1. INTENDED USE

BD CD19 (Leu-12) phycoerythrin (PE) is a single-color direct immunofluorescence reagent for the enumeration of B lymphocytes (CD19+) in peripheral blood, using fluorescence microscopy or flow cytometers such as the BD FACSCalibur™, BD FACSort™, BD FACScan™, or BD FACS™ Analyzer.

2. SUMMARY AND EXPLANATION

Human lymphocytes can be divided into three major groups: T, B, and natural killer (NK) lymphocytes, based on biological function and the expression of cell surface antigens. B lymphocytes express surface immunoglobulin (sIg), and upon antigen stimulation may proliferate and differentiate into plasma cells. The plasma cell is the most differentiated form of the B lymphocyte and is the major antibody secreting cell in the body.

In the early 1970s, B lymphocytes were recognized and quantified using polyclonal antibodies directed against sIg.1,2 The development of hybridoma technology3 has made it possible to develop immunologically pure antibodies highly specific for individual cell types, such as B lymphocytes. CD19 (Leu-12) is expressed at a very early stage of B-lymphocyte differentiation and continues to be expressed until the B lymphocyte becomes a plasma cell.4-6

See Table 1.

Clinical Applications

The peripheral blood B-lymphocyte count changes in many diseases. Some diseases are marked by lower than normal B-lymphocyte percentages, such as some
forms of immunodeficiency diseases and some types of autoimmune diseases.7

Table 1 B-lymphocyte maturation and cell surface antigen expressiona

<table>
<thead>
<tr>
<th>Stage No.</th>
<th>Description</th>
<th>Expressed</th>
<th>Newly Expressed</th>
<th>Not Expressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Progenitor cell (1)</td>
<td>HLA-DR CD45</td>
<td>CD34 TdT CD19</td>
<td>— — —</td>
</tr>
<tr>
<td>2</td>
<td>Pro-B cell (early pre-B) (2)</td>
<td>HLA-DR CD14 CD45</td>
<td>TdT CD10 CD19</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>Pre-B lymphocyte (3)</td>
<td>HLA-DR CD19 CD10 CD45</td>
<td>HLA-DR cµ</td>
<td>CD34 TdT</td>
</tr>
<tr>
<td>4</td>
<td>Immature B lymphocyte (4)</td>
<td>HLA-DR HLA-DP CD34 cµ</td>
<td>CD20 sIgM sIgD</td>
<td>HLA-DQ CD20</td>
</tr>
<tr>
<td>5</td>
<td>Mature B lymphocyte (resting) (5)</td>
<td>HLA-DR HLA-DP HLA-DQ CD34 CD19 CD20</td>
<td>CD21 CD22 sIgD</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>Activated B lymphocyte (6)</td>
<td>HLA-DR HLA-DP HLA-DQ CD34 CD20</td>
<td>CD23 CD38 sIgG</td>
<td>CD21</td>
</tr>
<tr>
<td>7</td>
<td>Plasma cell (Ig secretion) (7)</td>
<td>sIg CD38 CD45</td>
<td>sIgM sIgD cµ</td>
<td>CD19 CD20 CD22 CD23 HLA-DP HLA-DQ HLA-DR</td>
</tr>
</tbody>
</table>

a. This scheme subdivides a continuous biological process into a series of arbitrary stages. The reality is a continuum in which an earlier cell type evolves into a new immunoglobulin-producing plasma cell. This chart shows the expression of individual antigens on the cell surface. Expressed are the antigens continued from the previous stage; Newly Expressed are those emerging at the given stage; and Not Expressed are those that are no longer present.

b. The evidence is unclear whether the appearance of CD10 precedes or succeeds that of CD19 in B-lymphocyte ontogeny.

c. cµ = cytoplasmic immunoglobulin M

d. sIg = surface immunoglobulin

Table 1, column 2, shows a biological process that is depicted as a series of seven arbitrary stages (shown in the following figure). Each cell type evolves into a new immunoglobulin-producing plasma cell as antigens are expressed. See table note a for a more detailed explanation.
3. PRINCIPLES OF THE PROCEDURE

This is a monoclonal antibody–based, direct immunofluorescence staining method for use with fluorescence microscopy or flow cytometry for analysis of human B lymphocytes. The reagent consists of murine monoclonal antibody conjugated with PE, which upon excitation with light at 488 nm, emits light at a different wavelength. This permits the detection of B lymphocytes by combining their fluorescence property with their forward angle and wide-angle light scattering characteristics. Efficient detection of B lymphocytes in peripheral blood, using a PE-conjugated antibody, depends on the elimination of interfering cells. This can be accomplished by separating the mononuclear cells using a density gradient–separation method or by staining a whole blood sample with conjugated antibody and then lysing RBCs using BD FACS lysing solution.

4. REAGENT

Reagent Provided, Sufficient for 50 Tests

BD CD19 (Leu-12) PE reagent is a monoclonal antibody, provided in buffered saline with gelatin and 0.1% sodium azide, and is available in 50 tests. It contains PE-labeled CD19 (Leu-12), clone 4G7. The CD19 antigen, 95 kDa, is expressed on virtually all human B lymphocytes, which recognize 2% to 19% of peripheral blood lymphocytes. It is not expressed on T lymphocytes, granulocytes, or phytohemagglutinin-stimulated T lymphocytes.

Precautions

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

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 when disposing of all materials. Never pipette by mouth. Wear suitable protective clothing, eyewear, and gloves.

BD FACS lysing solution is required and contains diethylenes glycol and formaldehyde. Refer to the BD FACS Lysing Solution instructions for use (IFU) for warnings.

Concentration values are listed in the following table:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19 PE</td>
<td>12.5</td>
</tr>
</tbody>
</table>
5. INSTRUMENT
The BD CD19 (Leu-12) PE reagent is designed for use on a flow cytometer equipped with appropriate computer hardware and software or an epifluorescence microscope set up to excite phycoerythrin. The flow cytometer must be equipped to detect fluorescence using light-scatter properties. We recommend using software for data acquisition and analysis, such as BD CellQuest™ software, and a BD flow cytometer such as the BD FACSCalibur, BD FACSort, BD FACScan, or BD FACS Analyzer.

6. SPECIMEN COLLECTION AND PREPARATION
Blood samples may be prepared for flow cytometric analysis by using either lysed whole blood (LWB) or peripheral blood mononuclear cell (PBMC) preparation procedures. However, LWB is the recommended procedure since PBMC preparations yield more technique-dependent results.

Collect blood aseptically by venipuncture into a sterile BD Vacutainer® EDTA blood collection tube. A minimum of 1 mL of whole blood is required for LWB cell preparation and a minimum of 2 mL of whole blood is required for PBMC preparation. Follow the collection tube manufacturer’s guidelines for the minimum volume of blood to be collected. Blood should be stained within 6 hours of venipuncture for optimal results. Anticoagulated blood may be stored at room temperature (20°C–25°C) for up to 6 hours until ready for staining. Blood samples refrigerated prior to staining may give aberrant results.

A white blood cell (WBC) count and a differential white-cell count should be obtained from the same sample of whole blood before staining.

Interfering Conditions
Previously fixed and stored patient specimens should not be used. Whole blood samples refrigerated prior to staining may give aberrant results. Samples obtained from patients taking immunosuppressive drugs may yield poor resolution. The presence of blast cells may interfere with test results. In LWB preparations, the presence of unlysed or nucleated erythrocytes may interfere with test results. Hemolyzed samples should be rejected.

7. PROCEDURE
Reagent Provided
CD19 (Leu-12) PE

Reagents and Materials Required But Not Provided
- BD Calibrite™ beads (Catalog No. 349502). Refer to the BD Calibrite Beads IFU for instructions.
- BD Vacutainer EDTA blood collection tubes or equivalent.
- Dulbecco’s modified phosphate-buffered saline (PBS) without calcium and magnesium (Gibco), containing 0.1% sodium azide. To 10 mL 10X PBS, add 90 mL distilled water. Mix well, and add 100 mg sodium azide. Stir to dissolve. Store at 2°C–8°C.
- Falcon® disposable 12 x 75-mm polystyrene test tubes or equivalent.

* Falcon is a registered trademark of Corning Incorporated.
- Falcon disposable 15 mL polystyrene test tubes.
- Refrigerated centrifuge capable of 1400g (400g if Ficoll-Paque®† is used) with swinging-bucket rotor.
- Serological pipettes, 5- and 10-mL; Pasteur pipettes and bulbs.
- Vortex mixer.
- Hemacytometer chamber and coverslip.
- Precision micropipettes, with tips.
- Ice bath with cover.
- BD FACSFlow™ sheath fluid (Catalog No. 342003) or equivalent.

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

- Paraformaldehyde, 1% w/v in PBS without sodium azide. Refer to Paraformaldehyde Fixation of Hematopoetic Cells Stained with Fluorochromes, Monoclonal Antibodies Source Book, Section 2.10.
- Microscope slides and coverslips.

**Additional Materials for LWB Preparation**
- BD FACS lysing solution (10X), 100 mL (Catalog No. 349202). Refer to the BD FACS Lysing Solution IFU for dilution instructions and warnings.
- Reagent-grade (both distilled and deionized) water.

**Additional Materials for PBMC Preparation**
- LeucoPREP™ 13-mm cell separation tube or Ficoll-Paque separation medium for preparing a suspension of mononuclear cells. Refer to the appropriate IFU for instructions.
- Cell viability testing reagents and materials for the ethidium bromide/acridine orange method (refer to the BD procedure, Viability Staining using Ethidium Bromide and Acridine Orange, Monoclonal Antibodies Source Book, Section 2.3) or the trypan blue exclusion method.16

**Staining the Cells**
The staining procedures for LWB and PBMCs are described below. In either procedure, use care to protect the tubes from direct light. Both procedures should be performed at room temperature (20°C–25°C). For LWB preparation, use room temperature reagents. For PBMC preparation, use reagents at 2°C–25°C. See Precautions in Section 4, Reagent.

**LWB Procedure**
1. For each sample, add 20 µL of CD19 (Leu-12) PE to a tube labeled Reagent. Add 20 µL of PE-conjugated isotype control to a tube labeled Control.
2. Carefully add 100 µL of anticoagulated whole blood to the bottom of each tube, making sure that the blood does not run down the side of the tube.
3. Vortex at low speed for 3 seconds and incubate at room temperature for 15–30 minutes. Protect samples from light during this procedure.
4. Add 2 mL of 1X BD FACS lysing solution to the tube.

---

† Ficoll-Paque is a registered trademark of GE Healthcare.
6. Vortex to mix cells for 3 seconds and incubate at room temperature for 10 minutes. Protect samples from light during this procedure.

7. After incubation, centrifuge tubes at 300g for 5 minutes at room temperature.

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

9. Add 1 mL of 1X PBS with sodium azide to each tube. Vortex to suspend cells for 3 seconds, and centrifuge at 200g for 5 minutes at room temperature.

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

11. Add 0.5 mL of 1% paraformaldehyde to each tube. Vortex at low speed for 3 seconds.

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

13. For each sample, add 20 µL of CD19 PE-conjugated isotype control to a tube labeled Control. Add 20 µL of PBMCs at 2 x 10^7/mL to each tube.

14. Vortex for 2 seconds, and then incubate the tube for 20–30 minutes in an ice bath, covered, to prevent light exposure.

15. Add 2 mL of cold PBS to the tube and vortex for 2 seconds. Centrifuge the tube at 250g for 5 minutes at 2°C–8°C.

16. Aspirate the supernatant, leaving a small amount of fluid (approximately 50 µL) in the tube. Add 0.5 mL of 1% paraformaldehyde to the tube and immediately vortex for 2 seconds to suspend cells.

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

18. Flow Cytometry

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

19. Fluorescence Microscopy

Follow either the LWB procedure to step 10 or the PBMC procedure to step 8 and vortex to suspend the cells. Prepare a slide (wet mount) using 10 µL of cell suspension. Cover with a coverslip and protect the slide from light. If the slide will not be counted immediately, seal the coverslip and store at 2°C–8°C. Slide should be read within 24 hours.
Quality Control
For optimal results, we recommend using BD Calibrite beads and appropriate software for proper alignment of BD FACS brand instruments prior to any fluorescence determination employing fluorescein or phycoerythrin. Refer to the BD Calibrite Beads IFU for alignment procedure.

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

8. RESULTS
Lymphocytes that express CD19 (Leu-12) antigen will appear as a positive fluorescent cell peak in the FL2 histogram when analyzed by flow cytometry, or will display orange fluorescence when examined by fluorescence microscopy.

Flow Cytometry Using a BD FACSCalibur, BD FACS sort, BD FACScan, or BD FACS Analyzer Flow Cytometer
Refer to the operator’s manual for instrument setup. Figure 1 shows flow cytometry data obtained on a LWB sample from a normal subject stained with CD19 (Leu-12) PE and analyzed with a BD FACS brand flow cytometer.

Figure 2 shows representative flow cytometry data obtained on density gradient–separated cells stained with CD19 (Leu-12) PE.

NOTE Improper gate setting on the sample data can give incorrect results. See Ability of Flow Cytometry to Select Lymphocytes in Section 11, Performance Characteristics.

Fluorescence Microscopy
Count and classify at least 300 lymphocytes on a fluorescence microscope with phase contrast. Count only lymphocytes with surface staining.

Do not include lymphocytes with diffuse fluorescence, indicative of nonspecific staining, and exclude monocytes, granulocytes, and platelets by morphology in phase contrast. Lymphocytes which bear the CD19 (Leu-12) antigen will fluoresce orange. Record both the number of positive fluorescent and nonfluorescent (negative) lymphocytes.

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

\[
A = \frac{X}{Y} \times 100
\]

Where:

X = number of positively stained lymphocytes
Y = total number of lymphocytes
Absolute number of B lymphocytes/mL can be calculated from the white blood count and the differential count. The precision of this absolute count depends on the accuracy of the count of WBC, differential, and B lymphocytes.

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

\[
W = \frac{\text{Absolute number of B lymphocytes/mL}}{\text{WBC count}} \times \text{Differential count}
\]
W = B × C × D
Where:
B = total WBC/mL
C = % lymphocytes
D = % B lymphocytes

9. LIMITATIONS
General
Many abnormal states of health are not always associated with abnormal B-lymphocyte levels. These test results must be used in conjunction with other diagnostic procedures in assessing the clinical condition of the patient.

Determination of the B-lymphocyte population using CD19 (Leu-12) will not always give results identical to those obtained using polyclonal antibodies to sIg. In cases of Waldenström’s macroglobulinemia, high levels of circulating IgG may cause IgG adsorption on non-B lymphocytes. Other immune complex diseases and viral infections may give similar results. Also, in cases marked by predominance of a single clone of immature B lymphocytes in peripheral blood, the blood may exhibit CD19 (Leu-12) positivity with negative or very weak sIg staining.

Microscopy
Leucopenic patients or patients with low proportions of lymphocytes will sometimes fail to provide sufficient lymphocytes in a single sample of separated cells. If a total of 300 lymphocytes is not counted, the value determined for %B lymphocytes will be insufficiently precise for clinical use. If a WBC and a differential count are available for the patient, initial sample volume and/or final sample dilution should be adjusted to ensure that 300 lymphocytes can be counted on the slide.

The performance data was obtained using a properly aligned, high quality fluorescence microscope equipped for epifluorescence and phase contrast. The ability to switch easily from epifluorescence to phase contrast is required. Use of other microscope systems may not result in the same performance characteristics. In addition, the ability of the technician to discriminate monocytes from lymphocytes is critical.

Flow Cytometry
All flow cytometric performance data was obtained using a BD FACs Analyzer. Performance characteristics have not been established with any other flow cytometers.

Determination of B lymphocytes, by flow cytometry and single-stain in immunofluorescence, is not recommended for blood samples with high (>10 per 100 WBC) numbers of nucleated RBCs. These cells are incompletely separated by gradient methods and are not all lysed by the lysing procedures. Due to their size, they will be counted as lymphocytes in flow cytometry unless other controls besides size and scatter are used to discriminate cells of interest. If nucleated red cells are counted, the denominator will be artificially high and %B lymphocytes will be artificially low. See Section 11, Performance Characteristics, for more information.
Whole Blood Determination

BD FACS lysing solution is the lysing reagent recommended for use with BD FACS brand instruments. Samples from patients with high (>30 x 10^3 cells/mm^3) or low (<2 x 10^3 cells/mm^3) white cell counts will require adjustment of the amount of whole blood taken for analysis or erroneous results may occur.

Density Gradient Separation

Cells separated from whole blood by means of density gradients such as LeucoPREP or Ficoll-Paque will not have the same relative concentrations of B lymphocytes as lysed whole blood.\textsuperscript{19,20} Comparisons should only be made of samples prepared in the same manner. Additional limitations associated with gradient separation are:

- Prolonged contact of mononuclear cells with some lymphocyte separation media may reduce cell viability. Remove cells within 5 minutes after centrifugation.
- Results will not be valid if insufficient mononuclear cells are harvested.
- Incomplete gradient separation may occur in diseases marked by changes in cell density (burn patients with large numbers of immature granulocytes) or may be due to deficiencies in the separation technique. At times, a clear-cut interface of mononuclear cells may not appear following centrifugation. The sample may be contaminated by excessive erythrocytes, debris, granulocytes, or immature myeloid cells. These samples may not give valid results with CD19 (Leu-12) reagent. In such cases, consult the separation medium manufacturer’s literature for troubleshooting.

10. EXPECTED VALUES

As seen in Table 2, the normal ranges (central 95%) of B lymphocytes were determined on samples from healthy subjects using a BD FACS Analyzer and a fluorescence microscope. Expected values were also determined on 40–50 random hospital admissions. These values were combined with the values obtained from normal subjects.

Table 2 Normal ranges of B lymphocytes CD19 (Leu-12) expression\textsuperscript{a}

<table>
<thead>
<tr>
<th>Subjects</th>
<th>BD FACS Analyzer (WB)b</th>
<th>BD FACS Analyzer (PBMCs)c</th>
<th>Fluorescence Microscope (PBMCs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>2.0%–18.8%</td>
<td>2.1%–14.6%</td>
<td>2.0%–21.9%</td>
</tr>
<tr>
<td>95% Range</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>9.2%</td>
<td>8.1%</td>
<td>8.8%</td>
</tr>
<tr>
<td>N\textsuperscript{d}</td>
<td>39</td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td>Healthy + Hospital Admissions</td>
<td>1.2%–20.4%</td>
<td>2.1%–21.3%</td>
<td>2.6%–26.5%</td>
</tr>
<tr>
<td>95% Range</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>9.5%</td>
<td>10.9%</td>
<td>9.8%</td>
</tr>
<tr>
<td>N</td>
<td>87</td>
<td>87</td>
<td>95</td>
</tr>
</tbody>
</table>

\textsuperscript{a.} Values in this table were obtained using CD19 (Leu-12) plus Goat Anti Mouse Ig FITC. The equivalence of B-lymphocyte determinations obtained with direct or indirect immunofluorescence methods has been established.

\textsuperscript{b.} Whole blood

\textsuperscript{c.} Peripheral blood mononuclear cells

\textsuperscript{d.} N = number of samples

11. PERFORMANCE CHARACTERISTICS

Precision

The within-day precision of this array was determined using quadruplicate determinations from healthy subjects. All replicates were tested within 6 hours of
drawing blood, and the results are displayed in Table 3.

**Table 3** Within-day precision CD19 (Leu-12)

<table>
<thead>
<tr>
<th>Method</th>
<th>Sample Prep</th>
<th>N</th>
<th>Lab BD 1</th>
<th>Lab BD 2</th>
<th>Lab IND 1</th>
<th>Lab IND 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow Cytometry</td>
<td>Normal WB</td>
<td>4</td>
<td>17.5</td>
<td>14.3</td>
<td>15.9</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Normal PBMCs</td>
<td>5</td>
<td>10.0</td>
<td>8.3</td>
<td>10.2</td>
<td>—</td>
</tr>
<tr>
<td>Flow Cytometry</td>
<td>Abnormal WB</td>
<td>2</td>
<td>9.1</td>
<td>8.0</td>
<td>9.5</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Abnormal PBMCs</td>
<td></td>
<td>5</td>
<td>11.7</td>
<td>10.0</td>
<td>11.9</td>
</tr>
<tr>
<td>Fluorescence Microscopy</td>
<td>Normal WW</td>
<td>5</td>
<td>7.6</td>
<td>6.9</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Normal PBMCs</td>
<td>5</td>
<td>8.2</td>
<td>−</td>
<td>−</td>
<td>3.5</td>
</tr>
<tr>
<td>Fluorescence Microscopy</td>
<td>Abnormal WW</td>
<td>5</td>
<td>6.5</td>
<td>6.6</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Reproducibility

The between-laboratory reproducibility of the B-lymphocyte determination was estimated and the results are shown in Table 4. Whole blood samples (normal subjects) or PBMC suspensions (random hospital admissions) were determined in two laboratories at the same institution (BD Biosciences, San Jose, CA). In addition, prepared stained samples and slides from BD Lab 1 were supplied to an independent, outside laboratory for determination at 24 hours. Five normal subjects and five random hospital admissions were compared. Duplicate samples were determined at each site.

**Table 4** Between-laboratory reproducibility B-lymphocyte determination using CD19 (Leu-12)

<table>
<thead>
<tr>
<th>Method</th>
<th>Sample Prep</th>
<th>N</th>
<th>Lab BD 1</th>
<th>Lab BD 2</th>
<th>Lab IND 1</th>
<th>Lab IND 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow Cytometry</td>
<td>Normal WB</td>
<td>4</td>
<td>17.5</td>
<td>14.3</td>
<td>15.9</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Normal PBMCs</td>
<td>5</td>
<td>10.0</td>
<td>8.3</td>
<td>10.2</td>
<td>—</td>
</tr>
<tr>
<td>Flow Cytometry</td>
<td>Abnormal WB</td>
<td>2</td>
<td>9.1</td>
<td>8.0</td>
<td>9.5</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Abnormal PBMCs</td>
<td></td>
<td>5</td>
<td>11.7</td>
<td>10.0</td>
<td>11.9</td>
</tr>
<tr>
<td>Fluorescence Microscopy</td>
<td>Normal WW</td>
<td>5</td>
<td>7.6</td>
<td>6.9</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Normal PBMCs</td>
<td>5</td>
<td>8.2</td>
<td>−</td>
<td>−</td>
<td>3.5</td>
</tr>
<tr>
<td>Fluorescence Microscopy</td>
<td>Abnormal WW</td>
<td>5</td>
<td>6.5</td>
<td>6.6</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

**Comparison with Other Laboratory Techniques**

Comparisons between determinations of %B lymphocytes using CD19 (Leu-12) reagent vs the surface Ig method were obtained on normal subjects and/or abnormal or immune-suppressed patients. Table 5 shows that the comparison of test results with those obtained using a standard surface Ig technique gave good correlation. The surface Ig reagent employed was a commercially available polyclonal goat anti-human Ig (heavy-
and light-chain specific) conjugated to FITC.

**Table 5 CD19 (Leu-12) B-lymphocyte comparison with surface immunoglobulin (sIg) technique**

<table>
<thead>
<tr>
<th></th>
<th>BD FACS Analyzer (WB)</th>
<th>BD FACS Analyzer (PBMCs)</th>
<th>Fluorescence Microscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope (b)</td>
<td>0.94</td>
<td>0.98</td>
<td>1.01</td>
</tr>
<tr>
<td>Intercept</td>
<td>1.07</td>
<td>0.48</td>
<td>1.40</td>
</tr>
<tr>
<td>(R^2)</td>
<td>0.88</td>
<td>0.85</td>
<td>0.84</td>
</tr>
<tr>
<td>(N^d)</td>
<td>65</td>
<td>69</td>
<td>70</td>
</tr>
</tbody>
</table>

\(a\). B-lymphocyte values in this table were obtained using CD19 (Leu-12) plus Goat Anti Mouse IgG FITC.

\(b\). Slope: \(x = \text{sIg}; y = \text{mAb determination}.\)

\(c\). \(R = \text{regression coefficient computed using transformed values for percent positive}\).

\(d\). \(N = \text{number of samples}\).

**Ability of Flow Cytometry to Select Lymphocytes**

The flow cytometer's ability to eliminate platelets, red cells, debris, granulocytes, and monocytes from the lymphocyte count depends on the existence of a clear demarcation between these formed elements and lymphocytes on a side scatter vs forward scatter display. See Figure 1 and Figure 2 in Section 8, Results. For some patients, this demarcation is not clear and lymphocyte gating will be less effective.

Attempts to ensure a pure population of lymphocytes, by gating on only the center of the lymphocyte cluster, are not recommended, as relative proportion of B to T lymphocytes is not constant throughout the cluster.

Samples from ten normal subjects and nine hospital admissions, in which low-volume debris was clearly distinguishable, were compared to samples from four subjects, for which no discontinuity between the debris cluster and the lymphocyte cluster could be observed. All samples were treated by whole blood and gradient methods and were labeled with CD45 (Anti-HLe-1), a monoclonal antibody specific to white cells or platelets. Samples were run on a BD FACS Analyzer; the best lymphocyte gate was selected by a trained operator, and the percentage of cells positive with CD45 (Anti-HLe-1) FITC within the best...
lymphocyte gate was determined. Results are shown in Table 6.

Table 6 Percentage of leucocytes in best lymphocyte gate using CD45 (Anti-HLe-1) FITC (average + SD)

<table>
<thead>
<tr>
<th>Subjects</th>
<th>N</th>
<th>PBMCs</th>
<th>Lysed Whole Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>10</td>
<td>96.1 (+2.3)</td>
<td>95.8 (+1.8)</td>
</tr>
<tr>
<td>Random hospital admissions</td>
<td>9</td>
<td>93.7 (+5.5)</td>
<td>96.1 (+4.2)</td>
</tr>
<tr>
<td>Abnormal samples</td>
<td>4</td>
<td>68.5 (+22.3)</td>
<td>62.5 (+18.0)</td>
</tr>
</tbody>
</table>

a. SD = standard deviation
b. No discontinuity between debris and lymphocytes

Samples exhibiting poor separation between debris and lymphocytes may arise from patients with large numbers of nucleated red blood cells, erythroid blast cells, or from patients who are very leucopenic (<0.5 x 10^3 cells/mm^3).

In a second experiment, two trained flow cytometer operators used side scatter and forward scatter to select the best lymphocyte gate on cells stained with CD14 (Leu-M3) PE, which is specific for monocytes and does not stain lymphocytes. Table 7 shows the expected extent of monocyte contamination in the best lymphocyte gate.

Table 7 Percentage of monocytes in best lymphocyte gate BD FACS Analyzer (PBMCs) using CD14 (Leu-M3) PE (average + SD)

<table>
<thead>
<tr>
<th>Subjects</th>
<th>N</th>
<th>X (SD) Operator 1</th>
<th>X (SD) Operator 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>10</td>
<td>0.3 (+0.3)</td>
<td>0.4 (+0.3)</td>
</tr>
<tr>
<td>Random hospital admissions</td>
<td>7</td>
<td>1.5 (+1.8)</td>
<td>0.8 (+0.9)</td>
</tr>
<tr>
<td>Abnormal samples</td>
<td>6</td>
<td>2.2 (+3.0)</td>
<td>1.9 (+2.2)</td>
</tr>
</tbody>
</table>

a. No discontinuity between monocyte and lymphocyte clusters.

The presence of monocyte contamination of the sample may be inferred if no clear distinction between monocyte and lymphocyte clusters appears on the scatter vs volume dot plot. In such cases, the count will be slightly higher and the percentage of T and B lymphocytes will be slightly lower.

**WARRANTY**

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# Troubleshooting

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Possible Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow Cytometry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor resolution between debris and lymphocytes</td>
<td>Combination of mononuclear cells with other cells and platelets</td>
<td>Prepare and stain more cells.</td>
</tr>
<tr>
<td></td>
<td>Rough handling of cell preparation</td>
<td>Check cell viability; centrifuge cells at lower speed.</td>
</tr>
<tr>
<td></td>
<td>Cytometer not properly aligned</td>
<td>Troubleshoot instrument.</td>
</tr>
<tr>
<td>Negative peak of the stained sample moves over marker set with the negative control tube</td>
<td>Nonspecific staining (cytophilic Ig or Fc receptor)</td>
<td>If positive cell peak and negative cell peak can still be resolved, move marker to valley between two peaks. If two peaks cannot be resolved, prepare and stain more cells. NOTE: Sample must be at least 90% viable for data to be valid.</td>
</tr>
<tr>
<td>Staining dim or fading</td>
<td>Cell concentration too high at staining step</td>
<td>Check and adjust cell concentration; repeat staining with fresh sample of PBMCs.</td>
</tr>
<tr>
<td></td>
<td>Insufficient reagent</td>
<td>Check data sheet for correct amount of reagent.</td>
</tr>
<tr>
<td></td>
<td>Cells not analyzed within 24 hours of staining</td>
<td>Repeat staining with fresh sample of PBMCs.</td>
</tr>
<tr>
<td></td>
<td>Improper medium preparation (sodium azide omitted)</td>
<td>Use sodium azide in staining medium and washing steps.</td>
</tr>
<tr>
<td>Few or no cells</td>
<td>Cell concentration too low</td>
<td>Resuspend fresh sample of PBMCs at a higher concentration. Repeat staining and analysis.</td>
</tr>
<tr>
<td></td>
<td>Cytometer malfunctioning</td>
<td>Troubleshoot instrument.</td>
</tr>
<tr>
<td>Microscopy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staining dim or fading</td>
<td>Cell concentration too high at staining step</td>
<td>Check and adjust cell concentration with fresh sample of PBMCs.</td>
</tr>
<tr>
<td></td>
<td>Insufficient reagent</td>
<td>Check data sheet for correct amount of reagent.</td>
</tr>
<tr>
<td></td>
<td>Cells not analyzed within 24 hours of staining</td>
<td>Repeat staining with fresh sample of PBMCs.</td>
</tr>
<tr>
<td></td>
<td>Stained cells exposed to excess light</td>
<td>Repeat staining with fresh sample of PBMCs. Avoid exposure of stained cells to light.</td>
</tr>
<tr>
<td></td>
<td>Improper medium preparation (sodium azide omitted)</td>
<td>Use sodium azide in staining medium and washing steps.</td>
</tr>
<tr>
<td>Not enough cells on slide</td>
<td>Cell concentration too low</td>
<td>Check and adjust cell concentration by resuspending cells in smaller volume. Repeat staining and analysis.</td>
</tr>
<tr>
<td>Air bubbles in microscope field</td>
<td>Slide dried before analysis</td>
<td>Prepare new slide and seal correctly or read promptly</td>
</tr>
<tr>
<td>Considerable cytoplasmic staining</td>
<td>Large numbers of dead cells in preparation</td>
<td>Check viability of cell preparation (should be &gt;90% viable). Obtain fresh sample; separate mononuclear cells; stain and analyze.</td>
</tr>
</tbody>
</table>

## References


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