6602683 - 100 tests

PN 178467-A

MONOCLONAL ANTIBODY

For In Vitro Diagnostic Use

INTENDED USE

COULTER CLONE B4 is a murine monoclonal antibody reagent. In conjunction with a fluorescent label, it is used to identify and enumerate the percentage of CD19+ (B4)[1] lymphocytes in whole blood or mononuclear cell preparations by flow cytometry or fluorescence microscopy.

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.[2] These cell types are morphologically indistinguishable by microscopy but can be identified by characteristic antigenic differences in their cell membranes.

The CD19 antigen is a glycoprotein with a molecular weight of 95 kd.[1,3] It is an early, lineage-specific 'pan B cell' surface antigen and normally is present continuously from the earliest stages of B cell progenitor development until lost at the terminal stage of B lymphocyte differentiation into plasma cells.[1,3,4,5] It is found on over 90% of B lymphocytes isolated from either peripheral blood, spleen, lymph node or tonsil and on approximately 5% of bone marrow cells.[3] Expression within the hematopoietic system is restricted to normal and neoplastic B cells.[3] The CD19 antigen is not detected on peripheral blood T lymphocytes, monocytes, granulocytes or platelets.[3,5]

CLINICAL RELEVANCE

B lymphocyte percentage and absolute count may be used as aids to evalueate immune competency underlying known or unknown disease states[6-12] and to monitor lymphocyte levels following organ transplantation.[13,14]

To illustrate, identification of abnormal levels of B lymphocytes may aid in the diagnosis and/or prognosis of unidentified disease conditions in patients with low white blood cell counts. Measurement of B lymphocytes, in conjunction with CD4+ (inducer) and CD8+ (suppressor/cytotoxic) T lymphocytes and corresponding CD4+/CD8+ ratios, may aid in the diagnosis and/or prognosis of immunodeficiency disease such as infection with human immunodeficiency virus (HIV), the etiologic agent of acquired immunodeficiency syndrome (AIDS).[6,10-12] Altererd percentages of B lymphocytes recorded following organ (e.g., kidney, heart, liver, lung) transplantation suggest B lymphocyte quantitation may be useful as an aid in monitoring these cellular populations.

PRINCIPLES OF TEST

COULTER CLONE® monoclonal antibody B4 is a murine monoclonal antibody specific for the B cell surface antigen associated with surface immunoglobulin. Specific B cell staining is accomplished by incubating peripheral blood lymphocytes with the monoclonal antibody and washing the cells to remove unbound antibody. A second incubation with fluorescein-conjugated goat antiserum to mouse immunoglobulin, GAM-FITC is necessary. The percent of B cells may then be enumerated by either flow cytometry or fluorescence microscopy.

REAGENTS

COULTER CLONE® monoclonal antibody:
B4: PN 6602683 - 100 tests

CLONE:	Clone 89B was derived from the hybridization of mouse NS/1-Ag4-1 cells with spleen cells from BALB/c mice immunized with tumor cells from a patient with B cell CLL.5
Ig CHAIN COMPOSITION:	Mouse IgG1 heavy chain and Kappa light chains.
CYTOTOXICITY:	None by direct lysis
SOURCE:	Mouse ascites fluid and conditioned media
PURIFICATION:	Affinity chromatography

REAGENT CONTENTS

The final concentration of nonantibody reagents when reconstituted to 500 µL is 0.2% BSA, 0.01 M potassium phosphate, 0.15 M NaCl, and 0.1% NaN_3.

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® B4, 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 B4, Isotypic Control, or GAM-FITC Stock Solution Per Test	Volume of PBS**
100 tests	5 µL	Add 195 µL

*Diluted reagent must be used the same day as prepared.
**PBS - Phosphate Buffered Saline

4. Storage conditions of reconstituted B4
   Reconstituted and stored at 2-8°C..................6 months
   Reconstituted and stored at -70°C..................1 year

STORAGE CONDITIONS

Unreconstituted lyophilized B4 antibody may be stored at 2 to 8°C for 5 years from date of manufacture. 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, 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 (H3-A3), 3rd Edition" published by the National Committee for Clinical Laboratory Standards.

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 20-25°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, PN 6603152-300 tests, and follow the directions in the package insert. For each test, 100 µL of whole blood is required.

Coulter Procedure for Indirect Immunofluorescence Cell Surface Staining with COULTER CLONE® Antibodies (for microscopy or flow use)

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 antibody)
Phosphate Buffered Saline (PBS), 0.145 M NaCl, 0.01 M potassium phosphate, pH 7.2 (Coulter PN 6602489)
Formaldehyde (10% solution) in PBS
Ficoll-Paque® (Pharmacia PN 17-0840-03)
PBS containing 2% fetal or newborn calf serum and 0.01% NaN3 (wash media)
PBS containing 0.01% NaN3 (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
VACUTAINERS® 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 4°C PBS, mix and spin at 400 x g for 4 min. Aspirate and discard the supernatant.
6. Resuspend cells 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 4°C wash media or 4°C PBS, mix gently and determine cell concentration using a COULTER COUNTER® instrument (ZBI or S series) or hemocytometer.
8. Viability analysis is performed by one of two methods suggested below.

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.

9. Place a suspension of approximately 25,000 cells (25 µL of a 1 x 10^6 cells/mL suspension) onto a microscope slide.
10. Add 10 µL of a 0.01 mg/mL solution of propidium iodide and mix gently by stirring with a pipet tip.
11. Allow to stand for 30 s. Add 10 µL of a 0.005 mg/mL solution of acridine orange. Mix gently and allow to stand for 3 s.
12. Place a 24 x 50 mm coverslip on the slide, seal with stopcock grease, and examine by fluorescence microscopy.
13. Count 100 cells and report viability as the percent of viable cells which appear bright green as opposed to nonviable cells which 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.

9. Place 1 x 10^6 cells into a 12 x 75 mm siliconized test tube.
10. Wash one time with resuspension media and centrifuge.
11. Aspirate supernatant and add three drops of 0.05 mg/mL propidium iodide. Allow to stand for 1 min.
12. Wash two times in resuspension media.
13. 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 instrument manual.

CAUTION: If the laser on the flow cytometer is misaligned or the lymphocyte gates are improperly set, results may be erroneous.

PROCEDURE FOR INDIRECT IMMUNOFLUORESCENCE CELL SURFACE STAINING OF LYMPHOCYTES WITH COULTER CLONE B4 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% NaN3 (resuspension media)
10% formaldehyde in PBS
COULTER CLONE lyophilized GAM-FITC (conjugated second antibody)
PN 6602159 - 100 tests
COULTER CLONE MsIgG1 Isotypic Control,
PN 6602872 - 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, MsIgG1) should be run with each patient sample.

PROCEDURE

Test Tube Method

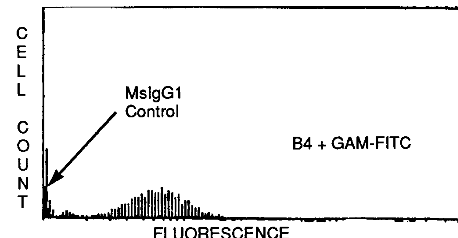
1. Into 12 x 75 mm test tubes place 1 x 106 cells from Ficoll-Paque® preparation, centrifuge 4 min at 400 x g at 4°C, aspirate supernatant.
2. Add 200 µL of COULTER CLONE B4 monoclonal antibody working solution to one test tube and control to the next test tube. Vortex gently. Incubate the reaction mixture at 4°C for 30±5 min.
3. 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.
4. After the second wash, aspirate supernatant and add 200 µL of GAM-FITC working solution to the cell pellet. Disrupt pellet (vortex) and incubate at 4°C for 30±5 min.
5. 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.
6. After third wash, resuspend pellet to 1 mL with resuspension media at 4°C for flow cytometry or fluorescence microscope analysis.

Microtiter Plate Method

1. Adjust the concentration of the cell suspension of mononuclear cells to 1 x 10^6 cells/200 µL and dispense that volume into each well.
2. Centrifuge microtiter plates at 4°C, at 400 to 450 x g for 5 min.
3. 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.
4. 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.
5. Add 200 µL of COULTER CLONE B4 monoclonal antibody working solution and control into alternate wells and gently agitate. Incubate at 4°C for 30 min.
6. Centrifuge the plate at 4°C at 400 to 450 x g for 5 min.
7. Aspirate the supernatant and disrupt the pellets on the vortex mixer.
8. Add 200 µL of wash media and centrifuge at 4°C at 400 to 450 x g for 5 min.
9. Aspirate the wash media and disrupt the pellets on the vortex.
10. Add 200 µL of GAM-FITC working solution. Mix gently. Incubate the reaction mixture at 4°C for 30 min.
11. Centrifuge the plate at 4°C at 400 to 450 x g for 5 min. Wash the cell pellet twice as performed previously.
12. Resuspend the pellets in 200 µL of resuspension media and transfer to appropriate containers for flow cytometric analysis or fluorescence microscopy.

Analysis of Cells for Both Methods

1. Transfer 200 µL of each final cell suspension to test tube containing 20 µL of 10% formaldehyde in PBS. Place one drop of fixed cells on a microscope slide. Cover with a 22 x 22 mm coverslip. Seal with stopcock grease and examine by fluorescence microscopy.
2. 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.
3. Analyze remaining unfixed cells on a flow cytometry according to the instrument manual.



PROCEDURE FOR FREEZING RECONSTITUTED COULTER CLONE MONOCLONAL ANTIBODIES

MATERIAL PROVIDED

COULTER CLONE Monoclonal Antibody:
B4: PN 6602683 - 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 µL of distilled water as described previously. Dilute the reconstituted 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 µL	100 µL

*These volumes 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 (20-25°C). Dilute with 100 µL 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 MsIgG1 control is used as a quality control reagent for surface staining procedures which use either labeled or unlabeled primary monoclonal antibody of the mouse IgG1 subclass. This reagent makes it possible to monitor levels of nonspecific staining in cell surface staining procedures. If background levels of the control are not acceptable, test results of the patient samples should be considered invalid.

Each lot of B4 is carefully screened on all cell types (lymphocytes, monocytes, granulocytes, erythrocytes, and thrombocytes) 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

1. Stored or refrigerated samples may give aberrant results.
2. Immunodeficient patients may present special problems due to altered or very low numbers of certain lymphocyte populations.
3. 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 five minutes after centrifugation.
4. 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.
5. 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.
6. Cryopreserved cells must have a viability of 85% to be used with these reagents.
7. 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 sIg readings, for example, in systemic lupus erythematosis.
8. 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.
9. Due to an unacceptable variance among the different laboratory methods for determining absolute lymphocyte counts, an assessment of the accuracy of the methods used is necessary.15

EXPECTED VALUES

Blood samples were collected from a population of 250 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® B4 monoclonal antibody expected values for peripheral blood mononuclear cells (monocytes and lymphocytes) range between 3-13% B4+ cells. Expected values for whole blood (gated on lymphocytes only) range between 7-15% B4+ cells. 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 specificity of the COULTER CLONE B4 monoclonal antibody for surface antigen of the B lymphocyte was determined by removal of the B4 positive population from peripheral blood by sorting the cell population that is induced to differentiate into Ig secreting plasma cells in a pokeweed mitogen driven system. COULTER CLONE B4 monoclonal antibody recognizes the B4 antigen on the human B lymphocytes.

Fc binding to lymphocytes can be negated using test samples stained with appropriate mouse isotypic control antibodies. The positive stained population can then be measured in gates set to exclude this low level of fluorescence. By microscopy, the nonspecific, dimly stained cells can be distinguished visually from the brightly stained positive cell populations. The Fc binding seen in monocytes can be excluded by proper gating on the flow cytometer and morphological characteristics on the microscope by phase contrast. Tests performed on five donors of various races on each lot of COULTER CLONE B4 monoclonal antibody demonstrate this test does not cross react with blood cell types other than lymphocytes.

CORRELATION

Comparison of COULTER CLONE B4 and B1 monoclonal antibody assay for B cells on 250 normal donors.

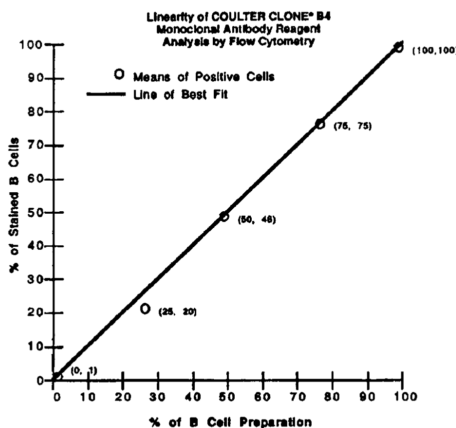
Method	Mean % B Cells	SD
CC B4 (microscopy)	5.47	3.82
CC B1	4.11	2.28
CC B4 (flow)	8.34	4.52
CC B1	4.13	2.21

Comparison of COULTER CLONE B4 and B1 monoclonal antibody assay for B cells on 64 abnormal donors.

Method	Mean % B Cells	SD
CC B4 (flow)	25.84	27.12
CC B1	20.14	23.81

LINEARITY

Dilutions were made to represent 100, 75, 50, 25, and 0 percent of the B-cell preparation in a standard reaction volume containing 106 total cells/mL. These cells were stained with the standard concentration of COULTER CLONE B4 monoclonal antibody in each tube and analyzed in triplicate by flow cytometry.



PRECISION

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

Method	Level	Mean%	SD
Flow Cytometer	HI	99.03	0.18
	MID	45.39	1.33
	LOW	15.26	0.68
Microscope	HI	97.42	1.78
	MID	44.81	6.02
	LOW	18.48	3.86

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PRODUCT AVAILABILITY

COULTER CLONE B4 Monoclonal Antibody
PN 6602683 - 100 tests

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

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