US 20040110122A1

(19) **United States**
(12) **Patent Application Publication** (10) **Pub. No.: US 2004/0110122 A1**
Kasinrerk (43) **Pub. Date: Jun. 10, 2004**

(54) **THREE-COLOR REAGENT FOR MEASUREMENT OF CD4 POSITIVE LYMPHOCYTES BY FLOW CYTOMETRY**

(75) Inventor: **Watchara Kasinrerk**, Amphur Muang (TH)

Correspondence Address:
Ms. Nuttina Netsuwan
113 Thailand Science Park
Paholyothin Rd., Klong 1
Klong Luang
Pathumthani 12120 (TH)

(73) Assignees: **National Science and Technology Development Agency**, Klong Luang (TH); **Thailand Research Fund**, Phayathai (TH)

(21) Appl. No.: **10/461,544**

(22) Filed: **Jun. 16, 2003**

(30) **Foreign Application Priority Data**

Jun. 14, 2002 (TH).. 074492

Publication Classification

(51) **Int. Cl.**^{7} **G01N 33/53**; C12Q 1/00
(52) **U.S. Cl.** **435/4**; 435/7.1

(57) **ABSTRACT**

The developed reagent is three-color immunophenotyping reagent for measurement of CD4 positive lymphocytes in peripheral blood. The reagent contains 7-aminoactinomycin D (7-AAD) which intercalates into double stranded DNA and is easily excited at 488 nm. The fluorescence emission of 7-AAD has peak at 670 nm that can be detected with FL3 detector of flow cytometer. The 7-AAD, therefore, stains white blood cells and discriminates it from red blood cells. The reagent also contains fluorescein isothiocyanate (FITC) labeled CD4 monoclonal antibody and phycoerythrin (PE) labeled CD14 monoclonal antibody which are detected with FL1 and FL2 detectors of flow cytometer, respectively. The developed reagent can be used to measure number of CD4 positive lymphocytes in lymphocyte population and monitor monocyte contamination simultaneously. This reagent therefore provides more accuracy results of CD4 positive lymphocyte enumeration.

THREE-COLOR REAGENT FOR MEASUREMENT OF CD4 POSITIVE LYMPHOCYTES BY FLOW CYTOMETRY

CROSS-REFERENCE TO RELATED APPLICATION

[0001] Not Applicable

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] Not Applicable

REFERENCE TO SEQUENCE LISTING

[0003] Not Applicable

BACKGROUND OF THE INVENTION

[0004] Human immunodeficiency virus (HIV) is a retrovirus that infects cells those possess the CD4 receptor. This infection causes the depletion of CD4 positive lymphocytes, which is a major clinical finding in progressive infection. In HIV infection, the absolute number of CD4 positive lymphocytes is an important marker for prognosis, classification of the state of disease, treatment decision and monitoring of therapy. Immunophenotyping using lysed whole blood stained with monoclonal antibody panels and analyzed by flow cytometry is the current standard method for determination of CD4 positive lymphocytes.

[0005] Recently several types of immunophenotyping reagents for measurement of CD4 positive lymphocytes have been developed and routinely used in clinical laboratory. The three-color immunophenotyping reagent is a common flow cytometric reagent for determining CD4 positive lymphocytes in routine laboratory. The available three-color reagents contain peridinin chlorophyll protein (PerCP) or phycoerythrin-cyanin 5.1 (PE-Cy5) labeled CD45 monoclonal antibody for discrimination of white blood cells and red blood cells and contain FITC labeled CD3 monoclonal antibody and PE labeled CD4 monoclonal antibody for enumeration of CD4 positive lymphocytes. Unlike two-color reagent, a disadvantage of three-color reagents is that it cannot indicate number of monocytes that are contaminated in the lymphocyte population gated during flow cytometric measurement. If significant numbers of the contaminated monocytes are presented in the acquisition lymphocyte gate, the obtained CD4 positive lymphocyte number will be erroneously decreased.

[0006] We have developed a three-color reagent that can enumerate percentage of CD4 positive lymphocytes in lymphocyte population and can determine the contamination of monocytes in the acquisition lymphocyte gate, simultaneously. This reagent contains 7-aminoactinomycin D (7-AAD) which can intercalate into double stranded DNA of white blood cells and allow white blood cells, but not red blood cells, to be detected with FL3 detector of flow cytometer. The reagent also contains FITC labeled CD4 monoclonal antibody and PE labeled CD14 monoclonal antibody, which allow CD4 positive cells and monocytes to be detected with FL1 and FL2 detector, respectively.

DETAILED DISCRIPTION OF THE INVENTION

[0007] 1. Production of Hybridomas Producing CD4 and CD14 Monoclonal Antibodies

[0008] For CD4 monoclonal antibody production, Balb/C mouse was immunized with SupT1 cell line. For CD14 monoclonal antibody production, Balb/C mouse was immunized with CD14 expressing COS cells. By using standard hybridoma technique, hydridoma producing CD4 monoclonal antibody (named MT4) and hybridoma producing CD14 monoclonal antibody (named MT14/3) were generated. MT4 and MT14/3 monoclonal antibodies are IgM and IgG1 isotype, respectively.

[0009] 2. Production of FITC Labeled CD4 Monoclonal Antibody and PE-Labeled CD14 Monoclonal Antibody

[0010] Ascitic fluids containing CD4 and CD14 monoclonal antibody were obtained by inoculating of MT4 and MT14/3 hybridoma clones into Balb/C mice. CD4 and CD14 monoclonal antibodies were purified from ascites by affinity chromatography using anti-mouse IgM coated sepharose column and protein G coated sepharose column, respectively. Purified CD4 monoclonal antibodies were conjugated with FITC and purified CD14 monoclonal antibodies were conjugated with PE.

[0011] 3. Immunofluorescence Staining

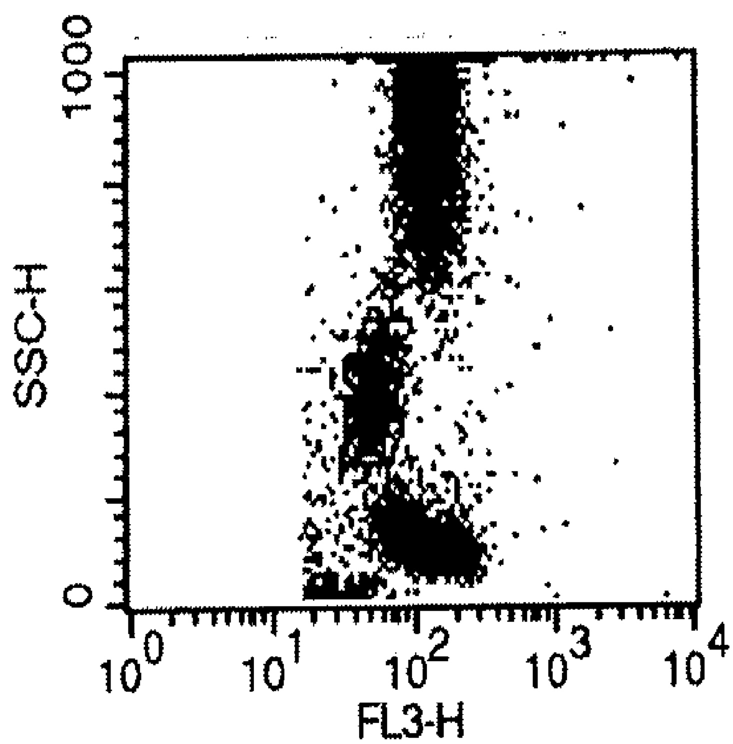
[0012] Ten microlitters of 7-AAD solution, FITC labeled CD4 monoclonal antibody and PE labeled CD14 monoclonal antibody are added into 100 μl of EDTA-whole blood in 12×75 mm tube (sample tube). For control, only 10 μl of 7-AAD solution is added into 100 μl of blood in 12×75 mm tube (control tube). All tubes are gently mixed and incubated at room temperature for 30 minutes in the dark. Following the incubation period, 2 ml of RBC lysing solution is added and incubated for another 10 minutes. After centrifugation at 500×g for 5 minutes and subsequent washed with 2 ml PBS containing 0.1% sodium azide, the cell pellets are resuspened in 0.5 ml of 1% paraformaldehyde in PBS. The stained cells are then analyzed by a flow cytometer.

[0013] 4. Flow Cytometric Analysis

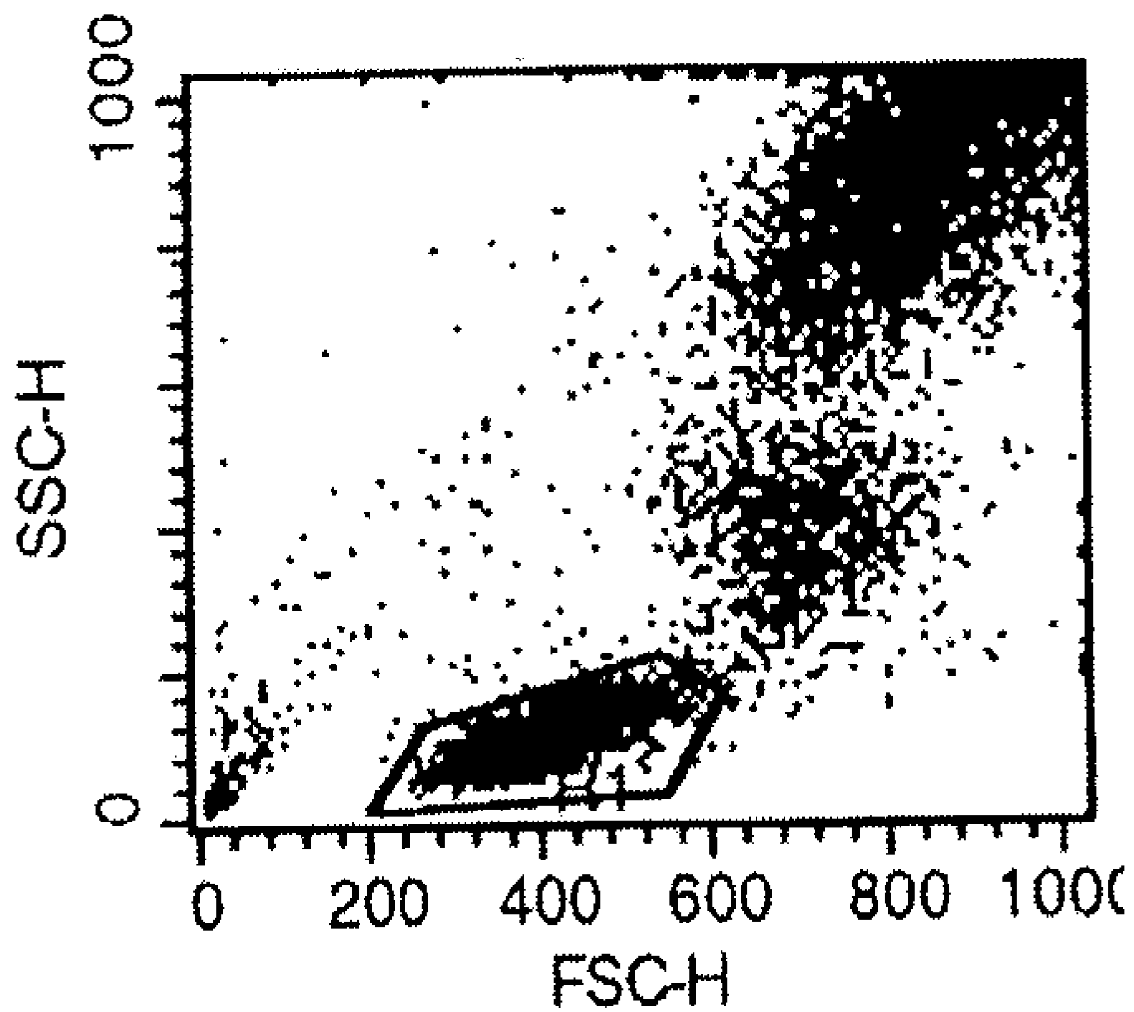
[0014] 4.1 Flow Cytometer Setting

[0015] The control tube is used firstly to set up the flow cytometer. By acquisition using FL3 and SSC, red blood cells are gated out from the 7-AAD stained white blood cells having bright red fluorescence by the FL3 threshold (FIG. 1). The remained white blood cells are analyzed using FSC/SSC and lymphocyte population is gated according to their size and granularity (FIG. 2). By monitoring of FL1 and FL2, cells in the gated lymphocyte population are set as fluorescence-negative cell populations (FIG. 3).

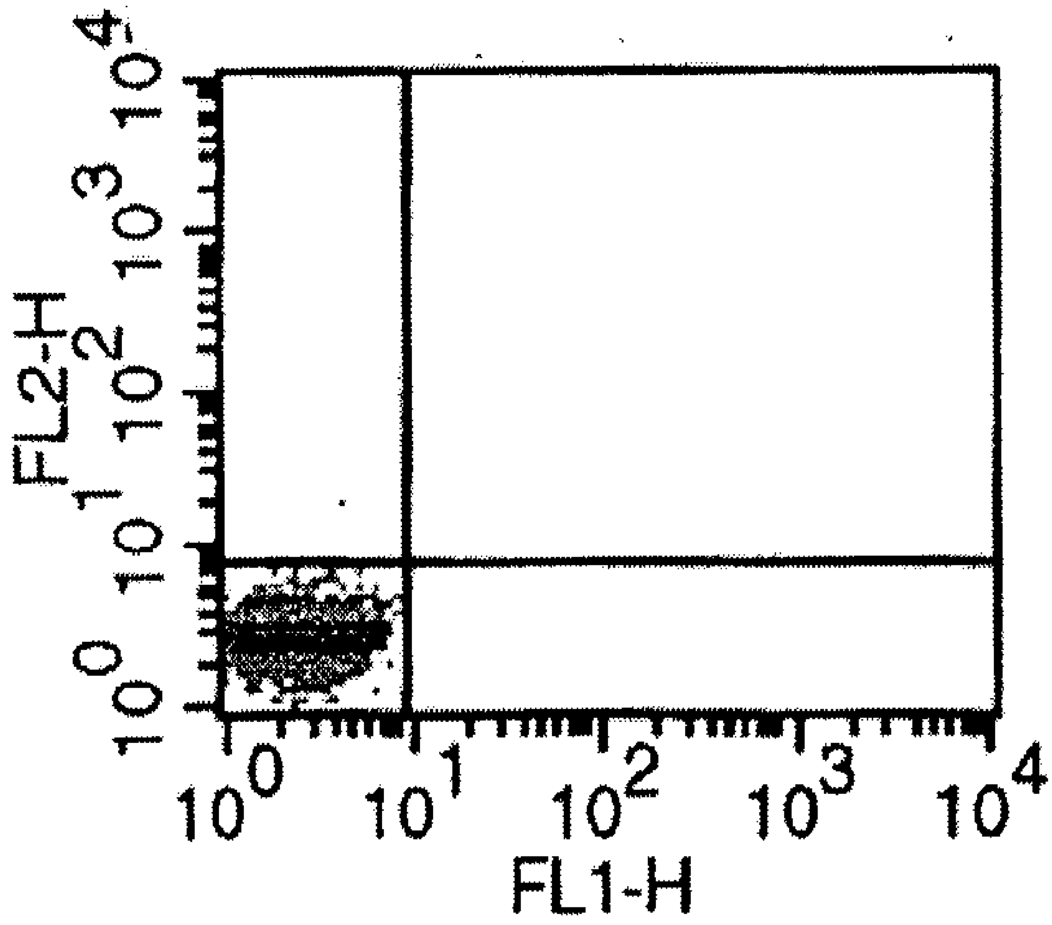
[0016] Then, tube containing cells stained with FITC labeled CD4 and PE labeled CD14 monoclonal antibodies is used to set up the flow cytometer. The red blood cells are removed and the lymphocyte population is gated as described above. By FL1 and FL2 detector, cells in the acquisition lymphocyte gate are used to set marker for distinguishing fluorescence-negative and positive cell populations (FIG. 4).


SSC-H
1000
0
10^0
10^1
10^2
10^3
10^4
FL3-H

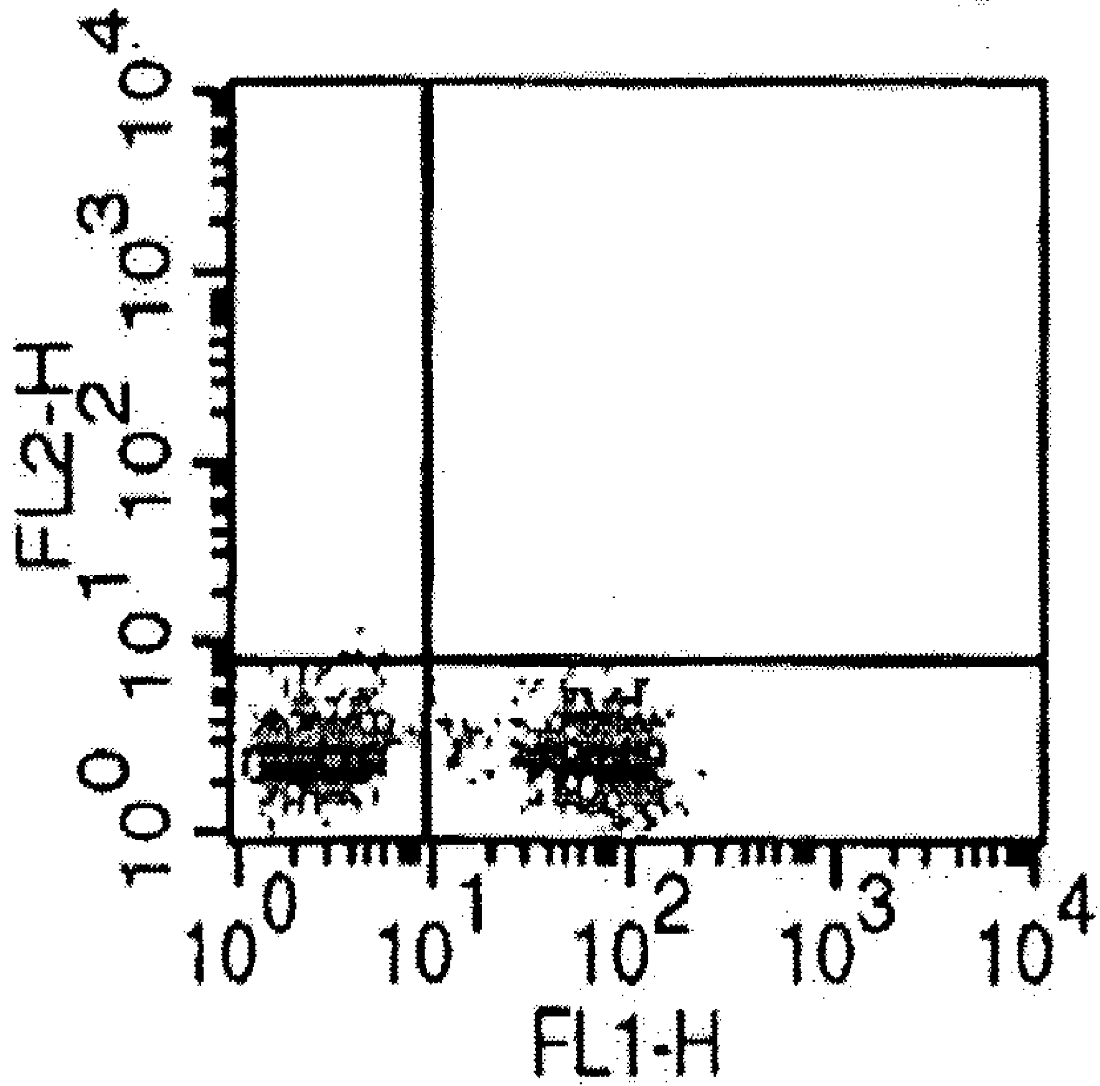
[**0017**] FIG. 1. By FL3 and SSC acquisition, the FL3 threshold is adjusted to gate out red blood cells from the 7-AAD stained white blood cells which having bright red fluorescence.


SSC-H
1000
0
0
200
400
600
800
1000
FSC-H

[**0018**] FIG. 2. By FSC and SSC monitoring, lymphocyte population is gated according to their size and granularity.


FL2-H
10^4
10^3
10^2
10^1
10^0
10^0
10^1
10^2
10^3
10^4
FL1-H

[**0019**] FIG. 3. By FL1 and FL2 monitoring, cells in the acquisition lymphocyte gate obtained from the control tube are set as fluorescence-negative cell populations.


FL2-H
10^0
10^1
10^2
10^3
10^4
FL1-H
10^0
10^1
10^2
10^3
10^4

[0020] FIG. 4. By FL1 and FL2 monitoring, cells in the acquisition lymphocyte gate obtained from sample tube are set for distinguishing fluorescence-negative and positive cell populations.

[0021] 4.2 Enumeration of CD4 Positive Lymphocytes

[0022] The minimum of **10,000** cells is measured for both control and sample tubes. By monitoring of FL3 and SSC, red blood cells are gated out from the 7-AAD stained white blood cells by the FL3 threshold. The remained white blood cells are analyzed using FSC/SSC and lymphocyte population is gated according to their size and granularity. By FL1 and FL2, cells in gated lymphocyte population in the control tube are used to determine non-specific binding and set marker for distinguishing fluorescence-negative and positive cell populations. The percentages of CD4 positive lymphocytes in gated lymphocyte population are determined from the sample tube by using FL1 and FL2. By this measurement, the CD4 positive lymphocytes are CD14−/CD4+ population. While the CD14+ population indicates the number of monocytes that contaminated in the gated lymphocyte population.

[0023] 5. Composition of the Developed Reagent

[0024] 1. 7-aminoactinomycin D (7-AAD) concentration of 100 μg/ml in phosphate buffer saline (PBS) pH 7.2

[0025] 2. FITC labeled CD4 monoclonal antibody and PE labeled CD14 monoclonal antibody

BRIEF SUMMARY OF THE INVENTION

[0026] The developed reagent is a three-color immunophenotyping reagent for measurement of CD4 positive lymphocytes in peripheral blood by flow cytometry. The reagent contains 7-aminoactinomycin D (7-AAD) which intercalates into double stranded DNA. The fluorescence emission of 7-AAD has peak at 670 nm that can be detected with FL3 detector of flow cytometer. The 7-AAD, therefore, stains white blood cells and discriminates it from red blood cells. The reagent also contains fluorescein isothiocyanate (FITC) labeled CD4 monoclonal antibody and phycoerythrin (PE) labeled CD14 monoclonal antibody which can be detected with FL1 and FL2 detectors of flow cytometer, respectively. The developed reagent can be used to measure number of CD4 positive lymphocytes in lymphocyte population and monitor monocyte contamination in the acquisition lymphocyte population, simultaneously. This reagent therefore provides more accuracy results of CD4 positive lymphocyte measurement.

ABSTRACT OF THE DISCLOSURE

[0027] The developed reagent is three-color immunophenotyping reagent for measurement of CD4 positive lymphocytes in peripheral blood. The reagent contains 7- aminoactinomycin D (7-AAD) which intercalates into double stranded DNA and is easily excited at 488 nm. The fluorescence emission of 7-AAD has peak at 670 nm that can be detected with FL3 detector of flow cytometer. The 7-AAD, therefore, stains white blood cells and discriminates it from red blood cells. The reagent also contains fluorescein isothiocyanate (FITC) labeled CD4 monoclonal antibody and phycoerythrin (PE) labeled CD14 monoclonal antibody which are detected with FL1 and FL2 detectors of flow cytometer, respectively. The developed reagent can be used to measure number of CD4 positive lymphocytes in lymphocyte population and monitor monocyte contamination simultaneously. This reagent therefore provides more accuracy results of CD4 positive lymphocyte enumeration.

DRAWINGS

[0028] Not applicable

We claim:

1. A reagent for measurement of CD4 positive lymphocytes in whole blood comprising at least of 7-aminoactinomycin D, CD4 monoclonal antibody and CD14 monoclonal antibody.

2. A reagent according to claim 1, wherein CD4 monoclonal antibody is for enumeration of CD4 lymphocytes.

3. Use of CD14 monoclonal antibody as defined in claim 1 for determining the contamination of monocytes in lymphocyte population.

4. A reagent according to claim 2, wherein CD4 monoclonal antibody is produced by MT4 hybridoma clone.

5. A reagent according to claim 3, wherein CD14 monoclonal antibody is produced by MT14/3 hybridoma clone.

6. Use of 7-aminoactinomycin D as defined in claim 1 for discrimination of red blood cells from white blood cells in the measurement of white blood cells by flow cytometry.

* * * * *

patsnap

专利名称(译)	用于通过流式细胞术测量CD4阳性淋巴细胞的三色试剂		
公开(公告)号	US20040110122A1	公开(公告)日	2004-06-10
申请号	US10/461544	申请日	2003-06-16
[标]申请(专利权)人(译)	泰国RES基金		
申请(专利权)人(译)	国家科学技术发展局 泰国研究基金		
当前申请(专利权)人(译)	国家科学技术发展局 泰国研究基金		
[标]发明人	KASINRERK WATCHARA		
发明人	KASINRERK, WATCHARA		
IPC分类号	G01N33/569 G01N33/53 C12Q1/00		
CPC分类号	G01N2333/70514 G01N33/56972 G01N33/533		
优先权	0201074492 2002-06-14 TH		
其他公开文献	US9285367		
外部链接	Espacenet USPTO		

摘要(译)

开发的试剂是用于测量外周血中CD4阳性淋巴细胞的三色免疫表型分析试剂。该试剂含有7-氨基放线菌素D（7-AAD），其嵌入双链DNA中，易于在488nm激发。 7-AAD的荧光发射在670nm处具有峰值，可以用流式细胞仪的FL3检测器检测。因此，7-AAD会污染白细胞并将其与红细胞区分开来。该试剂还含有异硫氰酸荧光素（FITC）标记的CD4单克隆抗体和藻红蛋白（PE）标记的CD14单克隆抗体，它们分别用流式细胞仪的FL1和FL2检测器检测。开发的试剂可用于测量淋巴细胞群中CD4阳性淋巴细胞的数量并同时监测单核细胞污染。因此，该试剂提供了CD4阳性淋巴细胞计数的更准确的结果。