MONOCLONAL ANTIBODIES TO HUMAN CELL SURFACE ANTIGENS

Mouse Anti CD3 Fluorescein (FITC)-labeled
Mouse Anti CD4 R-phycoerythrin (R-PE)-labeled

CATALOG No. CD3-4-A	50 tests	0.5 ml
CATALOG No. CD3-4-B	200 tests	2.0 ml

FOR *IN-VITRO* DIAGNOSTIC USE

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PI: L13003 (Rev 11/09) DCC-09-1769

I. INTENDED USE

INVITROGEN'S CD3/CD4 combination is a fluorescent reagent containing mouse anti-human CD3 and CD4 monoclonal antibodies conjugated to fluorescein and phycoerythrin, respectively. This reagent permits the simultaneous identification of $CD3^+$ mature T lymphocytes and $CD4^+$ helper/inducer T lymphocytes in human peripheral blood using flow cytometric methods.

II. SUMMARY AND EXPLANATION

The major cellular or cell-derived elements of human peripheral blood include lymphocytes, monocytes, granulocytes, red blood cells and platelets. The mature lymphocyte population contains functionally distinct cell types, that are referred to as T (thymus-derived), B and NK (Natural Killer) cells. Two major subsets of T lymphocytes have distinctly different functional properties, and can be identified by correspondingly different cell surface glycoprotein antigens (1). The $CD3^+$ mature T lymphocytes expressing the CD4 surface antigen are responsible for helper-inducer activity, while $CD3^+$ cells that express the CD8 antigen have suppressor and cytotoxic activity (2).

CD3 is a complex consisting of at least five glycoprotein chains, each having a molecular weight of 20-25 kDa. The gamma subunit, and probably other subunits, of this molecule are closely associated with the alpha and beta chains of the T cell receptor (TCR) molecule (3). The CD3/TCR complex is responsible for the recognition of antigens which are expressed in association with the major histocompatibility (MHC) antigens. The CD3 molecule is generally understood to initiate or mediate T cell activation through signals derived from the TCR molecule (4).

The CD3 molecule is present on the majority of resting and activated mature T lymphocytes, and has been used extensively to enumerate these cells in human peripheral blood. This molecule is also present on some natural killer (NK) cells. The CD3 molecule is a T cell lineage marker and is present on the majority of mature thymocytes. The expression of CD3 on thymocytes normally follows the expression of CD2, CD5 and CD7.

The CD4 molecule is a 62 kDa glycoprotein that is found on approximately 60% of peripheral blood T lymphocytes and a variable percentage of monocytes. This antigen is also expressed on most thymocytes, which are located primarily in the cortical area of the thymus (5).

The CD4 molecule is responsible for the recognition of type II major histocompatibility antigens (MHC-II). $CD4^+$ T cells function primarily as a helper or promoter of immune functions through T cell-T cell, T cell-B cell and T cell-macrophage interactions, and are considered to have a pivotal role in the regulation of the immune response. Among functions attributed to $CD4^+$ T cells are inducer activity for $CD8^+$ T suppressor cells (6), and inducer activity for the maturation of B lymphocytes (7).

III. CLINICAL RELEVANCE

INVITROGEN'S CD3 monoclonal antibody recognizes the CD3 antigen (8). The monoclonal antibody may be used to enumerate mature T lymphocytes in human peripheral blood. INVITROGEN'S CD3 monoclonal antibody may also be used, in combination with other indicators, for the diagnosis or prognosis of some immunodeficiency diseases, including agammaglobulinemia and the severe combined immunodeficiency disease (SCID), which exhibit decreased percentages of T lymphocytes (9,10). Decreased percentages of T lymphocytes may be observed in some autoimmune diseases, such as systemic lupus erythematosus (SLE), multiple sclerosis and Sjorgren's disease (11-13), as well as in certain viral diseases caused by cytomegalovirus and Epstein-Barr virus (14). In general, diseases that have decreased cellular immunocompetence as a component may exhibit decreases in T lymphocytes.

INVITROGEN'S CD4 monoclonal antibody recognizes the CD4 antigen (8). This antibody may be used, in combination with other indicators, for the diagnosis or prognosis of immunodeficiency diseases, including hypogammaglobulinemia, severe combined immunodeficiency (SCID) and the acquired immunodeficiency disease (AIDS) (10, 16-18).

The CD4 antigen domain is generally recognized to contain the binding site for the HIV retrovirus through interaction with the viral envelope glycoprotein (19). Infection of $CD4^+$ T lymphocytes with the HIV virus eventually results in a profound depletion of these cells with the progression of disease.

HIV Infected cells continue to serve as a reservoir for the replicating virus. A decrease in the population of $CD4^+$ cells is directly correlated with immune deficit and deterioration in infected patients (20).

The CD3/CD4 monoclonal antibody combination permits the simultaneous enumeration of $CD3^+$ $CD4^+$ T lymphocytes, and the exclusion of $CD3^-$ cells, which may not be members of the T cell lineage.

IV. PRINCIPLES OF THE TEST

INVITROGEN'S CD3/CD4 monoclonal antibody combination binds to the surfaces of blood cells that express the corresponding antigens. To identify cells bearing these antigenic determinants, peripheral blood leukocytes are incubated with the monoclonal antibodies and washed to remove unbound antibodies. Prior to removal of unbound antibody, a lysing solution is added to lyse red blood cells. An appropriate fixative solution is added to lysed and washed cells. Stained and fixed cells are subsequently analyzed by flow cytometric methods.

An appropriate combination isotypic control should be used in sample analysis to determine the amount of any background or nonspecific fluorescence. This isotype control should be of the same immunoglobulin isotypes and approximately the same antibody protein concentrations as the conjugated combination monoclonal antibodies.

An appropriate cell gating control should be used in sample analysis. The CD45 pan-lymphocyte and CD14 pan-monocyte reagents are intended to optimize the resolution of lymphocyte and monocyte populations (21).

V. REAGENTS

A. INVITROGEN MONOCLONAL ANTIBODY REAGENTS

Cluster Designation:	CD3
WHO Classification:	Leukocyte Workshop V(8)
Clone:	S4.1
Isotype:	IgG2a
Species:	Mouse
Composition:	IgG2a Heavy chain Kappa Light chain
Source:	Mouse ascites fluid
Method of Purification:	Column chromatography
Fluorochrome:	Fluorescein (FITC) Excitation Wavelength 488 nm Emission Wavelength 525 nm

Cluster Designation:	CD4
WHO Classification:	Leukocyte Workshop V(8)
Clone:	S3.5
Isotype:	IgG2a
Species:	Mouse
Composition:	IgG2a Heavy chain Kappa Light chain
Source:	Mouse ascites fluid
Method of Purification:	Column chromatography
Fluorochrome	R-Phycoerythrin (R-PE)* Excitation Wavelength 488 nm Emission Wavelength 575 nm

B. REAGENT CONTENTS

0.5 ml vial containing monoclonal antibody for 50 tests, 4 mg/ml BSA, 0.02 M sodium phosphate, 0.30 M sodium chloride, sucrose not more than 10% and 0.1% sodium azide.

2.0 ml vial containing monoclonal antibody for 200 tests, 4 mg/ml BSA, 0.02 M sodium phosphate, 0.30 M sodium chloride, sucrose not more than 10% and 0.1% sodium azide.

*** U.S. Patent No. 4,520,110, Canadian Patent No. 1,179,942, European Patent No. 76,695**

VI. STATEMENT OF WARNINGS

Reagents contain sodium azide. Sodium azide, under acid conditions yields, hydrazoic acid, an extremely toxic compound. Solutions containing sodium azide should be diluted with running water before being discarded. These conditions are recommended to avoid deposits in plumbing where explosive conditions may develop.

Light exposure should be avoided. Use dim light during handling, incubation with cells and prior to analysis.

Do not pipette by mouth.

Samples should be handled as if capable of transmitting infection. Appropriate disposal methods should be used.

The sample preparation procedure employs a fixative (formaldehyde). Contact is to be avoided with skin or mucous membranes.

Do not use antibodies beyond the stated expiration dates of the products.

FOR *IN VITRO* DIAGNOSTIC USE

VII. APPROPRIATE STORAGE CONDITIONS

Store reagents at 2-8°C. Do not freeze. Reagents should be brought to room temperature (22±3°C) before use. Protect cells from light sources during incubation with antibodies and prior to analysis.

VIII. EVIDENCE OF DETERIORATION

Reagents should not be used if any evidence of deterioration or substantial loss of reactivity is observed. The normal appearance of the CD3 FITC/CD4 R-PE-conjugated monoclonal antibody is a vivid light pink liquid.

IX. SPECIMEN COLLECTION

Collect venous blood samples into blood collection tubes using an appropriate anticoagulant. For optimal results the sample should be processed within 6 hours of venipuncture. EDTA, ACD or heparin may be used if the blood sample is processed for analysis within 30 hours of venipuncture. ACD or heparin, but not EDTA may be used if the sample is not processed within 30 hours of venipuncture. Samples that cannot be processed within 48 hours should be discarded (22, 23). If venous blood samples are collected into ACD for flow cytometric analysis, a separate venous blood sample should be collected into EDTA if a CBC is required. Unstained anticoagulated blood should be retained at 20-25°C prior to sample processing. Blood samples that are hemolyzed, clotted or appear to be lipemic, discolored or to contain interfering substances should be discarded.

Refer to "Standard Procedures For The Collection Of Diagnostic Blood Specimens", published by the National Committee For Clinical Laboratory Standards (NCCLS) for additional information on the collection of blood specimens.

X. SAMPLE PREPARATION

1. Collect blood into an appropriate anticoagulant.
2. Determine leukocyte count and, if necessary, dilute blood in an isotonic solution to a concentration of approximately 5 x 10^6 leukocytes per ml. Determine cell viability using Trypan Blue or Propidium Iodide. If the cell viability is not at least 85%, the blood sample should not be used.
3. Pipette 100 µl of well mixed blood into 12 x 75 mm polypropylene centrifuge tubes marked Unknown and Control.
4. Add 10 µl of INVITROGEN'S CD3/CD4 monoclonal antibody combination to tubes marked Unknown. Add 10 µl of INVITROGEN'S corresponding IgG2a FITC/IgG2a R-PE-conjugated isotypic control reagent to tubes marked Control. Mix gently.
5. Incubate all tubes for 15 minutes at room temperature (22 ±3°C) in the dark.
6. Add lysing solution to all tubes according to the manufacturer's directions.
7. Centrifuge all tubes at 400 x g for 3 minutes at room temperature.
8. Add fixing solution to all tubes according to the manufacturer's directions. Retain cells in fixing solution for not less than 30 minutes at room temperature (22 ±3°C).
9. Wash the cells in all tubes twice with 4 ml of PBS. Centrifuge at 400 x g for 3 minutes after each wash procedure.
10. Resuspend the cells from the final wash in 1 ml of PBS and store tubes at 2-8°C in the dark until flow cytometric analysis is performed. It is recommended that analysis be performed within 18 hours of fixation.
11. Analyze on a flow cytometer according to the manufacturer's instructions.

XI. MATERIALS REQUIRED BUT NOT SUPPLIED:

Serofuge or equivalent centrifuge
12 x 75 mm polypropylene centrifuge tubes
Micropipette capable of dispensing 20 µl, 100 µl, and 500 µl volumes
Blood collection tubes with anticoagulant
PBS
Trypan Blue or Propidium Iodide, 0.25% (w/v) in PBS for the determination of cell viability (24)
Lysing solution
Fixing solution
Flow cytometer; Becton Dickinson FACScan™, Coulter® Profile® or equivalent

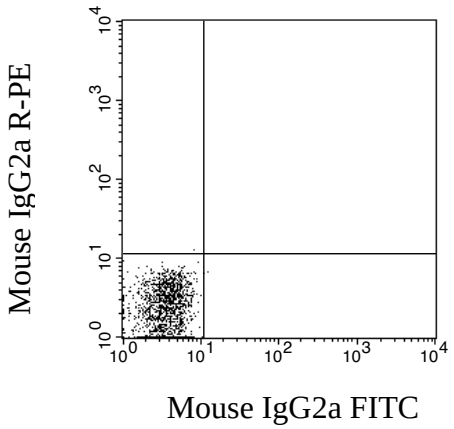
XII. INTERPRETATION OF RESULTS

FLOW CYTOMETRY

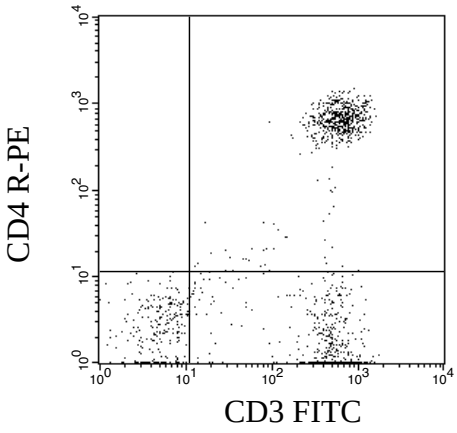
Analyze antibody-stained cells on an appropriate flow cytometer according to the manufacturer's instructions. The right angle (side) light scatter (SSC) versus forward angle light scatter (FSC) is collected to reveal the lymphocyte cell cluster. A gate is drawn for the lymphocyte cluster (lymphocyte bitmap). The fluorescence attributable to the FITC- and R-PE-conjugated monoclonal antibodies are collected, and the percentage of antibody-stained T lymphocytes is determined. An appropriate FITC- and R-PE-conjugated combination isotypic control of the same heavy chain immunoglobulin classes and antibody concentration must be used to estimate and correct for nonspecific binding to lymphocytes. An analysis region is set to exclude background fluorescence and to include positively stained cells.

The following histograms are representative of cells stained and gated on the lymphocyte region from a normal donor:

INVITROGEN IgG2a FITC/IgG2a R-PE ISOTYPE CONTROL



INVITROGEN CD3 FITC/CD4 R-PE MONOCLONAL ANTIBODY



ABSOLUTE COUNTS

Use the following formula for the calculation of Absolute Counts:

Absolute Counts = Total White Blood Cell Count x % Positive Lymphocytes x % Positively Stained Cells/10^4.

Due to an unacceptable variance among the different laboratory methods for determining absolute counts, an assessment of the accuracy of the method is necessary (25).

XIII. QUALITY CONTROL PROCEDURES

Nonspecific fluorescence identified by the FITC- and R-PE-conjugated isotypic controls is usually less than 2% in normal individuals. If the background level exceeds these values, test results may be in error. Increased nonspecific fluorescence may be seen in some disease states.

A blood sample from each normal and abnormal donor should be stained with the CD45 panlymphocyte and CD14 panmonocyte monoclonal antibodies (21). When used in combination, these reagents assist in identifying the lymphocyte analysis region, and distinguish lymphocytes from monocytes, granulocytes and unlysed or nucleated red cells and cellular debris.

A blood sample from a healthy normal donor should be analyzed as a positive control on a daily basis or as frequently as needed to ensure proper laboratory working conditions. Each laboratory should establish their own normal ranges, since values obtained from normals may vary from laboratory to laboratory. An appropriate combination isotype control should be used as a negative control with each patient sample to identify nonspecific Fc binding to lymphocytes.

An analysis region should be set to exclude the nonspecific fluorescence identified by the isotypic control, and to include the brighter fluorescence of the lymphocyte population that is identified by the specific antibody.

Refer to the appropriate flow cytometer instrument manuals and other available references for recommended instrument calibration procedures (26, 27).

XIV. LIMITATIONS OF THE PROCEDURE

1. Incubation of antibody with cells for other than the recommended time and temperature may result in capping or loss of antigenic determinants from the cell surface.
2. The values obtained from normal individuals may vary from laboratory to laboratory; therefore, it is recommended that each laboratory establish its own normal range.
3. Abnormal cells or cell lines may have a higher antigen density than normal cells. This could, in some cases, require the use of a larger quantity of monoclonal antibody than is indicated in the procedures for Sample Preparation.
4. Blood samples from abnormal donors may not always show abnormal values for the percentage of lymphocytes stained with a given monoclonal antibody. Results obtained by flow cytometric analysis should be considered in combination with results from other diagnostic procedures.

5. When using the whole blood method, red blood cells found in some abnormal donors, as well as nucleated red cells found in normal and abnormal donors may be resistant to lysis by lysis solutions. Longer red cell lysis periods may be needed to avoid the inclusion of unlysed red cells in the lymphocyte gated region.
6. Blood samples should not be refrigerated or retained at ambient temperature for an extensive period (longer than 24-30 hours) prior to incubating with monoclonal antibodies.
7. Accurate results with flow cytometric procedures depend on correct alignment and calibration of the laser, as well as proper gate settings.
8. Due to an unacceptable variance among the different laboratory methods for determining absolute lymphocyte counts, an assessment of the accuracy of the method used is necessary (25).

XV. EXPECTED VALUES

Blood samples were collected from a total of 159 apparently healthy normal donors in an age range of 15 to 82, for the determination of expected values of INVITROGEN'S CD3/CD4 monoclonal antibody combination. The population contained members of differing ethnic origins, including adult Caucasians, Blacks, Asians and Hispanics, and included males and females in approximately equal numbers. Donors in geographically diverse areas of the United States, including the Eastern, South Central and Western regions, participated in this study.

Summary of expected values for INVITROGEN CD3 FITC and CD4 R-PE components for 159 normal donors:

Procedure	Mean % Positive	S.D. ±2 S.D.	Range
CD3 FITC component	71.8	7.7	56-87
CD4 R-PE component	46.9	8.3	30-64

Comparison of CD3 FITC and CD4 R-PE components to the CD3 FITC and CD4 R-PE single antibodies. Blood samples were collected from a different randomly selected population of 130 adult normal donors having a similar age, gender and ethnic profile.

SINGLE ANTIBODIES

Procedure	Mean % Positive	S.D. ±2 S.D.	Range
CD3 FITC	71.8	6.9	58-86
CD4 R-PE	47.3	8.0	31-63

The values obtained from normal individuals may vary from laboratory to laboratory. Therefore, it is recommended that each laboratory establish its own normal range.

Expected values for pediatrics and adolescents have not been determined.

Rare hereditary CD4 epitope deficiencies have been reported for some CD4 monoclonal antibodies (28,29).

XVI. PERFORMANCE CHARACTERISTICS

SPECIFICITY

Blood samples were obtained from healthy normal donors of Caucasian, Black, Hispanic and Asian ethnic origins. Samples of each donor were stained with INVITROGEN'S CD3 FITC/CD4 R-PE monoclonal

antibodies. Cells contained in the lymphocyte, monocyte and granulocyte regions were selected for analysis. Separate samples from the same donors were prepared for analysis of red blood cells and platelets and stained with each of the INVITROGEN monoclonal antibodies.

CD3 FITC Component

Ethnic	Percent of Stained Cells				
Origin	Lymph.	Mono.	Gran.	Plt.	RBC
Caucasian	56.6	13.8	1.7	0.0	0.0
Caucasian	74.8	9.8	0.0	0.1	0.0
Hispanic	81.4	1.3	0.7	0.0	0.1
Asian	46.6	9.5	1.7	0.0	0.0
Black	76.4	5.7	0.5	0.1	0.0
Mean	67.2	8.0	0.9	0.0	0.0
±1 S.D.	14.8	4.7	0.8	0.0	0.0

CD4 R-PE Component

Ethnic	Percent of Stained Cells				
Origin	Lymph.	Mono.	Gran.	Plt.	RBC
Caucasian	29.5	93.7	1.7	0.0	0.0
Caucasian	45.2	94.0	2.5	0.0	0.0
Hispanic	48.4	50.7	1.2	0.1	0.1
Asian	21.8	93.8	1.4	0.0	0.0
Black	55.2	83.0	1.3	0.1	0.1
Mean	40.0	83.0	1.6	0.0	0.0
±1 S.D.	13.9	18.7	0.5	0.0	0.0

Specific and/or nonspecific antibody Fc binding to monocytes in a patient sample can be excluded by proper gating on lymphocytes on the flow cytometer.

CORRELATION

The Correlation study was performed on 67 donors, including 40 normal and 27 abnormal donors. Comparison of the INVITROGEN CD3 FITC conjugated monoclonal antibody component of CD3 FITC/CD4 R-PE with the Coulter CD3 FITC conjugated monoclonal antibody:

Procedure	Mean % Positive	S.D.	r value
CD3 FITC component	70.8	18.1	95.2
Coulter CD3 FITC	71.5	17.5	

CD3 FITC

Slope	+ 0.99
y intercept	+ 0.31
Linear regression	y = 0.99x + 0.31

Comparison of the INVITROGEN CD4 R-PE conjugated monoclonal antibody component of CD3 FITC/CD4 R-PE with the Coulter CD4 RD1 conjugated monoclonal antibody:

Pprocedure	Mean % Positive	S.D.	r value
CD4 R-PE component	41.9	18.7	93.9
Coulter CD4 RD1	41.5	18.1	

CD4 R-PE

Slope	+0.99
y intercept	+1.2
Linear regression	y = 0.99x + 1.2

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