping of erythrocyte-lysed whole blood (LWB). BD Simultest Control γ₁/γ₂a is used with BD Simulset software to set fluorescence-1 (FL1) and fluorescence-2 (FL2) quadrant markers around the unstained (negative) lymphocyte population and to estimate the amount of nonantigen-specific antibody binding (nonspecific staining) present, particularly that caused by Fc receptors. BD Simultest Control γ₁/γ₂a is for in vitro diagnostic use with BD Simultest in vitro diagnostic reagents.

2. SUMMARY AND EXPLANATION
Two procedures critical to performing human lymphocyte immunophenotyping with flow cytometric analysis are (1) setting fluorescence markers to establish a boundary between negatively and positively stained leucocyte events and (2) assessing nonspecific staining.1 After using BD Simultest™ Leucogate™ reagent with BD Simulset™ software to create a lymphocyte analysis gate, BD Simultest Control is used with BD Simulset software to analyze the events inside the gate, for fluorescence distribution, by setting markers for the fluorescence quadrants and estimating nonspecific staining. BD Simultest Control is used with BD Simulset software to divide events on the FL1 x-axis vs the FL2 y-axis into four quadrants that characterize cells according to the following properties: quadrant 1 (Q1) (upper left), PE-positive nonspecific staining; Q2 (upper right), PE-
and FITC-positive nonspecific staining; Q3 (lower left), PE- and FITC-negative unstained; and Q4 (lower right), FITC-positive nonspecific staining. Events in any quadrant other than Q3 are indicative of non-specific staining. The quadrant markers that show the boundaries of background and positive immunofluorescence from a hematologically normal patient sample are shown in Figure 1.

**Figure 1** BD FACScan LWB sample from a hematologically normal patient stained with BD Simultest Leucogate (tube A) and BD Simultest Control (tube B) reagents.

![Dot plot display](image)

**NOTE** Leucogate was used to reduce debris, monocytes, and granulocytes in the gate shown under tube A. A dot plot display of FL1 (x-axis) vs FL2 (y-axis) is shown for tube B.

The BD Simultest Control reagent contains FITC- and PE-labeled murine monoclonal antibodies that react specifically with keyhole limpet hemocyanin (KLH), an antigen not present on human leucocytes. Thus, when added to human whole blood, the control antibodies do not bind specifically to antigens on the surface of human leucocytes. However, murine antibodies may bind to human leucocytes through nonantigen-specific means, primarily by leucocyte Fc receptor interaction with the antibody Fc region. This nonspecific binding of monoclonal antibodies through surface membrane Fc receptors can create significant difficulty in determining true-positive reactions. By showing murine antibodies binding to human leucocytes through nonantigen-specific (Fc receptor–related) means, the control establishes a boundary between the negative and positive fluorescent populations and establishes the background fluorescence expected as a result of nonspecific staining.

BD Simulset uses the lymphocyte gate determined with the Leucogate tube and the fluorescence markers and limits of nonspecific staining determined with the control tube to analyze subsequent BD Simultest tube(s).

For additional information on how the lymphocyte gate is set, see the BD Simulset Software User’s Guide. For additional information and an overview of human lymphocyte immunophenotyping, see Section 2, Summary and Explanation, in the appropriate BD in vitro diagnostic reagent instructions for use (IFU).

### 3. PRINCIPLES OF THE PROCEDURE

The BD Leucogate reagent is added to the first whole blood patient sample tube to determine, with BD Simulset software, the settings for the lymphocyte gate. The BD Simultest Control reagent is added to
the second whole blood patient sample tube to determine, with BD Simulset software, the settings for the fluorescence markers. Lymphocyte immunophenotyping is performed using BD Simulset reagents on the third and subsequent whole blood patient sample tubes based on the parameters set by the Leucogate and control sample tubes. The BD Leucogate, BD Simulset Control, and BD Simultest reagent sample tubes are then treated with BD FACS™ lysing solution (to lyse erythrocytes), washed, and fixed prior to flow cytometric analysis.

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 they are excited by the laser beam and the emitted light is collected and processed by the flow cytometer. The use of two fluorochromes permits simultaneous two-color analysis because each fluorochrome emits light at a different wavelength when excited at 488 nm by an argon-ion laser. The FITC-stained lymphocytes emit yellow-green light (emission maximum approximately 515 nm) while the PE-stained lymphocytes emit red-orange light (emission maximum approximately 580 nm).

The cells also interact with the laser beam by scattering the light. The forward-scattered (FSC) light provides a measure of cell size, while the side-scattered (SSC) light is an indicator of cellular granularity. The BD FACScan™ flow cytometer used with BD Simulset software counts a sufficient number of cells to ensure that a minimum of 2,000 lymphocytes are included in the lymphocyte gate. The data should be saved as list mode files and given logical names to aid in retrieval for subsequent analysis or reanalysis by BD Simulset software.

BD Simulset Control is used with BD Simulset software to set fluorescence-1 (FL1) and fluorescence-2 (FL2) markers around the unstained lymphocyte population and to assess the amount of non-specific staining present, particularly that caused by Fc receptors. When greater than 5% of the control events are above the FL1 or FL2 negative control markers, an error message of “too much nonspecific staining” will appear on the computer display screen and the laboratory printout for the control tube. When the control tube is being processed by the software, the operator should check for error messages that would indicate nonspecific staining.

For each patient sample, the lymphocyte gate set with Leucogate and the fluorescence markers determined using BD Simultest Control are used to analyze patient samples stained with the BD Simultest reagents. Immunophenotyping results may be expressed as either percentages of lymphocytes in the gate or as percentages of total gated events. When the Percent Lymphocyte Conversion option has been selected from the main menu of BD Simulset software, the software subtracts nonlymphocytes (monocytes, granulocytes, and debris) from Q3 and then reports results as the percentage of positive, gated lymphocytes. If this software option is not selected, results will be expressed as percentages of the total gated events.
4. REAGENT

Reagent Provided, Sufficient for 50 Tests

BD Simultest Control IgG1/IgG2a is a combination of two murine monoclonal antibodies; one conjugated with FITC and one conjugated with PE. The reagent, sufficient for 50 tests, is provided in 1.0 mL of phosphate-buffered saline (PBS) with gelatin and 0.1% sodium azide. It contains FITC-labeled IgG1, clone X40, and PE-labeled IgG2a, clone X39. Both X40 and X39 react specifically with KLH, an antigen not expressed on human cells or human cell lines. Clones X40 and X39 are both derived from hybridization of mouse Sp2/0-Ag14 myeloma cells with spleen cells from BALB/c mice immunized with KLH. Clone X40 is composed of mouse IgG1 heavy chains and kappa light chains and clone X39 is composed of mouse IgG2a heavy chains and kappa light chains. The fluorescein-to-protein ratio (F:P) range for BD Simultest Control is 3 to 5. The F:P ratio for each reagent has been optimized for its intended use.

Precautions

- BD Simultest Control IgG1/IgG2a is for in vitro diagnostic use with BD Simultest in vitro diagnostic reagents.
- When stored at 2°C–8°C, antibody reagents are 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.
- Incubation or centrifugation times, or temperatures other than those specified may be a source of error.
- For optimal results, stain blood samples within 6 hours of venipuncture.
- 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 reagents contain sodium azide as a preservative. However, care should be taken to avoid microbial contamination, which may 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.

Concentration values are listed in the following table:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1 FITC</td>
<td>12.5</td>
</tr>
<tr>
<td>IgG2a PE</td>
<td>12.5</td>
</tr>
</tbody>
</table>

5. INSTRUMENT

BD Simultest Control IgG1/IgG2a is designed for use on a BD flow cytometer equipped with appropriate computer hardware, software, and gating electronics. The flow cytometer must be equipped to detect two-color fluorescence, FSC, and SSC.

The following instrument systems are recommended.
• BD FACScan flow cytometer system equipped for two-color fluorescence detection and two-parameter light-scatter detection. For detailed information on use, see the BD FACScan User’s Guide.

• BD CONSORT™ 30 or BD CONSORT 32 computer system and peripherals (included in the BD FACScan system). For detailed information on use, see the BD CONSORT 30 Software User’s Guide or the BD CONSORT 32 System User’s Guide.

• BD Simulset software. For detailed information on use, see the BD Simulset Software User’s Guide.

• BD Calibrite™ beads (Catalog No. 349502). These beads can be used for setting the photomultiplier tube (PMT) voltages, setting the fluorescence compensation, and checking instrument sensitivity on the BD FACScan flow cytometer. For detailed information on use, see the BD Calibrite Beads instructions for use (IFU).

• BD FACSComp™ software. For detailed information on use, see the BD FACSComp Software User’s Guide.

All performance characteristics were obtained using the aforementioned instrument systems. Other systems may have different characteristics.

6. SPECIMEN COLLECTION AND PREPARATION

Collect blood aseptically by venipuncture4,9 into a sterile BD Vacutainer® EDTA blood collection tube. A minimum of 1 mL of whole blood is required for this procedure. Blood should be stained within 6 hours of drawing 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 may give aberrant results. A white blood count (WBC) and a differential white-cell count should be obtained from the same sample of whole blood before staining. BD Simultest Control IgG1/IgG2a can be used on samples with WBCs in the usable range of any BD Simultest in vitro diagnostic reagent. Check the IFU of the specific BD Simultest reagent for the usable range of the reagent employed for phenotyping.

Interfering Conditions

Previously fixed and stored cells 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.10 The presence of abnormal (blast) cells or unlysed or nucleated red blood cells (RBCs) can interfere with test results. Hemolyzed samples or samples with less than 1 mL of whole blood in the collection tube should be rejected.

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, Sufficient for 50 Tests, and Precautions in Section 4, Reagent.
Reagents and Materials Required But Not Provided

- BD Simultest Leucogate (CD45/CD14), 1.0 mL (Catalog No. 340040). For determining a lymphocyte acquisition gate, see the BD Simulset Software User’s Guide. Store at 2°C–8°C.

- BD FACS lysing solution (10X), 100 mL (Catalog No. 349202). Store at 2°C–25°C. For use, dilute 1:10 with room temperature (20°C–25°C) reagent-grade water. Store in a glass container at room temperature. The prepared solution is stable for 1 month at room temperature. See the BD FACS Lysing Solution IFU.

  **WARNING** BD FACS lysing solution contains less than 50% diethylene glycol and less than 15% formaldehyde.

- BD Vacutainer EDTA blood collection tubes or equivalent.

- Falcon® disposable 12 x 75-mm polystyrene test tubes or equivalent.

- Vortex mixer.

- Low-speed centrifuge (200 g) with swinging bucket rotor and 12 x 75-mm tube carriers.

- Vacuum aspirator with trap.

- Micropipettor with tips.

- 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.

- BD FACSFlow™ sheath fluid (Catalog No. 342003) or equivalent.

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

- Paraformaldehyde (1%) for cell fixation. Dissolve 1 g of paraformaldehyde in 100 mL of PBS with 0.1% sodium azide by carefully heating at temperatures up to 56°C in a chemical fume hood. Adjust to pH 7.4 ±0.2 with either 0.1 mol/L NaOH or 0.1 mol/L HCl. Filter with a 0.45-µm filter. Store in glass at 2°C–8°C.

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

Staining and Fixing the Cells

Whole blood samples are first stained with Leucogate (tube A), BD Simultest Control (tube B), and the appropriate BD Simultest reagent (tube C) (and any other reagents in the panel in additional tubes). Diluted 1X BD FACS lysing solution is then used to lyse RBCs following staining. See Section 6, Specimen Collection and Preparation. Perform the procedure at room temperature (20°C–25°C) using room temperature reagents. The samples are then washed and fixed. See Precautions in Section 4, Reagent.

* Falcon is a registered trademark of Corning Incorporated.
1. For each patient sample, label three 12 x 75-mm tubes A, B, and C. If there are other reagents in the panel, label additional tubes as required. 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 into tube B, 20 µL of the appropriate immuno-phenotyping reagent into tube C, and 20 µL of each additional immuno-phenotyping reagent in the panel in separate tubes, as required.

3. For each patient sample tube, use a fresh micropipettor tip and carefully add 100 µL of the correct concentration of well-mixed, anti-coagulated whole blood patient sample into the bottom of each of the labeled tubes. Exercise care to prevent blood from running down the side of the tube. Vortex thoroughly at low speed for 3 seconds and incubate for 15–30 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 may 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 10–12 minutes at room temperature in the dark. Do not exceed 12 minutes.

   **NOTE** Avoid prolonged exposure of the cells to erythrocyte-lysing 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 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 1% paraformaldehyde to each tube. Vortex thoroughly at low speed for 3 seconds. Make sure that 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) before putting them through the flow cytometer to help reduce aggregation.
Flow Cytometry

Follow BD's instructions for two-color flow cytometric analysis and see Section 5, Instrument. The following general approach is recommended.

The BD FACScan flow cytometer is first prepared for sample analysis using BD Calibrite beads and BD FACSComp software. The stained sample tubes are then run on the flow cytometer and analyzed with the BD Simulset software. See the BD FACScan User's Guide, the BD Calibrite Beads IFU, the BD FACSComp Software User's Guide, and the BD Simulset Software User's Guide for detailed instructions for use. We recommend that patient data be stored in list mode to allow subsequent analysis of data files.

See the IFU for the specific in vitro diagnostic BD Simultest reagent for further instructions.

The BD Simultest Control tube (B) is used to set fluorescence intensity quadrant markers. BD Simulset software automatically uses the lymphocyte acquisition gate set with Leucogate and then establishes FL1 and FL2 markers. Fluorescence markers should be set around the negative population that appears as the cluster of events low in both yellow-green and red-orange fluorescence.

The software counts the number of events in each quadrant and then computes a percentage of positive lymphocyte events for Q1 (low yellow-green/high red-orange), Q2 (high yellow-green/high red-orange, or dual-fluorescence), Q3 (low yellow-green/low red-orange), and Q4 (high yellow-green/low red-orange) (see Figure 2).

Quality Control

See the Quality Control section of the IFU for the specific in vitro diagnostic BD Simultest reagent.

The BD Simultest Control is run with each patient sample to set FL1 and FL2 markers between positively stained and unstained events in the lymphocyte gate and to detect the presence of nonspecific staining that would indicate erroneous patient results.

After the markers are set, if more than 5% of the total counts for tube B remain in Q1, Q2, and Q4, nonspecific staining is suspected. The software notifies the operator with a message “too much nonspecific staining.” In this case, the results for tube C and any additional tubes in the reagent panel should be considered suspect.
If there is poor separation between negative and positive clusters as seen on visual inspection of the dot plots, nonspecific staining can be inferred and the run should be rejected. Nonspecific staining may be seen because of poor condition of the cells. See the Troubleshooting section if nonspecific staining is observed.

8. RESULTS

Nonantigen-Specific Antibody Binding (Nonspecific Staining)

Events falling in Q1 will have only FL2 fluorescence, those in Q4 only FL1 fluorescence, and those in Q2 will have both FL1 and FL2 fluorescence. Events in Q1, Q2, and Q4 are a measure of the nonspecific staining (see Table 1 and Figure 1). Events falling in Q3 have low fluorescence in FL1 (FITC) and FL2 (PE). Events in Q3 are a measure of the unstained lymphocytes and contaminating nonlymphocytes in the gate.

Examine the computer screen display and the Laboratory Report for control tube B. After the markers are set, if more than 5% of the total counts for tube B remains in Q1, Q2, and Q4, nonspecific staining is suspected. The software notifies the operator with a message "too much nonspecific staining." In this case, the results for tube C and any additional tubes in the reagent panel should be considered suspect. If this error message appears, a new sample should be obtained from the original aliquot of anticoagulated, whole blood, and the entire staining procedure should be repeated. See the Troubleshooting section at the end of this IFU.

Visual inspection of the dot plots obtained for tube C (and any additional tubes) is necessary to ensure that fluorescence markers are correctly set and that there is minimal nonspecific staining.

9. LIMITATIONS

- Use freshly drawn blood and stain within 6 hours of venipuncture. Prior to staining, store blood at room temperature (20°C–25°C) because cells that have been refrigerated before staining may give aberrant results. Previously fixed cells are not recommended for use.
- Stained and fixed cells should be assayed within 24 hours of staining.
- Confounding variables such as medications that affect properties of blood cells may yield inaccurate results. For example, poor resolution between positive and negative cells has been observed with transplant patients receiving immunosuppressive drugs. If there is poor separation between negative and positive clusters, the run

<table>
<thead>
<tr>
<th>Tube</th>
<th>Cell Type</th>
<th>Quadrant</th>
<th>%Lymphocytes (Converted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B (IgG1/ IgG2a)</td>
<td>PE-positive nonspecific staining</td>
<td>Q1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>PE- and FITC-positive nonspecific staining</td>
<td>Q2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>PE- and FITC-negative unstained</td>
<td>Q3</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>FITC-positive nonspecific staining</td>
<td>Q4</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 1: Data summary from Figure 1 and quadrants used to compute subsets
should be rejected (see the Troubleshooting section at the end of this IFU).

- Variation in either automatic or manual lymphocyte acquisition gate settings will change the relative amounts of subsets assayed. The BD Simulset software uses the BD Leucogate tube (A) to include at least 95% of the total lymphocytes in the sample to set the lymphocyte acquisition gate, and requires a visual inspection of the gate setting for validation.

- Samples with nucleated RBCs may show incomplete lysis because BD FACS lysing solution may not lyse nucleated erythrocytes. This may also occur when assaying blood samples from patients with certain hematologic disorders in which red cells are difficult to lyse (for example, myelofibrosis, spherocytosis). Nucleated erythrocytes will be counted as debris and, if debris is greater than 10%, the software will flag the sample as “too many nonlymphs in gate.” We recommend that such sample results be rejected. When the Percent Lymphocyte Conversion software option has been selected, nonlymphocytes such as nucleated or unlysed red cells, which are determined using BD Leucogate, are assumed to be unstained and are subtracted from Q3 by the software.

- BD Simulset software checks for nonspecific staining in the Control tube (B) only. The operator should examine the printout for any evidence of nonspecific staining in the tubes containing BD Simultest reagents (tube C and any additional tubes). Non-specific staining of the antibody may be indicated when there is poor separation between positive and negative clusters on the fluorescence dot plot for the tubes containing BD Simultest reagents (tube C and any additional tubes). The presence of such nonspecific staining may reflect a change in the reagent or an error in the preparation of the samples. Such samples should be repeated.

- Performance characteristics have been determined with BD FACScan and BD FACStrak flow cytometers using BD Simulset software. Performance characteristics have not been established with any other instrument system.

10. EXPECTED VALUES
For expected values obtained using BD Simultest Control IgG1/IgG2a, see the specific BD Simultest in vitro diagnostic reagent IFU.

11. PERFORMANCE CHARACTERISTICS
For lymphocyte immunophenotyping performance data obtained using BD Simultest Control IgG1/IgG2a, see the specific BD Simultest in vitro diagnostic reagent IFU.

Cross-Reactivity
Although the BD Simultest Control reagent is designed to detect nonspecific staining, nonspecific staining due to poor condition of cells may be observed with any antibody reagent. Nonspecific staining can be inferred if no clear
demarcation between negative and positive cells is seen on the fluorescence plot or if more than 5% of the counts from the control appear outside of Q3. Consult the Troubleshooting section if nonspecific staining is seen.

**Linearity-Recovery**

Linearity of response over a wide range of WBCs is determined for each BD in vitro diagnostic immunophenotyping reagent using BD Simultest Control in the assay procedure. See the specific in vitro diagnostic BD Simultest reagent IFU for the usable WBC range.

**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.

**TROUBLESHOOTING**

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insufficient separation between positive and negative fluorescent populations.</td>
<td>Decreased fluorescence due to incorrect PMT voltage or amplifier gain.</td>
<td>Run BD FACSComp software with BD Calibrite beads. Must meet specifications for intensity in fluorescence channels.</td>
</tr>
<tr>
<td>Insufficient antibody reagent.</td>
<td></td>
<td>Check pipet calibration, reagent volume added, as well as volume and concentration of cells added. Check reagent storage conditions.</td>
</tr>
<tr>
<td>No demarcation found between debris and lymphocyte clusters on light-scatter dot plot.</td>
<td>Presence of excess platelets or nucleated red blood cells or unlysed red blood cells.</td>
<td>Restain a fresh sample. Wash cells at lower speed (100g) for 15 minutes at room temperature (20°C–25°C).</td>
</tr>
<tr>
<td>Amplifier gain improperly set.</td>
<td></td>
<td>Adjust amplifier gain such that the left edge of the lymphocyte cluster begins at approximately channel 50 (256-channel scale). This is especially important when going from setup/compensation to whole blood determinations. See the appropriate user’s guide or instrument manual.</td>
</tr>
<tr>
<td>Sample not stained within 6 hours of collection.</td>
<td></td>
<td>Collect a fresh sample to stain.</td>
</tr>
<tr>
<td>FSC threshold set too high or too low.</td>
<td></td>
<td>Adjust FSC threshold so that there are approximately 10 channels of debris (256-channel scale).</td>
</tr>
<tr>
<td>Problem</td>
<td>Possible Cause</td>
<td>Solution</td>
</tr>
<tr>
<td>---------</td>
<td>----------------</td>
<td>----------</td>
</tr>
<tr>
<td>Suboptimal antibody-staining performance.</td>
<td>Sample not stained within 6 hours of collection.</td>
<td>Collect a fresh sample to stain.</td>
</tr>
<tr>
<td></td>
<td>Analysis gate selected by software included too many nonlymphocyte events.</td>
<td>Reprocess data using BD CONSORT 30, BD LYSYS II, or BD Simulset software and set a manual gate. <strong>Note</strong> A tight lymphocyte gate might exclude large lymphocytes (for example, NK lymphocytes, lymphoblasts). If possible, reacquire samples after optimization of scatter—adjust SSC PMT, FSC amplifier gain, and FSC threshold to separate lymphocyte clusters from debris. See the appropriate software user's guide.</td>
</tr>
<tr>
<td></td>
<td>Software unable to set an acceptable lymphocyte gate.</td>
<td>Sample is lymphopenic. Concentrate cells using a density-gradient method or reacquire a larger number of events using BD CONSORT 30 or BD LYSYS II software.</td>
</tr>
<tr>
<td></td>
<td>FSC threshold set too high or too low.</td>
<td>Adjust FSC threshold so that there are approximately 10 channels of debris (256-channel scale).</td>
</tr>
<tr>
<td></td>
<td>Amplifier gain improperly set.</td>
<td>Adjust amplifier gain such that the left edge of the lymphocyte cluster begins at approximately channel 30 (256-channel scale). This is especially important when going from setup/compensation to whole blood determinations. See the appropriate user's guide.</td>
</tr>
<tr>
<td></td>
<td>Sample not stained within 6 hours of collection.</td>
<td>Collect a fresh sample to stain.</td>
</tr>
<tr>
<td></td>
<td>Software marker setting not appropriate.</td>
<td>Reacquire data using BD CONSORT 30 or BD LYSYS II software.</td>
</tr>
<tr>
<td>Unstained cells in the stained sample move over marker set with control tube B into Q4 or Q1.</td>
<td>Nonspecific staining due to sample containing dead or damaged cells.</td>
<td>Collect a fresh sample to stain.</td>
</tr>
<tr>
<td></td>
<td>Nonspecific staining due to Fc receptors.</td>
<td>Check for excessive monocyte, granulocyte, or debris contamination in the lymphocyte acquisition gate.</td>
</tr>
</tbody>
</table>
Staining is dim or inconsistent.

- **Possible Cause**: Cell concentration too high at staining step.
- **Solution**: Check and adjust cell concentration. Repeat staining with fresh sample.

- **Possible Cause**: Insufficient reagent.
- **Solution**: Repeat staining with proper amount of reagent.

- **Possible Cause**: Cells not analyzed within 24 hours of staining.
- **Solution**: Repeat staining with fresh sample. Analyze promptly.

- **Possible Cause**: Improper medium preparation (sodium azide omitted).
- **Solution**: Use sodium azide in staining medium and washing steps.

- **Possible Cause**: Buffer or fixative at improper pH.
- **Solution**: Prepare fresh PBS with reagent-grade water and fresh fixative with PBS with sodium azide. Adjust to the proper pH. Filter through a 0.2-µm filter.

- **Possible Cause**: Possible bacterial contamination of PBS or fixative.
- **Solution**: Prepare fresh PBS with reagent-grade water and fresh fixative with PBS with sodium azide. Adjust to the proper pH. Filter through a 0.2-µm filter.

- **Possible Cause**: Few or no cells.
- **Solution**: Cell concentration too low. Resuspend fresh sample at a higher concentration. Repeat staining and analysis.

- **Possible Cause**: Flow cytometer malfunctioning.
- **Solution**: Troubleshoot instrument.

- **Possible Cause**: Increased autofluorescence.
- **Solution**: Prepare fresh PBS with reagent-grade water and fresh fixative. Filter PBS and fixative through a 0.2-µm filter.

- **Possible Cause**: Poor sample preparation.
- **Solution**: Collect a fresh sample and stain.

REFERENCES