

6602140 - 100 tests

PN 178465-A



**MONOCLONAL ANTIBODY**

**For In Vitro Diagnostic Use**

**INTENDED USE**

COULTER CLONE B1 monoclonal antibody is used to enumerate the percent of B lymphocytes in whole blood or Ficoll-separated cell suspensions by fluorescence microscopy and flow cytometry methods.

**SUMMARY AND EXPLANATION**

The lymphocyte population of human peripheral blood is composed of three cell types - T (thymus-derived), B (bone marrow-derived), and null cells. These cell types are morphologically indistinguishable by microscopy but can be identified by characteristic antigenic differences in their cell membranes.

B1 (CD20) antigen is found on all B cells isolated from the peripheral blood, lymph node, spleen, tonsil, and bone marrow.<sup>1</sup> It is not found on normal T cells, monocytes, or granulocytes.<sup>1,2</sup> Unlike surface immunoglobulin which may bind via the Fc receptors of B cells, monocytes and subsets of T cells, and give a spurious result, the B1 antigen is an integral component of the B cell membrane.<sup>1</sup>

**CLINICAL RELEVANCE**

B1 enumerates the percent of B1 positive lymphocytes in peripheral blood and single cell suspensions of lymphoid tissue in normal and disease states, e.g., elevated numbers of B lymphocytes in systemic lupus erythematosus.<sup>3</sup>

**PRINCIPLES OF TEST**

Specific B-cell staining is accomplished by incubating peripheral blood lymphocytes with the monoclonal antibody, washing the cells to remove unbound antibody, and then incubating with fluorescein-conjugated goat antiserum to mouse immunoglobulin, GAM-FITC. B cells may then be enumerated by either fluorescence microscopy or flow cytometry.

**REAGENTS**

COULTER CLONE Monoclonal Antibodies:  
PN 6602140 - 100 tests

**CLONE:** H299 (B1) was derived from the hybridization of mouse P3/NS1/1-Ag4-1 myeloma cells with spleen cells from BALB/c mice immunized with human B cell ascites Burkitt's lymphoma cells.<sup>1</sup>

**Ig CHAIN COMPOSITION:** Mouse IgG2a heavy chain and Kappa light chains<sup>2</sup>

**CYTOTOXICITY:** Complement dependent

**SOURCE:** Mouse ascites fluid or conditioned culture media

**PURIFICATION:** Ion exchange chromatography

**REAGENT CONTENTS**

The final concentration of nonantibody reagents when reconstituted is 0.2% BSA, 0.01 M potassium phosphate, 0.15 M NaCl, and 0.1% NaN<sub>3</sub>.

**NOTE:** (1) Avoid repeated freeze/thaw cycles. This will denature the antibody protein. (2) Do not store in a self-defrosting freezer. (3) If all the reagent is not to be used within six (6) months, follow the Procedure for Freezing.

**STATEMENT OF WARNINGS**

1. This reagent contains sodium azide. Sodium azide under acid conditions yields hydrazoic acid, an extremely toxic compound. Azide compounds should be flushed with running water while being discarded. These precautions are recommended to avoid deposits in metal piping in which explosive conditions can develop. If skin or eye contact occurs, wash excessively with water.
2. Do not use antibody beyond the expiration date on label.
3. Patient specimens and all material coming in contact with them should be handled as if capable of transmitting infection, and disposed of with proper precautions.
4. Never pipet by mouth and avoid contact with skin and mucous membranes.
5. Do not expose reagents to strong light during storage or incubation.
6. Incubation times or temperatures other than those specified may give erroneous results.
7. Avoid microbial contamination of reagents or incorrect results may occur.
8. Harmful if swallowed.
9. After contact with skin, wash immediately with plenty of water.

**RECONSTITUTION**

1. Reconstitute the lyophilized COULTER CLONE B1, GAM-FITC, and Isotypic Control by adding 500 µL of distilled water. This makes a stock solution.
2. Centrifuge the stock solutions at 100,000 x g for 10 min for optimization of staining results.
3. Prepare working solutions of the above reagents as follows:

| Vial Size | Volume of COULTER CLONE B1, Isotypic Control, or GAM-FITC Stock Solution Per Test | Volume of PBS* |
|-----------|---|----------------|
| 100 tests | 5 µL  | Add 195 µL     |

\*PBS - Phosphate Buffered Saline

4. Storage conditions of reconstituted B1
 

|                                   |          |
|-----------------------------------|----------|
| Reconstituted and stored at 2-8°C | 6 months |
| Reconstituted and stored at -70°C | 1 year   |

**STORAGE CONDITIONS**

Unreconstituted lyophilized B1 antibody may be stored at 2 to 8°C until date printed on the label. Do not expose reagents to strong light during storage or incubation. All reagents should be brought to 20 - 25°C prior to use.

**EVIDENCE OF DETERIORATION**

Any alteration of the physical appearance of the reagent either lyophilized or reconstituted or any major variations in values on control subjects may indicate deterioration.

**SPECIMEN COLLECTION AND PREPARATION**

**SPECIMEN COLLECTION**  
Collect venous blood sample aseptically by venipuncture into VACUTAINER® tubes, or equivalent using an appropriate anticoagulant (EDTA is the anticoagulant of choice). For detailed information on the collection of whole blood by venipuncture and interfering conditions, refer to "Procedures for the Collection of Diagnostic Blood

Specimens by Venipuncture 3rd Edition (H3-A3)," published by the National Committee for Clinical Laboratory Standards (1991) Villanova, PA.

**CAUTION:** The stability of abnormal specimens is quite variable. For optimal results, start the assay within 6 hours of venipuncture. Unstained, anticoagulated blood should remain at 20 to 25°C until processing is begun.

**SAMPLE PREPARATION**

**Whole Blood**

To prepare whole blood samples for analysis, use the appropriate Whole Blood Lysing Reagent kit, Coulter PN 6602764 - 100 tests, 6603154 - 300 tests, and follow the directions in the package insert. For each test, 100 µL of whole blood is required.

**Ficoll-separated Cell Suspensions**

**MATERIALS REQUIRED BUT NOT PROVIDED:**

- Flow cytometer or Fluorescence microscope (See manufacturer's reference manual for details)
- Stopcock grease
- Distilled or deionized water
- Microscope slides (3" x 1" x 1.0 mm)
- Coverslips (24 mm x 50 mm)
- Venous blood sample (1 to 2 mL required per tube)
- Phosphate Buffered Saline (PBS), 0.145 M NaCl, 0.01 M potassium phosphate, pH 7.2 (Coulter PN 6602485)
- Formaldehyde (10% solution) in PBS
- Ficoll-Paque® (Pharmacia PN 17-0840-03)
- PBS containing 2% fetal or newborn calf serum and 0.01% NaN<sub>3</sub> (wash media)
- PBS containing 0.01% NaN<sub>3</sub> (resuspension media)
- Propidium iodide 0.01 mg/mL, 0.05 mg/mL (Calbiochem PN 537059)
- Acridine orange 0.005 mg/mL (Baker PN A366-3)
- Low-speed refrigerated centrifuge with swinging bucket rotor
- VACUTAINER tubes, or equivalent, with an appropriate anticoagulant
- Siliconizing agent for glassware (Prosil®-28 PCR, Inc.)
- 15-mL siliconized glass conical centrifuge tubes
- 12 x 75 mm siliconized glass or plastic standard test tubes
- Transfer pipets
- Ice bath

1. Dilute blood 1:2 with 4°C PBS in an ice bath.

**NOTE:** If a larger volume of cells is desired, any number of tubes of blood may be pooled into a large siliconized glass tube or flask and treated as below.

2. Layer 8 mL of diluted blood over 4 mL of Ficoll-Paque in a 15-mL siliconized centrifuge tube.
3. Centrifuge at 4°C at 400-450 x g for 30 min. Mononuclear cells should form a visible, clean interface between the plasma and the Ficoll-Paque.
4. Aspirate the plasma and remove the mononuclear cell layer. Place cells in a clean 15-mL siliconized centrifuge tube. Fill the tube with 4°C PBS and gently mix the cells: spin at 400 x g for 8 min. Aspirate and discard the supernatant.
5. Resuspend cells in 4°C PBS, mix and spin at 400 x g for 4 min. Aspirate and discard the supernatant.
6. Resuspend cells in 4°C PBS, mix and spin at 400 x g for 3 min. Aspirate and discard the supernatant.
7. Resuspend cells with 5 mL of wash media or 4°C PBS, mix gently and determine cell concentration using a COULTER® instrument (ZBI or S series) or hemocytometer.
8. Viability analysis is performed by one of the two methods suggested below and.

## Fluorescence Microscopy:

Results obtained with fluorescence microscopes may vary due to the type of microscope used, the light source, the age of the bulb, filter assembly and filter thickness.

- Place a suspension of approximately 25,000 cells (25  $\mu\text{L}$  of a  $1 \times 10^6$  cells/mL suspension) onto a microscope slide.
- Add 10  $\mu\text{L}$  of a 0.01 mg/mL solution of propidium iodide and mix gently by stirring with a pipet tip.
- Allow to stand for 30 s. Add 10  $\mu\text{L}$  of a 0.005 mg/mL solution of acridine orange. Mix gently and allow to stand for 3 s.
- Place 24 x 50 mm coverslip on the slide, seal with stopcock grease, and examine by fluorescence microscopy.
- Count 100 cells and report % viability. Viable cells appear bright green. Nonviable cells appear red. If viability is not 85%, the cell preparation should not be used.

## Flow Cytometer Analysis:

Use an instrument that discriminates leukocytes and measures their fluorescence on a cell-by-cell basis.

- Place  $1 \times 10^6$  cells into a 12 x 75 mm siliconized test tube.
- Wash one time with resuspension media and centrifuge at 400 x g for 30 min at 4°C.
- Aspirate supernatant and add three drops of 0.05 mg/mL propidium iodide. Allow to stand for 1 min.
- Wash two times in resuspension media as in Step 10.
- Aspirate supernatant and resuspend to 1 mL. If viability is not 85%, the cell preparation should not be used. Analyze on a flow cytometer system according to the Product Manual.

## PROCEDURE FOR INDIRECT IMMUNOFLUORESCENCE CELL SURFACE STAINING OF LYMPHOCYTES WITH COULTER CLONE B1 MONOCLONAL ANTIBODY

### REAGENTS REQUIRED BUT NOT PROVIDED

Phosphate Buffered Saline (PBS): 0.145 M NaCl, 0.01 M potassium phosphate, pH 7.2 (Coulter PN 6602489)  
PBS containing 2% heat-inactivated fetal or newborn calf serum (2 mL calf serum diluted to 100 mL with PBS)  
PBS containing 0.01%  $\text{NaN}_3$  (resuspension media)  
10% formaldehyde in PBS  
COULTER CLONE GAM-FITC (conjugated second antibody) PN 6602159 - 100 tests  
COULTER CLONE MslgG2a Isotypic Control, PN 6602876 - 100 tests  
Siliconizing agent for glassware (Prosil-28 PCR, Inc.)

### EQUIPMENT REQUIRED FOR BOTH METHODS

Refrigerated centrifuge, capable of accurately achieving 400 x g.  
Ice bath  
Fluorescence microscope and/or flow system  
Ultracentrifuge (such as an airfuge)  
Vortex mixer

### Test Tube Method

Centrifuge fittings - 12 x 75 mm test tube holders  
12 x 75 mm siliconized glass or plastic test tubes  
Transfer pipets  
Coverslips (22 x 22 mm)

### Microtiter Plate Method

"V" bottom, vinyl, flexible, 96-well (8 x 12) microtiter plates  
Plastic plate covers  
Centrifuge carriers for microtiter plates

## REAGENT PREPARATION

Prepare the suspension of blood cells and stock solutions. (Refer to the Reconstitution and Specimen Collection and Preparation sections.) An appropriate isotypic control (in this case MslgG2a) should be run with each patient sample.

## PROCEDURE

### Test Tube Method

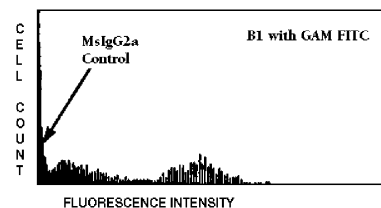
- Into 12 x 75 mm test tubes place  $1 \times 10^6$  cells from Ficoll-Paque preparation, centrifuge 4 min at 400 x g at 4°C, aspirate supernatant.
- Add 200  $\mu\text{L}$  of COULTER CLONE B1 monoclonal antibody working solution to one test tube and control to the next test tube. Vortex gently. Incubate the reaction mixture at 4°C for 30 $\pm$ 5 min.
- Wash the reaction mixture with 1 mL of wash media, centrifuge at 400 x g at 4°C for 4 min, aspirate supernatant carefully, and vortex gently. Repeat.
- After the second wash, aspirate supernatant and add 200  $\mu\text{L}$  of GAM-FITC working solution to the cell pellet. Disrupt pellet (vortex) and incubate at 4°C for 30 $\pm$ 5 min.
- At the end of 30 min, wash three times with resuspension media, centrifuging each time for 4 min at 400 x g at 4°C.
- After third wash, resuspend pellet to 1 mL with resuspension media at 4°C for flow cytometry or fluorescence microscopy analysis.

### Microtiter Plate Method

- Adjust the concentration of the cell suspension of mononuclear cells to  $1 \times 10^6$  cells/200  $\mu\text{L}$  and dispense that volume into each well.
- Centrifuge microtiter plates at 4°C, at 400 to 450 x g for 5 min.
- Remove the supernatant from each well by aspiration with a Pasteur pipet having a fire-polished and slightly bent tip. Insert the pipet tip into the well only as far as the lower ledge which permits efficient removal of all supernatant without disturbing the pellet.
- Disrupt the cell pellets by carefully placing the lid on the tray and gently, vigorously pressing the microtiter plate "V" bottom onto the top of a vortex at an approximate setting of 8 or 9. All areas of the plate should be moved so that they come in contact with the vortex head. Vortex mixing should continue until all pellets are resuspended.
- Add 200  $\mu\text{L}$  of COULTER CLONE B1 monoclonal antibody working solution and control into alternate wells and gently agitate. Incubate at 4°C for 30 min.
- Centrifuge the plate at 4°C at 400 to 450 x g for 5 min.
- Aspirate the supernatant and disrupt the pellets on the vortex mixer.
- Add 200  $\mu\text{L}$  of wash media and centrifuge at 4°C at 400 to 450 x g for 5 min.
- Aspirate the wash media and disrupt the pellets on the vortex.
- Add 200  $\mu\text{L}$  of GAM-FITC working solution. Mix gently. Incubate the reaction mixture at 4°C for 30 min.
- Centrifuge the plate at 4°C at 400 to 450 x g for 5 min. Wash the cell pellet twice as performed previously.
- Resuspend the pellets in 200  $\mu\text{L}$  of resuspension media and transfer to appropriate containers for flow cytometric analysis or fluorescence microscopy.

### Analysis of Cells for Both Methods

- Transfer 200  $\mu\text{L}$  of each final cell suspension to test tube containing 20  $\mu\text{L}$  of 10% formaldehyde in PBS. Place one drop of fixed cells on microscope slide. Cover with 22 mm x 22 mm coverslip. Seal with stopcock grease and examine by fluorescence microscopy.
- Count all the cells in the field. Record the number of lymphocytes by phase-contrast differential. Examine the cells under fluorescent light (488-nm filter). Record the percentage of fluorescent lymphocytes by switching between phase and fluorescent illumination.
- Analyze remaining unfixed cells on a flow cytometer according to the Product Manual.



## PROCEDURE FOR FREEZING RECONSTITUTED COULTER CLONE MONOCLONAL ANTIBODIES

In the event some unused reagent is left from your testing and will not be used within six months, freeze the remaining amount as described below.

### MATERIAL PROVIDED

COULTER CLONE Monoclonal Antibodies  
B1 PN 6602140 (100 tests)

### REAGENTS REQUIRED BUT NOT PROVIDED

Phosphate Buffered Saline (PBS): 0.145 M NaCl, 0.01 M potassium phosphate, pH 7.2 (Coulter PN 6602489)  
PBS containing 2% heat-inactivated fetal or newborn calf serum (2 mL calf serum diluted to 100 mL with PBS)

### PROCEDURE

Reconstitute the lyophilized COULTER CLONE monoclonal antibody by adding 500  $\mu\text{L}$  of distilled water as described previously. Dilute the COULTER CLONE monoclonal antibody with PBS containing 2% heat-inactivated calf serum prior to the freezing as follows:

| Vial Size | *Volume of Reconstituted Monoclonal Antibody/Test | **Volume of PBS with 2% Calf Serum |
|-----------|---|------------------------------------|
| 100 tests | For each: 5 $\mu\text{L}$                         | 100 $\mu\text{L}$                  |

\*This volume may be frozen in multiple test volumes.

\*\*This yields one-half the working dilution of the monoclonal antibody.

Freeze the diluted monoclonal antibody. Stable for one year when reconstituted, stabilized with a solution of fetal or newborn calf serum in PBS, and stored at -70°C. Do not freeze and thaw repeatedly. Store in aliquots.

Prior to use, allow the diluted monoclonal antibody to reach room temperature. Dilute with 100  $\mu\text{L}$  PBS.

### QUALITY CONTROL PROCEDURE

A normal, apparently healthy donor should be run as a control to ensure proper working conditions. Normal ranges should be established within a local population of normal donors. An appropriate COULTER CLONE isotypic mouse control is used to monitor the levels of nonspecific staining. If the background levels of the control are not acceptable, test results of the patient samples should be considered invalid.

Each lot of B1 is carefully screened on blood cell types including lymphocytes, monocytes, and granulocytes during the rigid quality control in-process testing protocols. The specimens used for this quality control testing are drawn from our employee population that includes Black, White, Oriental and Hispanic.

### LIMITATIONS

- Stored or refrigerated samples may give aberrant results. Immunodeficient patients may present special problems due to altered or very low numbers of certain lymphocyte populations.
- Results are dependent on proper isolation of lymphocytes. Prolonged contact of mononuclear cells with lymphocyte separation media may reduce cell

viability. Cells should be removed within 5 min after centrifugation.

- Cells separated from whole blood by means of density gradients such as Ficoll-Paque may not have the same relative concentrations of T and B cells as unseparated blood. This alteration is believed relatively insignificant for samples of blood from subjects with normal white blood counts. However, in leukopenic patients or patients with low proportions of lymphocytes, the selective loss of specific subsets may affect the accuracy of the determination.
- Incomplete gradient separation may occur in diseases marked by changes in lymphocyte size or may be due to the separation technique. At times, a clear-cut interfacial layer of mononuclear cells may not appear following centrifugation, or the sample may have excessive erythrocytes, debris, immature myeloid cells, or granulocyte contamination. If this occurs, do not use the preparation. Redo the procedure.
- Cryopreserved cells must have a viability of 85% to be used with these reagents.
- Determination of B-cell population using monoclonal antibodies will not always give results identical to those obtained using polyclonal antibodies to surface Ig. Discrepant results have been observed in cases where high levels of circulating IgG may cause aberrantly high slg readings, for example, in systemic lupus erythematosus.
- Abnormal states of health are not always represented by abnormal percentages of T or B lymphocytes. That is an individual who may be in an abnormal state of health may exhibit the same T or B lymphocyte percentage as a healthy individual. Test results should be used in conjunction with information available from the clinical evaluation and other diagnostic procedures.

### EXPECTED VALUES

Blood samples were collected from a population of 102 apparently healthy males and females over a period of one year. This population includes a variety of races, adults (ranging in age from 19 to 65 years) and pediatrics. COULTER CLONE B1 monoclonal antibody expected values for peripheral blood mononuclear cells (monocytes and lymphocytes) range between 3 and 11%. Expected values for whole blood (gated on lymphocytes only) range between 4 and 20%. Pediatric values were similar to those found for adult donors. These are intended as representative values only. Each laboratory should establish its own expected values from the local population of normal donors.

### PERFORMANCE CHARACTERISTICS SPECIFICITY

The B1 (CD20) associated antigen on a human B cell has a molecular weight of 33-35 kd.<sup>7</sup> The specificity of the COULTER CLONE B1 monoclonal antibody was determined by removal of the B1 positive population from peripheral blood by sorting or complement-mediated lysis eliminating the cell population that is induced to differentiate into Ig secreting plasma cells in a pokeweed mitogen driven system. COULTER CLONE B1 monoclonal antibody recognizes the B1 antigen on the human B lymphocytes.

Binding of the B1 antibody nonspecifically via the Fc receptor of normal lymphocytes is evaluated by inclusion of an isotypic mouse immunoglobulin control with each patient. This staining is limited to 1-2% in normal individuals. The Fc binding seen in monocytes can be excluded by proper gating on the flow cytometer (refer to the Product Manual) and morphological characteristics on the microscope by phase contrast. Tests performed on 3 (three) donors of various races at Coulter Immunology on each lot of COULTER CLONE B1 monoclonal antibody demonstrates these tests do not cross react with cell types other than lymphocytes.

### CORRELATION

Comparison of COULTER CLONE B1 monoclonal antibody assay with the slg assay for B cells on Ficoll-separated cells of normal donors.

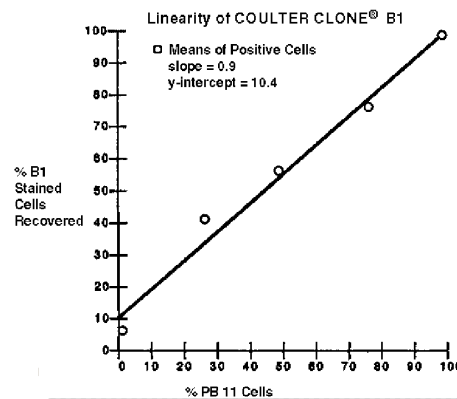
| Method             | Mean % B Cells | SD   | n  |
|--------------------|----------------|------|----|
| CC B1 (microscopy) | 8.65           | 5.14 | 69 |
| slg                | 11.51          | 6.35 | 69 |
| CC B1 (flow)       | 4.59           | 2.92 | 39 |
| slg                | 8.18           | 4.35 | 39 |

Comparison of COULTER CLONE B1 monoclonal antibody assay with the slg assay for B cells on Ficoll-separated cells of abnormal donors.

| Method             | Mean % B Cells | SD   | n  |
|--------------------|----------------|------|----|
| CC B1 (microscopy) | 6.75           | 4.78 | 59 |
| slg                | 12.17          | 8.62 | 59 |
| CC B1 (flow)       | 5.97           | 4.90 | 59 |
| slg                | 12.17          | 8.62 | 59 |

### LINEARITY

Dilutions were made to represent 100, 75, 50, 25, and 0 percent of B cells in a standard reaction volume containing 106 total cells/mL. These cells were stained with the standard concentration of COULTER CLONE B1 monoclonal antibody in each tube and analyzed by the EPICS® flow cytometer. This study was performed using two cell lines, PB 11 that strongly expresses the B-cell antigen and PB 55 that has no B-cell expression.



### PRECISION

Thirty-one replicate measurements were performed within the same day for each of the three levels of B-cell concentrations by flow cytometry and fluorescence microscopy.

| Method         | Level | Mean% | SD   | CV%   |
|----------------|-------|-------|------|-------|
| Flow Cytometer | HI    | 63.05 | 8.99 | 14.26 |
|                | MID   | 42.39 | 7.14 | 16.84 |
|                | LOW   | 22.76 | 3.76 | 14.32 |
| Microscope     | HI    | 66.54 | 6.05 | 9.09  |
|                | MID   | 46.12 | 6.71 | 14.55 |
|                | LOW   | 25.96 | 4.98 | 19.18 |

### REFERENCES

- Stashenko P, Nadler LM, Hardy R, and Schlossman SF: 1980. Characterization of a human B lymphocyte-specific antigen. *J. Immunol.* 125:1678-1685.
- Leucocyte Typing II: 1986. Volume 2. Springer - Verlag NY, NY Reinherz EL, Haynes BF, Nadler LM, and Bernstein ID. editors. p. 8, 20.
- Morimoto C, Reinherz EL, Nadler LM, Distaso JA, Steinberg AD, and Schlossman SF: 1982. Comparison of T and B cell markers in patients with Sjogrens syndrome and systemic lupus erythematosus. *Clinical Immunol. Immunopath.* 22:270.

### PRODUCT AVAILABILITY

COULTER CLONE B1 Monoclonal Antibody  
PN 6602140 - 100 tests (0.5 mL)

For additional information in the US, call 1-800-526-7694. Outside the US, contact your local Beckman Coulter Representative.

### TRADEMARKS

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