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(54) **A FLOW-THROUGH CELL COUNTING ASSAY**

TEST ZUM ZÄHLEN VON DURCHFLUSSZELLEN

DOSAGE DE COMPTAGE DE CELLULES À ÉCOULEMENT TRAVERSANT

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EP 2 972 345 B1

Description

GOVERNMENT SUPPORT

[0001] The present invention was made with a US Government support under SBIR Grant No. 1 R43 HL097933 entitled "A FLOW-THROUGH CELL COUNTING ASSAY" awarded by the National Institutes of Health, National Heart Lung and Blood Institute. The government has certain rights in this invention.

BACKGROUND

[0002] The present invention relates to methods for determination of a leukocyte count and/or leukocyte subset count in biological fluids.

[0003] The tests for white blood cell, WBC (leukocyte) count and leukocyte subsets count are widely used in clinical practice. In general, laboratory methods for quantification of WBC or WBC subsets are based on the use of automated cell counting instruments and require 3- 5 mL of venous blood samples, which are first diluted and then cells of different sizes and shapes are counted in a flow chamber [1]. Instruments which are utilized for automated cell counting are complex and costly, and only available in very few central hospitals and other larger laboratories. In addition, they require professionally trained personnel to operate the instruments.

[0004] At present, there is an increasing demand to reduce the turnaround time of test results, and new point-of-care testing (POCT) technologies are being developed for rapid diagnosis at or near patients' bedside. POCT devices are portable and user-friendly to operate, and, therefore, are of great value in both developing and developed countries, where access to automated cell counters is restricted, for instance in rural clinics or general practices.

[0005] Rapid measurements of leukocyte and their subsets are important in many clinical situations (see for example WO2004/072097). They can be useful when physicians need to make decisions regarding the initiation (or monitoring) of treatment when the patient is still at clinical setting. The ability of POCT to provide results within minutes allows physicians to do it and use the results right away.

[0006] In hematology testing, there are three types of technology to support POCT: small bench-top analyzers, hand-held devices and manual tests. The bench-top systems are often smaller versions of laboratory analyzers, providing a full blood count (FBC) with red cell indices and either a five-part white cell differential or a partial three-part differential. Bench-top analyzers, however, are not useful at the patient's bedside and are not truly POCT devices because they are designed for use in clinics or small laboratories. The hand-held test devices and manual methods include measurement of hemoglobin (Hb) concentration, WBC and platelet count, detection of malaria and enumeration of CD4+ T-lymphocytes for human-immunodeficiency-virus (HIV) diagnosis and treatment monitoring [2; 3; 4].

[0007] Clinicians routinely use WBC and differentials (subsets) as biomarkers for acute infection/ inflammation in various clinical settings from primary to critical care. An increased WBC count occurs in infection, allergy, systemic illness, inflammation, tissue injury, and leukemia. A low WBC count may occur in viral infections, immunodeficiency states, post chemotherapy, acute leukemia. For example, patients who are on chemotherapy need to check their WBC count frequently to ensure that they are eligible for the next treatment.

[0008] An increased or decreased total white blood cell count (WBC) could be due to abnormal bone marrow pathology [5]. Leukocytosis with an associated neutrophilia or lymphocytosis could infer the presence of a microbial or viral infection [3]. Leukocytosis is also a prognostic marker of patients who are at a higher risk of hospital mortality [6; 7] and identifies patients at increased risk for excessive bleeding [8]. Thus, clinicians can use the WBC biomarker to improve risk prognostication and identify patients in need of immediate treatment and a closer follow-up [9].

[0009] At present, there are several commercially available methods for the WBC count that can be used as POCT tests: 1) HemoCue WBC (Angelholm) [10], 2) the Chempaq XBC analyser (Chempaq A/S; Hirsemarken 1B, Farum, Denmark) [11], 3) PortaWBC™ (PortaScience, Moorestown, NJ), and 4) the traditional procedure for the total and differential WBC count by manual microscopy [12].

[0010] The HemoCue WBC device measures total WBC without giving any differential. It consists of a microscopic image detector, a cuvette holder and an LCD display unit. The method is based on drawing peripheral capillary blood or venous blood into a plastic cuvette containing a reagent where the red cells are haemolysed and the nuclei of the white cells stained by methylene blue. Then, an image is captured, and the image analysis program counts WBC.

[0011] The Chempaq XBC hematology analyzers comprise a disposable cartridge and an instrument, and use impedance cell counting and measurement of Hb by a spectrophotometric method on 20 μl of blood. Thus, the aforementioned two instruments use rather complex instrumentation.

[0012] The PortaWBC™ method for quantitatively measuring white blood cell count involves capture of white blood cells from a fluid sample by a retainer that has a dye substrate immobilized therein, washing, and reading the result of a color reaction in which an ester which is present on the white blood cells cleaves a chromogenic substrate which produces a water insoluble dye. The signal is read using a glucose-like meter which measures the sample reflectance.

[0013] The total WBC count is obtained by lysing red blood cells in 2% acetic acid solution and counting WBCs in the hemacytometer chamber. For the differential WBC

count, a stained smear is examined under microscope in order to determine the percentage of each type of leukocyte present. Stains for preparing whole blood smears are available from numerous manufacturers. Standard manual cell counting methods are time consuming and subjective. It is a common occurrence to obtain cell counts with wide inconsistencies in total cell counts. Likewise, the traditional procedure for the differential WBC count has a poor statistical reliability. In addition to that, it is time consuming, requires experience to make technically adequate smears consistently, and therefore is one of the most expensive routine tests in the clinical hematology laboratory [13]. Although the latter two methods are simpler and use less expensive equipment than the first two methods, they are labor-intensive and time-consuming. In addition, the PortaWBC™ method does not allow for determination of leukocyte subsets such as neutrophils, eosinophils, basophils, lymphocytes, monocytes, macrophages, dendritic cells, and granulocytes.

[0014] Very important parameters which are measured for diagnosis of HIV infection and monitoring of HIV patients are CD4+ T-lymphocyte levels for adults and CD4 percentage for pediatric patients.

[0015] HIV infection is one of the major problems in public health. It is estimated that more than 1.1 million HIV-infected persons are living in the United States and roughly 33.3 million people are living with HIV worldwide. In the recent years much importance is being given to a wider HIV screening and identification of infected individuals for implementation of intervention strategies. HIV testing has gained immense therapeutic relevance: starting the highly active antiretroviral therapy (HAART) early may improve quality of life and considerably prolong life. Treatment with HAART has dramatically improved survival rates in HIV since its introduction in 1995.

[0016] Individuals with HIV infection and AIDS exhibit abnormalities of the immune system, reflected primarily in their CD4 T lymphocytes, which are targeted by the virus. In adults, the evaluation of CD4 levels provides an important assessment of immunologic competency and has proven to be the most important test for HIV progression. Results from the measurement of CD4 lymphocyte levels provide information that guides therapy and predicts disease outcome. For example, new US guidelines favor antiretroviral therapy for patients at 350-500 CD4 cells/μL. New WHO recommendations are to start HAART for HIV patients with CD4 cell counts at or below 350 cells/μL, instead of a CD4 cell count of 200 cells/μL, the threshold which the WHO recommended in its 2006 guidelines. Normally, T lymphocytes (CD4 and CD8) account for 60-90% of all lymphocytes. Their numbers are about 1,600 cells/μl, with CD4 cells accounting for approximately 1,000 and CD8 cells approximately 500. CD4 cells are the main target of HIV and the number of CD4 cells will gradually decrease during HIV infection (50-100 cells/μl per year). HIV-positive individuals who are successfully treated with HAART demonstrate an increase in the CD4 cell count.

[0017] In pediatric patients, the high variability of absolute CD4+ T count occurs within the first 5 years of age. In addition, incurrent illnesses may affect CD4+ counts and the "normal" reference ranges for CD4 T+ cell absolute counts in African children differ from those reported for populations in Europe and North America [14; 15]. Therefore, in pediatric patients aged less than 5 years, it is strongly recommended that percentage of CD4+ T cells is determined within the total lymphocyte population (%CD4/ly) [16; 17]. If available, the percentage of CD4+ T cells is utilized to establish the level of immunodeficiency and make decisions when to start cotrimoxazole (CTX) prophylaxis and/or ART in all HIV infected children less than 5 years of age [18].

[0018] Relative to adults, very high absolute lymphocyte counts are seen in neonates (6,500 ± 2,200 (SD) lymphocytes/μL) with a gradual decrease to near adult levels in children greater than 5 years of age (1,900 ± 550 (SD) lymphocytes/μL) [19]. For classification of the HIV-associated immunodeficiency, the following %CD4/ly values are considered: mild immunodeficiency is suggested if %CD4/ly is 30-35% in infants, 25-30% in children aged 12-35 months, and 20-25% in children between 36-59 months of age. Severe immunodeficiency occurs when the %CD4/ly values in the age groups mentioned above drop below 25%, 20%, and 15%, respectively [18]. World Health Organization (WHO) recommends initiation of CTX prophylaxis in all HIV-exposed children under the age of 1 year irrespective of their %CD4/ly measurement, particularly, in resource limited settings where infant HIV status may not be established until the age of 18 months due to the lack of proper technology [18]. In settings where %CD4/ly measurements are available, WHO recommends to initiate CTX prophylaxis in the 1-4 year age group if %CD4+/ly is less than 25%. The initiation of ART is determined by clinical presentation of disease and %CD4/ly measurements. ART is indicated for all HIV-infected individuals with an AIDS defining illness [18]. In pediatric patients <5 years of age with non-AIDS defining illness, ART is initiated at severe level of immune deficiency as determined by %CD4/ly [18].

[0019] *Strategies to Assess Lymphocytes.* Lymphocyte subsets are typically measured by immuno-fluorescent labeling of cells with fluorochromes conjugated to specific monoclonal antibodies and quantifying the proportion of specifically labeled cells by flow cytometry. Manual alternatives to flow cytometry are also available to quantify CD4 cells. They are simple light or fluorescence microscopy methods that just require cell counting.

[0020] *Flow Cytometry.* In flow cytometry, specific monoclonal antibodies made against the specific CD antigens present on the cells are labeled with fluorescent dyes. The labeled monoclonals are allowed to react with the mononuclear cells (lymphocytes and monocytes), and the cells that react can be classified by the flow cytometer into subpopulations depending on which monoclonals are bound. Flow cytometry generally gives the

percentage of CD4+ or CD8+ cells. To obtain absolute cell counts, dual and single platform technologies are used. A dual platform technology employs a flow cytometer and a hematology analyzer. CD4 absolute count using dual platform approach is a product of three measurements: the white blood cell count, the percentage of white blood cells counts that are lymphocytes (differential), and the percentage of lymphocytes that are CD4 cells (determined by flow cytometry). If a single platform technology is used, absolute counts of lymphocyte subsets are measured in a single tube by a single instrument. Usually it is accomplished by spiking a fixed volume of sample with a known number of fluorescent beads (bead-based systems) or by precisely recording the volume of the sample analyzed. Recent recommendations suggest that single platform technology should be the gold standard for the CD4 absolute count.

[0021] Several varieties of flow cytometers are available, with the FACSCalibur (Becton Dickinson) and EPICS XL (Beckman Coulter) being the most popular. These instruments offer high sample throughput, workflow management through automation, and simple software applications. Both instruments can detect four colors and measure relative cell size and cellular complexity. The systems are designed to use whole blood, collected in liquid EDTA. Besides using the traditional flow cytometers (open platforms that can employ dual or single platform technology), the simplified dedicated platforms are developed for CD4+ T-cell counts. The commercially available dedicated platforms include FACScount (Becton Dickinson), CyFLow Counter (Partec), and Guava Auto CD4/CD8% (Millipore/Merck). The dedicated platforms allow CD4+ T-cell counting with reduced technical complexity. It produces absolute CD4 counts and a CD4/CD8 ratio without requiring an external computer. The system uses whole blood, eliminates the need for lysis and wash steps, and has a unique software algorithm that automatically identifies the lymphocyte populations of interests. However, the instrument costs about $25,000, with each assay costing $3-20 depending on volume of tests performed in the laboratory.

[0022] Flow cytometry, even though the "gold standard" reference method for determining lymphocyte subpopulations, has several disadvantages: the method requires an expensive instrument ($25,000-90,000), an expensive service contract, and well-trained personnel. Highly trained personnel and costly equipment make it difficult for small laboratories or those in developing countries to routinely provide this testing.

[0023] *Manual Methods to Quantify Lymphocytes.* Manual alternatives to flow cytometry available on the market are: the Cyto-Spheres (Coulter Corporation, USA) and the Dynabeads (Dynal AS, Norway). The Dynal T4 kit (the Dynabeads) is used to manually count CD4 cells in a cell counting chamber under a microscope. This method measures CD4 absolute count; no lymphocyte percentages can be determined. It requires an epifluorescent microscope (recommended), although it can be performed with only a light microscope; a hemacytometer, a vortex, a tube rocker, a timer, and a magnet. Magnetic beads are coated with monoclonal antibodies as a solid phase to isolate CD4 and CD8 cells from whole blood, whereas CD4-positive monocytes are pre-depleted using CD14 magnetic beads. After isolation of CD4 cells, the cells are lysed, stained, and counted. Blood samples should be fresh, preferably not older than 24 hours. The Coulter Manual CD4 Count Kit (cytospheres method) requires a light microscope, timer, and a hemacytometer and measures CD4 absolute counts (no percentages) from whole blood collected in EDTA tubes [20]. Antibody-coated latex particles are used to bind CD4 cells resulting in a "rosette" of latex beads around each CD4 cell; the rosette is readily recognized by light microscopy. A monocyte blocking reagent minimizes the interference from monocytes that contain CD4 antigens because they can be recognized during CD4 cell counting. Blood should be tested within 6 hours of collection and should not be refrigerated. Both manual assays cost between $5-6 per test and are designed to operate with low sample throughput in resource-limited laboratories. However, the manual methods are labor-intensive; require many manual steps, and an experienced microscopist. Large intra- and inter-operator variations are the norm rather than the exception and these methods have been proven very difficult to implement in most settings.

[0024] *New CD4 Technologies.* Recent development of CD4 technologies is concentrated on creating an accurate point-of-care (POC) assay that could be used for CD4 testing in resource-limited countries in any health care setting or in the field. Inverness Medical (now Alere) has a PIMA CD4 test based on static image analysis and counting principles. A disposable cartridge containing dried reagents and a portable analyzer is used. The analyzer is easy to operate: no extensive training is required. The cost of analyzer is $5,500; an estimated cost per test is $6. The assay is already available in select markets. Daktari Diagnostics is developing a microfluidic-based system to capture CD4 cells and to count cells using simple electrical impedance measured by a small portable device. The estimated device cost is $800 and the single test cost is estimated to be $8. mBio Diagnostics, a division of the Precision Photonics Corporation has a new patent-protected CD4 count technology that uses an integrated fluidic cartridge and a portable fluorescence imaging device. Zyomyx is another company involved in the development of a POC CD4 test: Zyomyx CD4 counter is similar to a thermometer where CD4 cell stacking height highly correlates with CD4 count in the blood sample. The major advantage of this assay is that it does not require any instrument for reading the results. The estimated cost of the assay is $6-7, however, this assay is still under development. A different new CD4 technology developed by Burnet Institute in Australia is based on the measurement of CD4 protein on T-cells, rather than measuring CD4 cells using a lateral flow assay. The test, which is expected to cost about $2, is semi-

quantitative: it will be able to determine, whether a patient's CD4 count is above or below a threshold of 350 CD4 cells/μL, but it will not give full quantitative results. The reader of the test is expected to cost about $1,200. The above mentioned POC tests are promising, however, they still need to be evaluated in the clinical trials: peer reviewed published data is available only for PIMA CD4 test. All other tests still need to stand up to robust assessment.

[0025] Current flow cytometric methods to measure CD4 cells are expensive, require significant technical expertise, the instrumentation has high service demands, and these methods are difficult to support in resource-limited countries. The aforementioned manual CD4 tests have their advantages and limitations. Indeed, Dynabeads (Dynal Biosciences) and Cytospheres (Coulter), methods are multi-step assays and require a microscope for counting targeted cells, what leads to increasing of the assay cost. In addition, these manual methods are laboratory-based and their procedures are not conducive for point-of-care workers such as nurses, counselors, and physicians. Moreover, currently there are no manual methods on the market that provide %CD4/ly measurement. Thus, the rationale is to develop and validate a truly point-of-care CD4 method that can be used by non-laboratory personnel in a wide variety of health care settings and in the field to provide anti-retroviral treatment decisions immediately and without the need for infected persons to return for results. Serious limitations of the aforementioned methods hinder their wide use in clinical practice. Therefore, the development of an alternative manual assay that is free from the above drawbacks is of utmost relevance.

SUMMARY

[0026] It is an object of the present invention to overcome limitations of the methods cited above.

[0027] It is another object of the present invention to provide a method for quantification of white blood cell (leukocyte) and their subsets count in a biological fluid.

[0028] Disclosed is also a device for estimating WBC and their subsets count in a biological fluid.

[0029] The method of the present invention, the flow-through cell counting assay (FTCA), employs a special filter that selectively captures white blood cells (WBC) and a flow-through cassette to measure cell count status.

[0030] The methods of the present invention in general include the following steps: (a) adding to said biological fluid sample a sole antibody specific to anyone of said respective lymphocytes, said sole antibody conjugated with a an optically detectable marker, said sole antibody is selected from the group consisting of an anti-CD4 antibody for determination of said CD4+ T-cell lymphocytes count, anti-CD8 antibody for determination of said CD8+ T cell lymphocytes count, and an anti-CD45 antibody for determination of said total lymphocytes count; (b) incubating said biological fluid sample mixture of step (a) for duration from 5 min to 60 min and at a temperature ranging from 20 degrees Celsius to 37 degrees Celsius; (c) filtering said biological fluid sample mixture through a leukocyte-reduction retainer filter, said filter is configured for capturing thereon of said leukocytes including those which bound to said antibody of said biological fluid sample mixture, said filter is further configured for all captured leukocytes to contain at least 80 percent or more lymphocytes and at least 20 percent or less of any other subsets of leukocytes; (d) washing said leukocyte-reduction retainer filter to remove weakly-bound substances; and (e) optically detecting said bound antibody conjugated to said optically detectable marker which is retained on said leukocyte-reduction retainer filter in step (c) for determining of said at least one respective CD4+ T-cell lymphocytes count, CD8+ T-cell lymphocytes count, or total lymphocytes in said biological fluid.

[0031] In embodiments, another variant of the assay may be based on the selective adsorbtion of white blood cells by the retainer filter. In this version of the test, however, magnetic beads carrying specific antibodies against interfering substances may be added to the biological fluid and after incubation may be removed before filtering the biological fluid through retained filter.

[0032] In further embodiments, another variant of the assay may be based on the selective adsorbtion of white blood cells by the retainer filter. In this version of the test, however, interfering substances may be adsorbed by several upper layers of the multilayer retainer filter, made of the same or different materials, and configured to be removed before reading the signal from the surface of the remaining layers of the retainer filter.

[0033] The device may include a cartridge comprising a cartridge housing supporting a fluid sample acceptance tube, an absorbent pad, a removable strip containing a retainer filter configured for binding of leukocytes of biological fluid sample.

BRIEF DESCRIPTION OF THE DRAWINGS

[0034] Subject matter is particularly pointed out and distinctly claimed in the concluding portion of the specification. The foregoing and other features of the present disclosure will become more fully apparent from the following description and appended claims, taken in conjunction with the accompanying drawings. Understanding that these drawings depict only several embodiments in accordance with the disclosure and are, therefore, not to be considered limiting of its scope, the disclosure will be described with additional specificity and detail through use of the accompanying drawings, in which:

FIG. 1 shows a block-diagram of conducting FTCA for enumeration of CD4+ T-lymphocytes cells.

FIG. 2 shows a front cross-section view of the device of the present invention.

FIG. 3 shows a side cross-section view of the device of the present invention.

FIG. 4 illustrates a general view of the device of the present invention.

FIG. 5 shows the FTCA calibration curve for determination of CD4+ T-cell count for patients greater than five years of age and HIV positive samples with low CD4 counts.

FIG. 6 shows the FTCA calibration curve for determination of CD4+ T-cell count for patients less than 5 years of age.

FIG. 7 shows calibration curves obtained using two FTCA methods for determination of total lymphocyte count for patients less than 5 years of age and signals for clinical samples.

FIG. 8 shows the FTCA calibration curve for determination of CD8+ T-cell count for patients less than 5 years of age.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

[0035] The following description sets forth various examples along with specific details to provide a thorough understanding of claimed subject matter. The scope of protection is defined by the appended claims. The illustrative embodiments described in the detailed description, drawings, and claims are not meant to be limiting. It will be readily understood that the aspects of the present disclosure, as generally described herein, and illustrated in the figures, can be arranged, substituted, combined, and designed in a wide variety of different configurations, all of which are explicitly contemplated and make part of this disclosure.

[0036] The method of the present invention may be used for enumeration of the total leukocyte and/or at least one leukocyte subset such as for example neutrophils, eosinophils, basophils, lymphocytes, monocytes, macrophages, dendritic cells, granulocytes, and their respective subsets as well as one or more ratios of one desired subset count to another desiredsubset count.

[0037] The method is further described as it applies to determination of specific WBC subset count, which is meant only to be illustrative of the method and not to limit the scope of the claims that follow. One skilled in the art would recognize the utility of the present invention in other WBC subset count assays utilizing this method. The description of some other such assays is also provided.

[0038] For example, Figure 1 shows one of the general approaches of conducting FTCA for enumeration of CD4+ T-lymphocytes cells. This variant of assay comprises the following steps: 1) An anti-CD4 antibody conjugated with enzyme may be added to whole blood; 2) After several minutes of incubation, the sample may be delivered to the flow-through cassette. The cassette may comprise a retainer filter and an absorbent pad. The retainer filter may be configured to selectively retain white blood cells, including CD4 antibody-T-cell complexes; 3) A washing buffer solution may be poured through the cassette to remove substances from the sample unbound (or weakly bound) by the retainer, such as for example red blood cells, enzymes, enzyme inhibitors, proteins, and lipids; 4) Then, an enzyme substrate may be added, and the result of a color-forming reaction of the enzyme with an insoluble chromogenic substrate may be read either by eye or by an instrument.

[0039] In order to determine other than CD4+ T-cell subset of WBC, specific antibodies to the particular WBC subset may be used. For instance, anti-CD8 antibody may be used for determining of CD8+ T-cell lymphocytes; and anti-CD45 antibody (or other antibodies such as anti-CD52, CD 81, CD96, CDw137, CD200 antibodies) may be used for determining of total leukocytes or total lymphocytes.

[0040] The methods of the present invention may be based on the use of filters that selectively capture white blood cells whereas other potentially interfering weakly-bound to the filter substances such as red blood cells, enzymes, enzyme inhibitors, proteins, and lipids can be removed by washing. The selective capturing of white blood cells onto the filter occurs because white blood cells (leukocytes) are negatively charged, whereas the retainer filter has a net positive charge. Filters that selectively bind WBC are known to be used for filtering white blood cells from blood intended for transfusions. These filters can capture 30-80% of the white blood cells from whole blood due to their well-controlled surface charge and pore sizes. Commercially available filters used for leukocyte reduction apply selective filtration technology combining depth and adsorption filtration to achieve the highest efficiency. The features of filters for leukocyte reduction also include high leukocyte absorption rate and ease-of-use. Depth filters are usually composed of densely packed fibers to remove particles, either by adherence or absorption onto the fibers, or by entrapment between fibers as particles pass through the filter. Adsorption filters utilize the properties of white blood cells, which selectively adhere to filter fibers.

[0041] Several types of the filters (membranes) may be used for the methods of the present invention:

a. Leukosorb type filters from Pall Life Sciences, the surface characteristics of which was grafted by compounds containing an ethylenically unsaturated group, such as an acrylic moiety combined with a hydroxyl group. Monomers such as HEMA or acrylic acid are used;

b. Certain grades of cellulose paper that possess positively charged surfaces;

c. 3 μm Nuclepore polycarbonate, track-etch filters (Whatman, Florham Park, NJ and Sterlitech Corp., Kent, WA). These membranes selectively capture lymphocytes and provide for the removal of red blood cells without sample processing because the more rigid leukocytes are retained by the porous membrane while the more flexible erythrocytes, as well as plasma are relegated to waste; and

d. Sepacell R-500 (Asahi Medical Co Ltd, Tokyo, Japan).

[0042] Although some of the aforementioned filters may be used successfully for detection of some WBC subsets such as total WBC count, they may not be suitable for detection of some of the WBC subsets such as CD4+ T-cell lymphocytes, CD8+ lymphocytes, and total lymphocytes. The reason for this is that different WBC subsets may carry the same antigen, and, therefore, the use of a one set of antibodies to this antigen may lead to detection of not only the targeted cells but, also, to detection of potentially interfering cells.

[0043] For example, one of the main problems of all manual tests for CD4+ T-lymphocyte cells is the interference of monocytes. In embodiments, an anti-CD4 antibody conjugated with a marker may be used for determination of CD4+ T-lymphocytes. However, monocytes as well as CD4+ T-cells carry CD4 antigens (although about 10 times less), and anti-CD4 antibodies used for enumeration of CD4 antigens may bind to monocytes, which results in erroneous increase in CD4 count. Therefore, in most CD4 manual tests using anti-CD4 antibody, monocytes may be first removed from blood, and then CD4 cells may be counted. The additional step of monocyte removal obviously complicates the test procedure.

[0044] In embodiments, CD4 percentage, that is the ratio of CD4+ T-cell count to total lymphocyte count, may be calculated. In this case, both CD4+ T-cell and total lymphocyte counts have to be determined. In the method of the present invention, anti-CD45 antibody may be used for determination of the total lymphocyte count. However, not only lymphocytes but also other WBC subsets such as neutrophiles (granulocytes) and monocytes contain CD45 antigen.

[0045] Thus, in order for the membrane filter to be suitable for FTCA, it needed to possess the following three main properties:

a. ability to selectively capture as many white blood cells (WBC) as possible,

b. ability to allow cells other than WBC blood cells to be washed out; and

c. the ratio of captured (adsorbed) lymphocytes to monocytes and granulocytes needs to be as high as possible in the case when CD4+ T-lymphocytes and total lymphocytes are detected. In particular, the amount of lymphocytes captured on the filter needs to be sufficient to register the signal obtained using anti-CD4 or anti-CD45 detection reagents. This signal also needs to be specific in order to avoid detection of other types of CD4+ or CD45+ carrying cells, such as granulocytes and monocytes.

[0046] Examples of commercially available leukocyte-reduction filters, which provide the highest ratio of adsorbed lymphocytes to monocytes, and gave the best results for enumeration of CD4+ T-cells in whole blood (see results below) include LRF10S filter (Pall Inc., Long Island, NY). This retainer filter is configured for capturing leukocytes in such a way that the captured leukocytes retained on said preferential retainer filter consist of approximately 80 percent or more of the lymphocytes and approximately 20 percent or less of any other subset of the leukocytes. Still in another aspect of the invention, the potentially interfering cells may be removed by adding magnetic beads carrying at least one antibody selected to bind to at least one interfering substance to biological fluid sample; incubating the sample for a certain period of time; and removing magnetic beads together with at least one interfering substance from biological fluid sample using a magnet. Still in another aspect of the invention, the potential interference of other than targeted cells may be diminished by using more specific antibodies to the targeted cell. For example, in the method of the present invention, other than anti-CD45 antibody such as but not limited to anti-CD52 antibody, anti-CD81 antibody, anti-CD96 antibody, anti-CDw137 antibody, and anti-CD200 antibody may be used for determination of the total lymphocyte count.

[0047] Still in another aspect of the invention, potentially interfering cells may be removed by using a multilayer retainer filter. Different layers of the retainer filter may be made of the same or different materials. In this version of the assay, biological fluid sample containing an antibody conjugate to certain WBC antigen may be (i) added to the multilayer retainer filter; (ii) incubated for a certain period of time; (iii) washed and (iv) one or several upper layers of the multilayer retainer filter may be removed together with at least one interfering substance adsorbed to said upper one or several layers from biological fluid sample.

[0048] The antibody markers of different origins may be used for the methods of the present invention such as for example: a) an enzyme, b) an enzyme complex, c) a plurality of gold microparticles, d) a plurality of quantum dot microparticles, e) a plurality of fluorescent latex microparticles, f) a plurality of fluorescent liposomes, g) a fluorescent dye, h) a photochemical dye, i) an chemiluminescent compound, and j) an electrochemiluminescent compound. In another aspect of the invention, the marker is a biotin and a streptavidin conjugate or an avidin conjugate of a material selected from a group consisting of an enzyme, an enzyme complex, a plurality of gold microparticles, a plurality of quantum dot micropar-

ticles, a plurality of fluorescent latex microparticles, a plurality of fluorescent liposomes, a fluorescent dye, a photochemical dye, a chemiluminescent compound, and a electrochemiluminescent compound.

[0049] In yet another embodiment of the invention, the marker may be an Alkaline Phosphatase (AP) or a complex of biotin and streptavidin- or avidin- Alkaline Phosphatase conjugate. In this case, before reading the signal, an Alkaline Phosphatase substrate solution may be added. The AP substrate may be of different origins as well, such as for example: a) a chromogenic substrate, b) a fluorogenic substrate, c) a chemiluminescent substrate, and d) an electrochemiluminescent substrate.

[0050] A chromogenic substrate may be selected from a group consisting of 5-Bromo-4-chloro-3-indolyl phosphate/ p-iodonitrotetrazolium (BCIP/INT); 6-chloro-3-indoxyl phosphate, p-toluidine/ NBT (BCIP pink/NBT); 3-indoxyl phosphate/ NBT; Fast Red TR/ Naphthol AS-MX phosphate; Fast Red TR/ Naphthol AS-TR phosphate; and p-nitrophenol phosphate (pNPP).

[0051] A fluorogenic substrate may be selected from a group consisting of 4-methylumbelliferyl phosphate (4-MUP); 6,8-Difluoro-4-methylumbelliferyl phosphate (DiFMUP); Fluorescein diphosphate (FDDP); 2'-[2-benzthiazoyl]-6'-hydroxy-benzthiazole (BBTP); 2-(5'-chloro-2-phosphoryloxyphenyl)-6-chloro-4(3H)-quinazolinone (ELF 97); Acridinium (N-sulfonyl) carboxamide and acridinium ester-based substrates; and Rhodamine 110-based phosphates.

[0052] A chemiluminescent substrate may be selected from a group consisting of Acridinium (N-sulfonyl) carboxamide and acridinium ester-based substrates; 1,2 Dioxetane- based substrates (4-methoxy-4-(3-phosphatephenyl)spiro [1,2-dioxetane-3,2'-adamantane]; and disodium salt- based substrates (AMPPD).

[0053] An electrochemiluminescenct substrate may be a ruthenium salt-based substrate.

[0054] The marker may be detected visually by eye or by using an optical instrument or by using an electrochemical instrument. Indeed, if the enzyme and a chromogenic insoluble substrate or chromogenic gold or colored latex particles are used as markers, the signal may be detected either by eye or by a reflectometer. If the enzyme chromogenic soluble substrate is used, a photometer (optical density reader) may be used. If fluorescent materials such as fluorochromes, dyes, fluorescent latex particles are used as markers and enzyme fluorescent substrates are used, then a fluorimeter may be utilized for reading the signal. When a chemiluminescent signal is generated, a chemiluminometer may be used for signal detection. Electrochemical detection may also be possible when an electrochemical signal is generated using enzyme substrate and registered by using a reader of electrical parameters.

[0055] The methods of the present invention may be used for determination of WBC and their subset counts in biological fluid samples such as whole blood, a blood fraction, a cerebrospinal fluid, urine, saliva, and perspiration.

Construction of the Flow-Through Cell-Counting Assay cartridge

[0056] The general concept of a disposable cartridge design and manipulation technique for whole blood testing are illustrated in Figure 2 (front cross-section view), Figure 3 (side cross-section view) and Figure 4 (general view). The main idea of the FTCA cartridge is that a strip can be inserted into the cartridge, and pulled out from the cartridge for subsequent optical detection analysis. The cartridge generally comprises a cartridge housing supporting a fluid sample acceptance tube, an absorbent pad, a removable strip containing a preferential retainer filter configured for binding of leukocytes thereto and a disengaging mechanism. The preferential retaining filter may be placed between the fluid sample acceptance tube and the absorbent pad and may be configured to retain at least 80 percent or more of the lymphocytes and not more than 20 percent or less of any other subsets of the leukocytes. The disengaging mechanism may be configured to lift the fluid sample acceptance tube from the retaining filter to release the latter for subsequent removal from the cartridge housing using the removable strip.

[0057] In embodiments, an examplary cartridge may be further described as applied to determination of WBC subsets count. One skilled in the art would recognize the utility of other designs of the cartridge for WBC subset count assays. Discussing this examplary design now in greater detail, the cartridge **10** may contain a string disengagement mechanism (comprising a spring **2** and the lever 8) and an absorbent pad **6** enclosed in a plastic cartridge housing **7**. The FTCA cartridge **10** further contains a side slot for inserting and pulling out a removable strip **5** eqipped with a retaining filter **3**. The removable strip **5** is normally kept in place by the spring **2** pushing down the fluid sample acceptance tube and binding the strip **5** against the absorbent pad **6**. The plastic lever **8** plays a role of a leverage. It may be placed through the slot at the side panel of the cartridge housing 7 and attached (glued) to the upper part of the string disengagement mechanism (spring **2)** to interact with the lower end of the fluid sample acceptance tube **1**. Pushing down on the external end of the lever **8** causes the other end to lift up the spring **2** along with the tube **1** thereby releasing the removable strip **5**. Lifting of the string disengagement mechanism **2** may be carried out by holding the cartridge housing **7** down while pressing the lever **8** down as shown in Figure 4.

[0058] Other designs of the disengagement mechanism are also contemplated as part of the present invention, for example, comprising a metal or plastic wire spring **2** connected to a stair-lift platform including a wheel, which upon its turning runs up and down the platform.

[0059] In embodiments, a commercially available disposable pipette may be used to collect the exact desired

volume of blood (up to 40 μl). After performing a finger stick, blood sample may be taken by the pipette. The blood may then be squeezed out of the pipette into the plastic fluid sample acceptance tube **1**.

[0060] In embodiments, the fluid sample acceptance tube **1** may contain either a solution or lyophilized anti-CD4 AP - antibody conjugate or a mixture of anti-CD4-biotinilated antibody and streptavidin-AP conjugate (position **4** in Figures 2 through 4). After incubation for several minutes, the mixture may be poured into the fluid sample acceptance tube **1** of the FTCA cartridge by pipette, and may go through the preferential retainer filter **3** of the removable strip **5**. After washing and adding the substrate solution (and stop reagent, if necessary), the removable strip **5** may be pulled out and analyzed by a blood glucose-type meter (reflectometer) or by eye using a reference color chart.

[0061] In alternate embodiments, the surface of the removable strip **5** may be made very smooth and may be coated with a hydrophobic polymer material, for example by silane or ethylcellulose derivatives[21] except, of course, the round area containing the retainer filter **3** (Figures 2, 3, 4). This may be done for the following reason: in the initial position of the removable strip **5** inside the cartridge **10**, the fluid sample acceptance tube **1** may touch the removable strip **5** not in the area where the retainer filter **3** is placed, but in the area which is several millimeter farther to the end of the strip (Fig. 4, dashed line on the strip). This area may contain lyophilized anti-CD4 AP-antibody conjugate **4** (or mixture of anti-CD4-biotinylated antibody and streptavidin-AP conjugate), sprinkled (positioned) through the plastic cylinder during FTCA cartridge manufacturing. As mentioned above, whole blood may be poured into the plastic cylinder of the FTCA cartridge directly. In order for the blood to stay and not to leak through the bottom of the fluid sample acceptance tube 1 (since several minutes of incubation reagents may be required), the strip surface must be hydrophobic. After incubation, the strip may be pulled out for several millimeters up to the strip broad gray line (Fig. 4). In this case, the removable strip **5** may be released as the fluid sample acceptance tube **1** may be lifted above the preferential retainer filter **3**.

[0062] In further embodiments, after whole blood is poured through the fluid sample acceptance tube **1**, all reagents including anti-CD4 conjugates, wash and stop solutions may be added sequentially. This may be feasible, however, only in the case when high affinity CD4 antibodies are used. Indeed, in this version of the assay, the incubation time may be very short (it equals to the passing time of the solution through the filter), and very high affinity antibodies may be used for the antibody-antigen reaction to occur.

[0063] In embodiments, after washing and adding the stop reagent, the removable strip 5 may be pulled out and analyzed in the same manner as described above, that is by blood glucose-type meter or by eye. If a spectrophotometer is available, the removable strip 5 may be placed into the test tube (or cuvette) containing alkaline phosphatase substrate solution in a way that the retainer filter 3 may be completely covered by the substrate solution, and after incubation, the optical density of the sample may be measured.

[0064] In some embodiments, one or several layers of the retainer filters may be attached (glued) to the bottom of the sample acceptance tube **1** and after adding the biological fluid sample containing antibody conjugate, incubating and washing, may be detached from the remaining layers of the retainer filter **3** by lifting the sample acceptance tube **1**. In the case of using enzymatic antibody label, the enzyme substrate solution may be added before or after removing upper filter layers.

Quantitative objective assessment of results by optical detection

[0065] In a further embodiment of the FTCA, the retainer filter **3** of certain diameter may be made integral or attached to the disposable removable paper strip **5**. One of the unique features of the FTCA methods is that they allow using several modes of signal detection. For example, the signal may be detected using the following optical methods: 1) visual reading using a chart of colored spots; 2) detection of light reflectance using an inexpensive meter - similar to that used for blood glucose measurement; and 3) detection of optical density of the samples using spectrophotometric methods. The option of using different modes of detection provides flexibility for clinical practitioners in different settings. Indeed, the chart of colored spots and inexpensive optical reader may be successfully used in the field conditions, whereas spectrophotometric method may be used in clinical laboratories. Advantageously, very simple and inexpensive battery-operated spectrophotometers for use in field conditions are readily available. They also may be used for the purposes of the present invention.

[0066] Although the described below methods pertain to determination of CD4+ T-cell count, readily understood modifications may be apparent to those skilled in the art allowing adapting the present invention for such purposes as determination of WBC count, total lymphocytes count, CD8+ lymphocytes count and others. The following non-limiting examples for determination of CD4+ T-lymphocytes, total lymphocytes count, and %CD4/ly further illustrate the principles behind the present invention.

EXAMPLE 1

Determination of CD4+ T-lymphocyte count for people greater than 5 years of age

[0067] In order to be able to determine CD4+ T lymphocyte count in clinical samples of patients older than five years old, the calibration (or standard) curve must be prepared. The targeted clinically significant count range for people older than five years old is between 200

and 1000 cells/μL. For the preparation of the FTCA calibration curve, real fresh whole blood samples from three healthy donors were used. Since all of these samples contain normal levels of CD4 T-cells, samples containing decreased levels of CD4 T-cells were prepared by other means as described below.

[0068] Whole blood samples containing decreased CD4 T-cells concentrations may be prepared by depletion of CD3+ cells or CD4+ cells. Since CD4+ depletion leads to reduction of both CD4+ lymphocytes and monocytes, depletion of CD3+ cells was used to keep monocyte concentration in blood samples unchanged. Whole blood samples with reduced CD4 count were obtained by using magnetic beads coated with anti-CD3 antibodies (Dynabeads® CD3) as described in manufacturer's instructions. The final CD4 count in these samples was determined using the manual Dynal® T4 Quant Kit.

[0069] The following protocol 1 was used: Undiluted anti-CD4 antibody was premixed at 2:1 with Streptavidin-AP (Sigma, Saint Louis, MO) diluted in TRIS-buffered saline (TBS, pH 7.4) buffer to the concentration of 24 μg/mL and incubated at room temperature for 30 min. Seventy microliters of WBC-enriched blood, Whole blood or CD3-depleted blood was incubated with 30 μL Strep-AP/anti-CD4 "conjugate" (anti-CD4 and Strep-AP premix) and the samples were incubated for 20 min at RT. Thirty microliters of each sample were placed with a pipette onto the LRF10S membrane incorporated into the flow-through device. After each addition of a sample, 500 μL of wash solution (10 mM TRIS (pH 7.4), 50 μg/mL NBT) were placed with a pipette onto the device five times to wash out the red blood cells. One hundred μL of alkaline phosphatase (AP) substrate (Hercules, CA) was added. Ten min after adding AP substrate, 200 μL of stop solution (0.1 N H_2SO_4) was added onto each of the membranes. Membranes were removed from the flow-through device and the color intensity was read using portable reflectance colorimeter (FIA Biomed, Germany). A plot of the color measurements (reflectance) versus the CD4+ T lymphocyte count obtained using Dynal® T4 quantification method (Invitrogen, Grand Island, NY) in a range of 200 to 1,000 cells/μL is shown in Figure 5. This plot may be used as the FTCA calibration curve. As seen in Figure 5, the calibration curve is linear in the entire clinically significant range of CD4 T-cells concentrations. The most important feature of the FTCA calibration curve is that it allows one to accurately measure signals in the region of CD4 T-cell counts equal to 350 cells/μl, the cut-off CD4 concentration used for making a decision as to whether or not initiate an antiretroviral treatment (ART) for HIV positive patients. Figure 5 also shows that the error of the measurements (coefficient of variation, CV) did not exceed 20% which is acceptable for this kind of assays [22; 23].

[0070] As mentioned above, CD4+ monocytes may interfere with the enumeration of CD4+ T-cells if not removed from whole blood before testing for CD4 count. In order to prove that the presence of monocytes in whole blood at high concentrations does not affect the performance of the FTCA test, the following experiment was conducted. The FTCA calibration curve similar to that shown in Figure 5 was prepared using whole blood samples diluted in 1) the mixture of red blood cells and plasma (1:1) and 2) Phosphate buffer saline (PBS) + plasma (1:1). Thus, unlike the samples used for the preparation of the calibration curve shown in Figure 5, which contained decreased amounts of CD4 cells and high initial concentrations of monocytes, the samples prepared by simple dilution in the aforementioned solutions contained decreased amounts of CD4 cells and also decreased amounts of monocytes. The calibration curve was then prepared using these samples and showed that it is practically identical to that shown in Fig. 5. Figure 5 also demonstrates that signals obtained for HIV positive samples containing decreased CD4 counts and normal monocyte concentration (~400-800 cells/μl), as determined by Flow cytometry (FC), were in accordance with those obtained for the samples used for the preparation of the calibration curve. Thus, the above described experiments show that the presence of CD4 monocytes in whole blood does not affect the performance of the FTCA.

EXAMPLE 2

Determination of CD4+ T-lymphocyte percentage for pediatric patients

[0071] The percentage of CD4+ T-cells within the total lymphocyte population (%CD4/ly) may be calculated by finding the ratio of CD4+ T-cells and total lymphocytes counts and multiplied by 100. These parameters may be determined using corresponding calibration curves, which have to cover the clinically significant concentration range between of about 350 to about 3,500 cells/μL for CD4+ T-cells and about 2,000 to about 9,000 cells/μL for total lymphocytes [17].

[0072] The calibration curve for CD4+ T-cells determination was prepared as follows (protocol 2). Undiluted anti-CD4 antibody was premixed at 2:1 with Streptavidin-AP (Sigma, Saint Louis, MO) diluted in TRIS-buffered saline (TBS, pH 7.4) buffer to the concentration of 24 μg/mL and incubated at room temperature for 30 min. Fifty microliters of WBC-enriched blood, whole blood or CD3-depleted blood was incubated with 30 μL Strep-AP/anti-CD4 "conjugate" (anti-CD4 and S-AP premix) and the samples were incubated for 20 min at RT. Fifty microliters of each sample was placed with a pipette onto the LRF10S membrane incorporated into a flow-through device. After each addition of sample, 500 μL of wash solution (10 mM TRIS (pH 7.4), 50 μg/mL NBT) was placed with a pipette onto the device five times to wash out the red blood cells. One hundred μL of alkaline phosphatase (AP) substrate (Hercules, CA) was added after washing. Five min after adding the AP substrate, 200 μL of stop solution (0.1 N H_2SO_4) was added onto each of the membranes. Membranes were removed from the

flow-through device and the color intensity was read using a portable reflectance colorimeter (FIA Biomed, Germany) as described in the Example 1. CD4+ T cell counts were obtained using Dynal® T4 quantification method (Invitrogen, Grand Island, NY). A plot of the color measurements (reflectance) versus the CD4+ lymphocyte counts in a range of 324-3,610 is shown in Figure 6.

[0073] The calibration curve for the total lymphocyte determination was prepared using the following protocol 3. Whole blood samples with elevated white blood cell counts were prepared by mixing buffy coat layer with red blood cell (RBC) and plasma mixture at a 1:1 ratio. Whole blood samples with low WBC counts were prepared by mixing red blood cells with plasma at a 1:1 ratio. Samples with various concentrations of lymphocytes were prepared by mixing whole blood with samples containing elevated WBCs with RBC/plasma at a 1:1 mixture ratio. For flow-through assay experiments, the prepared blood samples were diluted in WBC-free serum at a 1:1 ratio. Undiluted anti-CD45 antibody (BD Pharmingen, San Diego, CA) was premixed at a 2:1 ratio with Streptavidin-AP (Sigma, Saint Louis, MO) diluted in TRIS-buffered saline (TBS, pH 7.4) buffer to the concentration of 120 μg/mL. Seventy five μL of the artificially prepared blood samples containing different concentrations of lymphocytes were incubated with 25 μL anti-CD45/ Strep-AP premix and the samples were incubated for 15 min at RT. After incubation, 30 μl of each sample were placed using a pipette onto the 6-layer LRF10S membrane (Pall, Port Washington, NY) incorporated into the flow-through device. After each addition of sample, 500 μL of wash solution (10 mM TRIS (pH 7.4), 50 μg/mL NBT) were placed with a pipette onto the device five times to wash out the red blood cells. One hundred μL of alkaline phosphatase (AP) substrate (Hercules, CA) was added after washing. Ten min after adding the AP substrate, 200 μL of stop solution (0.1 N H_2SO_4) were added onto each of the membranes. Membranes were removed from the flow-through device and the color intensity was read utilizing a portable reflectance colorimeter (FIA Biomed, Germany) using two methods. In the first method, reading was done as described in the Example 1, that is, from the top layer of intact retainer filter. It should be noted that in order to eliminate the effect of high amounts of granulocytes in some samples, the second modified procedure of performing of the FTCA was used. Namely, after finishing the assay, two upper layers of the 6-layer LRF10S filter were removed and the signals were recorded.

[0074] Total lymphocyte counts (CD45+ lymphocyte counts) were also obtained using the traditional procedure for the total and differential WBC count by manual microscopy and flow cytometry method. The total WBC counts using manual microscopy were determined by lysing red blood cells in 2% acetic acid solution and counting WBCs in the hemacytometer chamber. For the lymphocyte count, a blood smear stained with Wright solution (EMS, Hatfield, PA) was examined under microscope and the determined percentage of lymphocytes was used to calculate absolute count (Total lymphocyte count = Total WBC count x (% lymphocytes)/100). The calibration curve for calculation of CD4+ T-cell count is presented in Fig. 6. Plots of the color measurements (reflectance) versus the total lymphocyte count in the range of 2,000 to 9,000 counts/μL are shown in Figure 7.

[0075] In order to cover a broad range of possible lymphocyte concentrations in blood of pediatric patients (2,300 - 9,000 cells/μl), whole blood samples with normal concentrations of WBC enriched with WBC were used. For this purpose, a buffy coat obtained from these samples was diluted in different proportion in the mixture of red blood cells (RBS) and plasma, 1:1 obtained from the same sample. The calibration curve prepared using the above samples by reading the signals of intact retainer filter is shown in Figure 7, dashed line. As can be seen in Fig. 7, dashed line, the calibration curve is linear over the clinically significant range of lymphocyte concentrations. It should also be noted that samples used for the preparation of the calibration curve contain different amount of potentially interfering cells such as granulocytes and monocytes.

Possible interference of granulocytes and monocytes on CD45 lymphocyte count

[0076] As mentioned above, granulocytes and monocytes, which carry CD45 antigens just as lymphocytes do, may interfere with the enumeration of lymphocytes if not removed from whole blood before testing for CD45 count. In order to examine if the presence of high concentrations of granulocytes and monocytes in whole blood does or does not affect the performance of the FTCA test, signals (reflectance) measured for samples containing approximately the same amount of lymphocytes (at low concentrations) were compared with those containing low or high concentrations of granulocytes and monocytes. It should be noted that in pediatric populations, values for the absolute granulocyte counts vary from 10,000 to 1,000 cells/μL in 1 week to 5 year old patients [24], and the reported monocyte range in this population is 1,800 - 300 cells/ μL [25].

[0077] It was demonstrated that the signals obtained for samples containing low total lymphocytes (~2,000 cells/μl) with low (600 cells/μl) or very high (2,500 cells/μl) concentrations of monocytes are practically the same. Thus, this experiment shows that the presence of monocytes in whole blood does not affect the performance of the FTCA for enumeration of lymphocytes. This could be due to the following: 1) monocyte adsorption onto the LRF10S filter is negligible compared to that for lymphocytes; 2) the real maximal monocyte numbers in whole blood samples (2,500 cells/μl) are not high enough to interfere with lymphocyte count.

[0078] Unlike monocytes, the presence of granulocytes at high concentrations in whole blood samples does affect the performance of the FTCA if performed without

modifications. Indeed, Figure 7 shows the signals obtained for samples containing low (<3,000 cells/μl), medium (~5,000 cells/μl) and high (>10,000 cells/μl) amount of granulocytes in whole blood samples (markers are triangles (not filled), rhombus (filled gray) and squares (filled black), respectively). One can see in Fig. 7 that the signals obtained for samples with high granulocytes amount are much higher than those for the samples with low granulocytes amount at low lymphocyte counts.

[0079] The most successful way to eliminate the interference caused by the presence of granulocytes may be based on the following. WBC depletion filters are in fact multi-layer membranes made of polyester and polyamide materials [26]. In this example, LRF10S filter consisting of 6 layers was used. As a result, after conducting the FTCA total lymphocytes test the 2 upper layers of the LRF10S filter are removed and the reflectance (signal) is read from the surface of the 3rdlayer, the interference of granulocytes is abolished. Thus, the two upper layers of the filter LRF10S play a role of a pre-filter, which retains granulocytes and removes them from the FTCA reading zone (3rd layer).

[0080] As described above, some samples used for the preparation of the FTCA calibration curve prepared using the unmodified method contained very high granulocyte cell counts. In order to prepare a more precise FTCA calibration curve, the modified FTCA procedure was used including removing of two upper layers from the corresponding filters before reading the signals and the reflectance from the 3-rd top layer was measured. The calibration curve prepared using this modified procedure is shown in Figure 7, solid line. As can be seen in Figure 7, the calibration curves prepared using the unmodified (dashed line) and modified (solid line) FTCA protocols differ insignificantly.

[0081] The CD4+ T-cell count obtained using calibration curves presented in Figs. 6 and 7 respectively can be used for calculation of the percentage of CD4+ T-cells for the given sample. The %CD4/ly obtained using the FTCAs and Flow cytometry was calculated for five clinical samples and showed that the data obtained by two methods correlate well (standard errors did not exceed 7%).

EXAMPLE 3

Determination of CD8+ T-lymphocyte count for pediatric patients

[0082] Sometimes, CD4/CD8 ratio is used to establish the level of immunodeficiency and make decisions when to start cotrimoxazole (CTX) prophylaxis and/or ART in all HIV infected children younger than 5 years of age. In order to do that, CD4+ and CD8+ T-cells counts must be determined. In this example, the calibration curve for determination of CD8+ T-cells was prepared using the following protocol 4: Undiluted anti-CD8antibody (BD Pharmingen, San Diego, CA) was premixed at 2:1 with Streptavidin-AP (Sigma, Saint Louis, MO) diluted in TRIS-buffered saline (TBS, pH 7.4) buffer to the concentration of 24 μg/mL and incubated at room temperature for 30 min. Seventy microliters of WBC-enriched blood, whole blood or CD3 depleted blood was incubated with 30 μL Strep-AP/anti-CD4 "conjugate" (anti-CD4 and Strep-AP premix) and the samples were incubated for 15 min at RT. Thirty microliters of each sample were added using a pipette onto the LRF10S membrane incorporated into the flow-through device. After each addition of the sample, 500 μL of wash solution (10 mM TRIS (pH 7.4), 50 μg/mL NBT) were added using a pipette onto the device five times to wash out the red blood cells. One hundred μL of alkaline phosphatase (AP) substrate (Hercules, CA) was added after washing procedure. Ten min after adding AP substrate, 200 μL of stop solution (0.1 N H_2SO_4) was added onto each of the membranes. Membranes were removed from the flow-through device and the color intensity was read using a portable reflectance colorimeter (FIA Biomed, Germany) as described in the Example 1. A plot of the color measurements (reflectance) versus the CD8+ T lymphocyte count obtained using Dynal® T4 quantification method (Invitrogen, Grand Island, NY), using CD8 magnetic beads is shown in Figure 8

[0083] Although the invention herein has been described with respect to particular embodiments, it is understood that these embodiments are merely illustrative of the principles and applications of the present invention.

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Claims

1. A method for determining of at least one of CD4+ T-cell lymphocytes count, CD8+ T-cell lymphocytes count, or total lymphocytes count in a biological fluid sample, the method comprising the steps of:

   (a) adding to said biological fluid sample a sole antibody specific to anyone of said respective lymphocytes, said sole antibody conjugated with an optically detectable marker, said sole antibody is selected from the group consisting of an anti-CD4 antibody for determination of said CD4+ T-cell lymphocytes count, anti-CD8 antibody for determination of said CD8+ T-cell lymphocytes count, and an anti-CD45 antibody for determination of said total lymphocytes count;
   (b) incubating said biological fluid sample mixture of step (a) for duration from 5 min to 60 min and at a temperature ranging from 20 degrees Celsius to 37 degrees Celsius;
   (c) filtering said biological fluid sample mixture through a leukocyte-reduction retainer filter, said filter is configured for capturing thereon of said leukocytes including those which bound to said antibody of said biological fluid sample mixture, said filter is further configured for all captured leukocytes to contain at least 80 percent or more lymphocytes and at least 20 percent or less of any other subsets of leukocytes;

(d) washing said leukocyte-reduction retainer filter to remove weakly-bound substances; and
(e) optically detecting said bound antibody conjugated to said optically detectable marker which is retained on said leukocyte-reduction retainer filter in step (c) for determining of said at least one respective CD4+ T-cell lymphocytes count, CD8+ T-cell lymphocytes count, or total lymphocytes in said biological fluid.

2. The method of claim 1, wherein the optically detectable marker is selected from the group consisting of an enzyme, an enzyme complex, a plurality of gold microparticles, a plurality of quantum dot microparticles, a plurality of fluorescent latex microparticles, a plurality of fluorescent liposomes, a fluorescent dye, a photochemical dye, an chemiluminescent compound, and an electrochemiluminescent compound.

3. The method of claim 1, wherein said optically detectable marker is a biotin and said step (a) further includes adding of a streptavidin conjugate or an avidin conjugate of a material selected from the group consisting of an enzyme, an enzyme complex, a plurality of gold microparticles, a plurality of quantum dot microparticles, a plurality of fluorescent latex microparticles, a plurality of fluorescent liposomes, a fluorescent dye, a photochemical dye, a chemiluminescent compound, and a electrochemiluminescent compound.

4. The method of claim 1, wherein in said step (d) said optically detectable marker is detected by analyzing color intensity by eye or by using an optical instrument or by using an electrochemical instrument.

5. The method as in claim 4, wherein said optical instrument or said electrochemical instrument is selected from the group consisting of a reflectometer, a photometer, a fluorimeter, a chemiluminometer, and an electrochemical reader.

6. The method of claim 1 further comprising a step of calculating at least one of a ratio of CD4+ T cells count to total lymphocytes count or a ratio of CD4+ T cells count to CD8 lymphocytes count or a ratio of CD8 lymphocytes count to total lymphocytes count.

7. The method of claim 1, wherein said optically detectable marker is an Alkaline Phosphatase.

8. The method of claim 1, wherein said optically detectable marker is a biotin and said step (a) further includes adding of a streptavidin - Alkaline Phosphatase or an avidin - Alkaline Phosphatase conjugate to said biological fluid sample mixture.

9. The method of claim 8, wherein after said step (d) of washing said retainer filter and before said step (e) of optically detecting said marker there is included an additional step of adding an Alkaline Phosphatase substrate solution.

10. The method as in claim 9, wherein said Alkaline Phosphatase substrate solution is selected from the group consisting of a chromogenic substrate, a fluorogenic substrate, a chemiluminescent substrate, and a electrochemiluminescent substrate.

11. The method as in claim 10, wherein:

said chromogenic substrate is selected from the group consisting of 5-Bromo-4-chloro-3-indolyl phosphate/ p-iodonitrotetrazolium (BCIP/INT); 6-chloro-3-indoxyl phosphate, p-toluidine/ NBT (BCIP pink/NBT); 3-indoxyl phosphate/ NBT; Fast Red TR/ Naphthol AS-MX phosphate; Fast Red TR/ Naphthol AS-TR phosphate; and p-nitrophenol phosphate (pNPP);
said fluorogenic substrate is selected from the group consisting of 4-methylumbelliferyl phosphate (4-MUP); 6,8-Difluoro-4-methylumbelliferyl phosphate (DiFMUP); Fluorescein diphosphate (FDDP); 2'-[2-benzthiazoyl]-6'-hydroxybenzthiazole (BBTP); 2-(5'-chloro-2-phosphoryloxyphenyl)-6-chloro-4(3H)-quinazolinone (ELF 97); Acridinium (N-sulfonyl) carboxamide and acridinium ester-based substrates; and Rhodamine 110-based phosphates;
said chemiluminescent substrate is selected from the group consisting of Acridinium (N-sulfonyl) carboxamide and acridinium ester-based substrates; 1,2 Dioxetane- based substrates (4-methoxy-4-(3-phosphatephenyl)spiro [1,2-dioxetane-3,2'-adamantane]; and disodium salt-based substrates (AMPPD); and
said electrochemiluminescenct substrate is a ruthenium salt-based substrate.

12. The method of claim 9, wherein said step (e) including detecting a chromogenic signal or a fluorogenic signal or a chemiluminescent signal or an electrical signal, said detecting is done either visually by eye or by using an optical instrument or by using an electrochemical instrument.

13. The method as in claim 1, wherein said biological fluid sample is selected from the group consisting of a whole blood and a blood fraction.

14. The method of claim 13, wherein said blood fraction is selected from the group consisting of a buffy coat, and a whole blood cell fraction.

15. The method of claim 1, wherein said antibody is add-

ed in the step (a) in a lyophilized freeze-dried form.

Patentansprüche

1. Verfahren zum Bestimmen wenigstens eines aus einem CD4+ T-Zellen-Lymphozyten-Zählwert, einem CD8+ T-Zellen-Lymphozyten-Zählwert oder einem Lymphozyten-Zählwert insgesamt in einer biologischen Fluidprobe, wobei das Verfahren die folgenden Schritte umfasst:

   (a) Hinzufügen eines einzelnen Antikörpers, der für jeden der entsprechenden Lymphozyten spezifisch ist, zu der biologischen Fluidprobe, wobei der einzelne Antikörper konjugiert ist mit einem optisch nachweisbaren Marker, wobei der einzelne Antikörper ausgewählt ist aus der Gruppe bestehend aus einem Anti-CD4-Antikörper zur Bestimmung des CD4+ T-Zellen-Lymphozyten-Zählwerts, einem Anti-CD8-Antikörper zur Bestimmung des CD8+ T-Zellen-Lymphozyten-Zählwerts und einem Anti-CD45-Antikörper zur Bestimmung des Lymphozyten-Zählwerts insgesamt;
   (b) Inkubieren des biologischen Fluidprobengemischs aus Schritt (a) über einen Zeitraum von 5 Minuten bis 60 Minuten und auf einer Temperatur zwischen 20 °C und 37 °C;
   (c) Filtern des biologischen Fluidprobengemischs durch einen Leukozytenreduktions-Rückhaltefilter, wobei der Filter so gestaltet ist, dass er daran von den Leukozyten diejenigen erfasst, die an den Antikörper des biologischen Fluidprobengemischs binden, wobei der Filter ferner so gestaltet ist, dass alle erfassten Leukozyten wenigstens 80 Prozent oder mehr Lymphozyten und wenigstens 20 Prozent oder weniger jeder anderen Subpopulationen von Leukozyten enthalten;
   (d) Waschen des Leukozytenreduktions-Rückhaltefilters, um schwach gebundene Substanzen zu entfernen; und
   (e) optisches Nachweisen des gebundenen Antikörpers, der an den optisch nachweisbaren Marker konjugiert ist, der in Schritt (c) an dem Leukozytenreduktions-Rückhaltefilter zurückgehalten wird, um wenigstens eines aus einem CD4+ T-Zellen-Lymphozyten-Zählwert, einem CD8+ T-Zellen-Lymphozyten-Zählwert oder einem Lymphozyten-Zählwert insgesamt in dem biologischen Fluid zu bestimmen.

2. Verfahren nach Anspruch 1, wobei der optisch nachweisbare Marker ausgewählt ist aus der Gruppe bestehend aus einem Enzym, einem Enzymkomplex, einer Mehrzahl von Goldmikropartikeln, einer Mehrzahl von Quantenpunktmikropartikeln, einer Mehrzahl von fluoreszierenden Latexmikropartikeln, einer Mehrzahl von fluoreszierenden Liposomen, einem fluoreszierenden Farbstoff, einem fotochemischen Farbstoff, einer chemilumineszierenden Verbindung und einer elektrochemilumineszierenden Verbindung.

3. Verfahren nach Anspruch 1, wobei der optisch nachweisbar Marker ein Biotin ist, und wobei Schritt (a) ferner das Hinzufügen eines Streptavidin-Konjugats oder eines Avidin-Konjugats einer Substanz aufweist, die ausgewählt ist aus der Gruppe bestehend aus einem Enzym, einem Enzymkomplex, einer Mehrzahl von Goldmikropartikeln, einer Mehrzahl von Quantenpunktmikropartikeln, einer Mehrzahl von fluoreszierenden Latexmikropartikeln, einer Mehrzahl von fluoreszierenden Liposomen, einem fluoreszierenden Farbstoff, einem fotochemischen Farbstoff, einer chemilumineszierenden Verbindung und einer elektrochemilumineszierenden Verbindung.

4. Verfahren nach Anspruch 1, wobei in Schritt (d) der optisch nachweisbare Marker nachgewiesen wird durch Analysieren der Farbintensität mit dem Auge oder unter Verwendung eines optischen Instruments oder unter Verwendung eines elektrochemischen Instruments.

5. Verfahren nach Anspruch 4, wobei das optische Instrument oder das elektrochemische Instrument ausgewählt ist aus der Gruppe bestehend aus einem Reflektometer, einem Chemiluminometer und einem elektrochemischen Lesegerät.

6. Verfahren nach Anspruch 1, ferner umfassend einen Schritt des Berechnens wenigstens eines der folgenden Verhältnisse: eines Verhältnisses des CD4+ T-Zellenzählwerts zu dem Zählwert der Lymphozyten insgesamt oder eines Verhältnisses des CD4+ T-Zellenzählwerts zu einem CD8-Lymphozyten-Zählwert oder eines Verhältnisses des CD8-Lymphozyten-Zählwerts zu dem Zählwert der Lymphozyten insgesamt.

7. Verfahren nach Anspruch 1, wobei der optisch nachweisbare Marker eine alkalische Phosphatase ist.

8. Verfahren nach Anspruch 1, wobei der optisch nachweisbare Marker ein Biotin ist, und wobei Schritt (a) ferner das Hinzufügen eines Konjugats aus Streptavidin - alkalischer Phosphatase oder aus Avidin - alkalischer Phosphatase zu dem biologischen Fluidprobengemisch aufweist.

9. Verfahren nach Anspruch 8, wobei nach Schritt (d) des Waschens des Rückhaltefilters und vor Schritt (e) des optischen Nachweisens des Markers ein zu-

sätzlicher Schritt des Hinzufügens einer alkalischen Phosphatase-Substratlösung enthalten ist.

10. Verfahren nach Anspruch 9, wobei die alkalische Phosphatase-Substratlösung ausgewählt ist aus der Gruppe bestehend aus einem chromogenen Substrat, einem fluorogenen Substrat, einem chemilumineszierenden Substrat und einem elektrochemilumineszierenden Substrat.

11. Verfahren nach Anspruch 10, wobei:

das chromogene Substrat ausgewählt ist aus der Gruppe bestehend aus 5-Brom-4-chlor-3-indolylphosphat/p-Iodonitrotetrazolium (BCIP/INT); 6-Chlor-3-indoxylphosphat, p-Toluidin/ NBT (BCIP pink/NBT); 3-Indoxylphosphat/ NBT; Fast Red TR/ Naphthol AS-MX-Phosphat; Fast Red TR/ Naphthol AS-TR-Phosphat; und p-Nitrophenolphosphat (pNPP);
wobei fluorogene Substrat ausgewählt ist aus der Gruppe bestehend aus 4-Methylumbelliferylphosphat (4-MUP); 6,8-Difluor-4-methylumbelliferylphosphat (DiFMUP); Fluoresceindiphosphat (FDDP); 2'-[2-Benzthiazoyl]-6'-hydroxy-benthiazol (BBTP); 2-(5'-Chlor-2-phosphoryloxyphenyl)-6-chlor-4(3H)-chinazolinon (ELF 97); Acridinium (N-Sulfonyl)carboxamid- und Acridinium-Ester-basierten Substraten; und Rhodamin 110-basierten Phosphate; wobei das chemilumineszierende Substrat ausgewählt ist aus der Gruppe bestehend aus Acridinium (N-Sulfonyl)carboxamid- und Acridinium-Ester-basierten Substraten; 1,2-Dioxetan-basierten Substraten (4-Methoxy-4-(3-phosphatephenyl)spiro [1,2-dioxetan-3,2'-adamantan]; und Dinatriumsalz-basierten Substraten (AMPPD); und wobei das elektrochemilumineszierende Substrat ein Rutheniumsalz-basiertes Substrat ist.

12. Verfahren nach Anspruch 9, wobei Schritt (e) das Nachweisen eines chromogenen Signals oder eines fluorogenen Signals oder eines chemilumineszierenden Signals oder eines elektrischen Signals aufweist, wobei das Nachweisen entweder visuell mit dem Auge oder unter Verwendung eines optischen Instruments oder unter Verwendung eines elektrochemischen Instruments erfolgt.

13. Verfahren nach Anspruch 1, wobei die biologische Fluidprobe ausgewählt ist aus der Gruppe bestehend aus einem Vollblut und einer Blutfraktion.

14. Verfahren nach Anspruch 13, wobei die Blutfraktion ausgewählt ist aus der Gruppe bestehend aus Buffy Coat und einer Vollblutzellenfraktion.

15. Verfahren nach Anspruch 1, wobei der Antikörper in Schritt (a) in lyophilisierter, gefriergetrockneter Form hinzugefügt wird.

Revendications

1. Procédé de détermination d'au moins un nombre de lymphocytes de cellules T CD4+, un nombre de lymphocytes de cellules T CD8+, ou un nombre total de lymphocytes de cellules T dans un échantillon de fluide biologique, le procédé comprenant les étapes consistant à :

(a) ajouter audit échantillon de fluide biologique un unique anticorps spécifique à chacun desdits lymphocytes respectifs, ledit anticorps unique conjugué à un marqueur optiquement détectable, ledit anticorps unique est choisi parmi le groupe constitué d'un anticorps anti-CD4 pour la détermination dudit nombre de lymphocytes de cellules T CD4+, d'un anticorps anti-CD8 pour la détermination dudit nombre de lymphocytes de cellules T CD8+, et d'un anticorps anti-CD45 pour la détermination dudit nombre totale de lymphocytes ;
(b) incuber ledit mélange d'échantillons de fluides biologiques de l'étape (a) pendant une durée allant de 5 minutes à 60 minutes et à une température comprise entre 20 degrés Celsius et 37 degrés Celsius ;
(c) filtrer ledit mélange d'échantillons de fluides biologiques à travers un filtre conservateur de la réduction des leucocytes, ledit filtre est configuré pour capturer sur celui-ci lesdits leucocytes y compris ceux qui sont liés auxdits anticorps dudit mélange d'échantillons de fluides biologiques, ledit filtre est en outre configuré pour tous les leucocytes capturés pour qu'il contienne au moins 80 % ou plus de lymphocytes et au moins 20 % ou moins de n'importe quel sous-ensemble de leucocytes ;
(d) laver ledit filtre conservateur de la réduction des leucocytes pour éliminer les substances faiblement liées ; et
(e) détecter de manière optique ledit anticorps lié conjugué audit marqueur optiquement détectable qui est retenu sur ledit filtre conservateur de la réduction des leucocytes de l'étape (c) pour déterminer ledit au moins un nombre de lymphocytes de cellules T CD4+, un nombre de lymphocytes de cellules T CD8+, ou un nombre total de lymphocytes respectif dans ledit fluide biologique.

2. Procédé selon la revendication 1, dans lequel le marqueur optiquement détectable choisi parmi le groupe constitué d'une enzyme, d'un complexe enzymati-

que, d'une pluralité de microparticules d'or, d'une pluralité de microparticules de point quantique, d'une pluralité de microparticules de latex fluorescentes, d'une pluralité de liposomes fluorescents d'un colorant fluorescent, d'un colorant photochimique d'un composé chimioluminescent, et d'un composé électrochimioluminescent.

3. Procédé selon la revendication 1, dans lequel ledit marqueur optiquement détectable est une biotine et ladite étape (a) consiste en outre à ajouter un conjugué de streptavidine ou un conjugué d'avidine d'un matériau choisi parmi le groupe constitué d'une enzyme, d'un complexe enzymatique, d'une pluralité de microparticules d'or, d'une pluralité de microparticules de point quantique, d'une pluralité de microparticules de latex fluorescentes, d'une pluralité de liposomes fluorescents d'un colorant fluorescent, d'un colorant photochimique d'un composé chimioluminescent, et d'un composé électrochimioluminescent.

4. Procédé selon la revendication 1, dans lequel dans ladite étape (d) ledit marqueur optiquement détectable est détecté par l'analyse de l'intensité de couleur par l'oeil ou en utilisant un instrument optique ou en utilisant un instrument électrochimique.

5. Procédé selon la revendication 4, dans lequel ledit instrument optique où ledit instrument électrochimique est choisi parmi le groupe constitué d'un réflectomètre, d'un photomètre, d'un fluorimètre, d'un chimioluminomètre, et d'un lecteur électrochimique.

6. Procédé selon la revendication 1, comprenant en outre une étape de calcul d'au moins un d'un rapport du nombre de cellules T CD4+ au nombre total de lymphocytes ou un rapport du nombre de cellules T CD4 + au nombre de lymphocytes CD8 ou un rapport du nombre de lymphocytes CD8 au nombre total de lymphocytes.

7. Procédé selon la revendication 1, dans lequel ledit marqueur optiquement détectable est une Phosphatase Alcaline.

8. Procédé selon la revendication 1, dans lequel ledit marqueur optiquement détectable est une biotine et ladite étape (a) consiste en outre à ajouter un conjugué streptavidine - Phosphatase Alcaline ou un conjugué avidine - Phosphatase Alcaline audit mélange d'échantillons de fluides biologiques.

9. Procédé selon la revendication 8, dans lequel après ladite étape (d) de lavage dudit filtre conservateur et avant ladite étape (e) de détection optique dudit marqueur, on inclut une étape supplémentaire d'addition d'une solution de substrat de Phosphatase Alcaline.

10. Procédé selon la revendication 9, dans lequel ladite solution de substrat de Phosphatase Alcaline est choisie parmi le groupe constitué d'un substrat chromogène, d'un substrat fluorogène, d'un substrat chimioluminescent, et d'un substrat électrochimioluminescent.

11. Procédé selon la revendication 10, dans lequel :

ledit substrat chromogène est choisi parmi le groupe constitué du phosphate de 5-bromo-4-chloro-3-indolyle / p-iodonitrotétrazolium (BCIP/INT) ; du phosphate de 6-chloro-3-indoxyle, p-toluidine/ NBT (BCIP rose/NBT) ; du phosphate de 3-indoxyle / NBT ; du Fast Red TPv/ phosphate de Naphthol AS-MX ; du Fast Red TR/ phosphate de Naphthol AS-TR ; et du phosphate de p-nitrophénol (pNPP) ;
ledit substrat fluorogène est choisi parmi le groupe constitué du phosphate de 4-méthylbelliféryle (DiFMUP) ; du diphosphate de fluorescéine (FDDP) ; du 2'-[2-benzthiazoyl]-6'-hydroxybenzthiazole (BBTP) ; du 2-(5'-chloro-2-phosphoryloxyphényl)-6-chloro-4(3H)-quinazolinone (ELF 97) ; de l'acridinium (N-sulfonyl) carboxamide et de substrats d'acridinium à base d'ester ; et de phosphates à base de Rhodamine 110 ;
ledit substrat chimioluminescent est choisi parmi un groupe constitué de l'acridinium (N-sulfonyl) carboxamide et de substrats d'acridinium à base d'ester ; de substrats à base de 1,2 Dioxétane (4-méthoxy-4-(3-phosphatephényl)spiro [1,2-dioxétane-3,2'-adamantane] ; et de substrats à base de sels de disodium (AMPPD) ; et
ledit substrat électrochimioluminescent est un substrat à base de sel de ruthénium.

12. Procédé selon la revendication 9, dans lequel ladite étape (e) comprenant la détection d'un signal chromogène ou d'un signal fluorogène ou d'un signal chimioluminescent ou d'un signal électrique, ladite détection est effectuée soit visuellement par l'oeil soit en utilisant un instrument optique ou en utilisant un instrument électrochimique.

13. Procédé selon la revendication 1, dans lequel ledit échantillon de fluide biologique est choisi parmi le groupe constitué d'un sang total ou d'une fraction sanguine.

14. Procédé selon la revendication 13, dans lequel ladite fraction de sang est choisie parmi le groupe constitué d'une couche leucocytaire, et d'une fraction cellulaire de sang total.

15. Procédé selon la revendication 1, dans lequel ledit anticorps est ajouté dans l'étape (a) sous une forme

lyophilisée à froid.

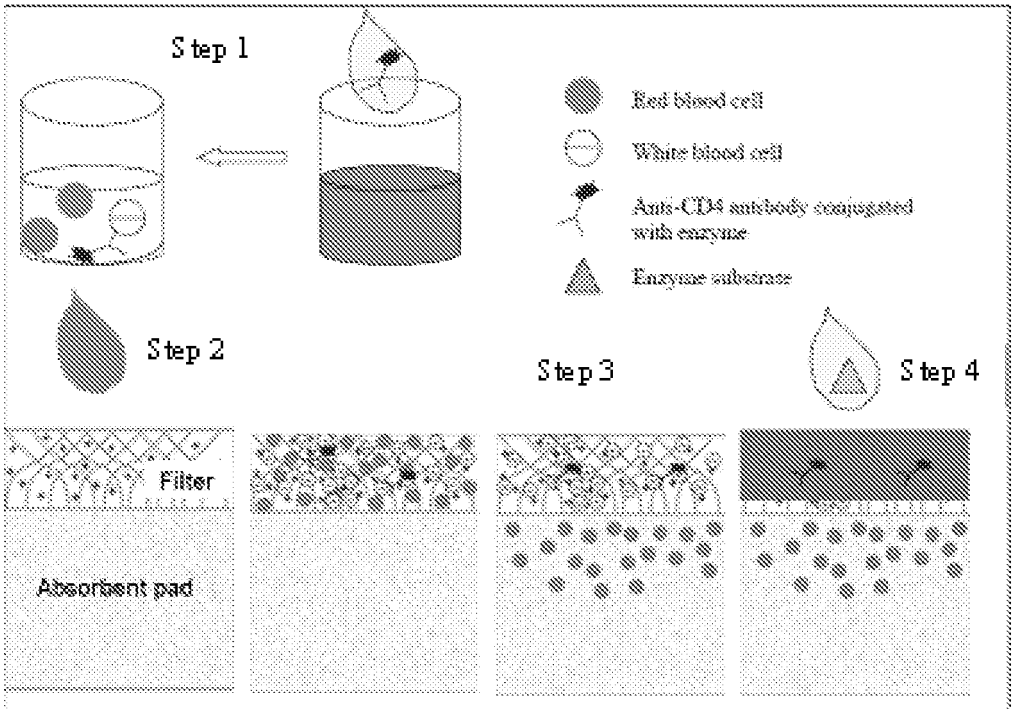
Step 1
Red blood cell
White blood cell
Anti-CD4 antibody conjugated with enzyme
Enzyme substrate
Step 2
Step 3
Step 4
Filter
Absorbent pad

Figure 1

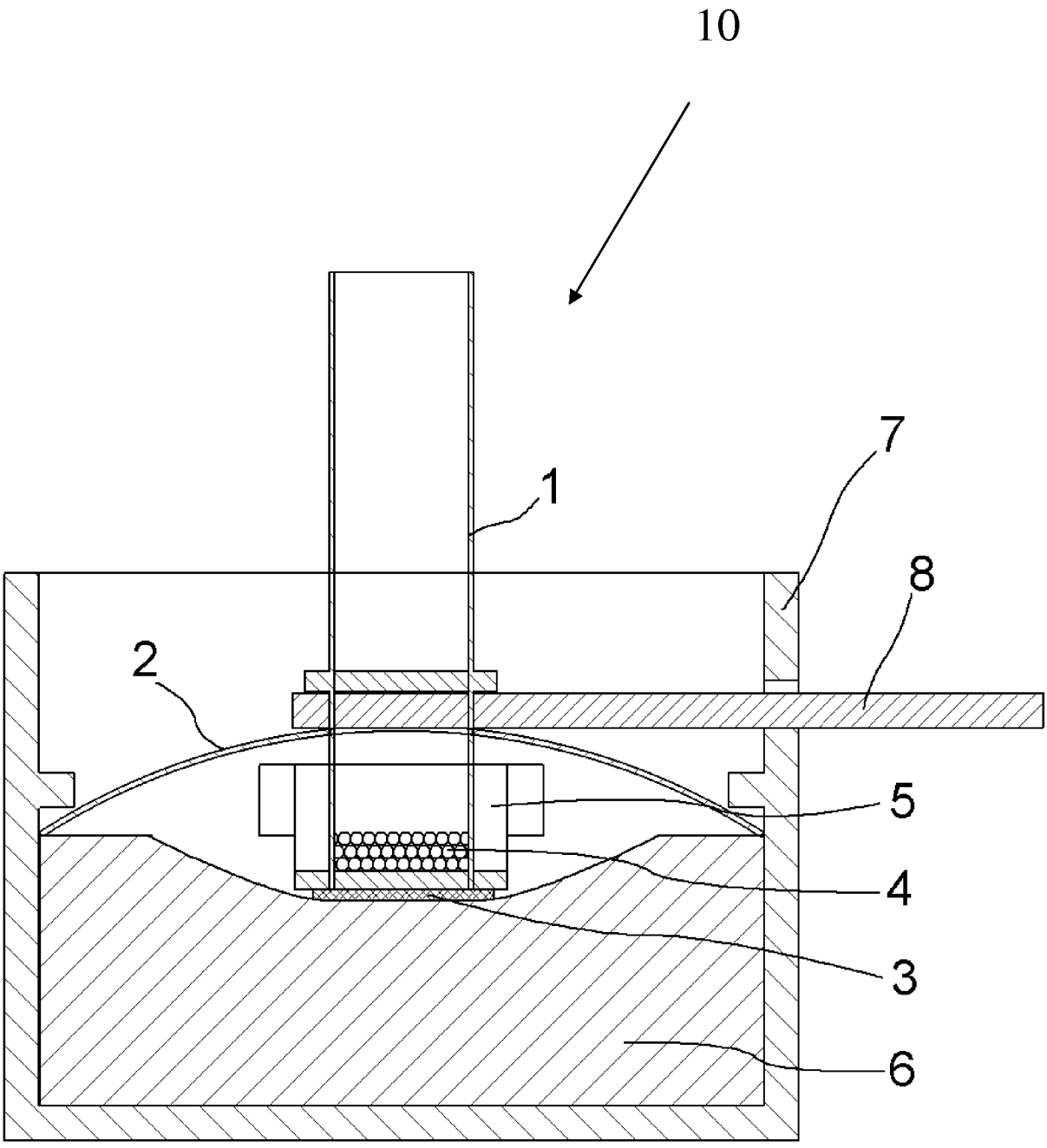

Figure 2

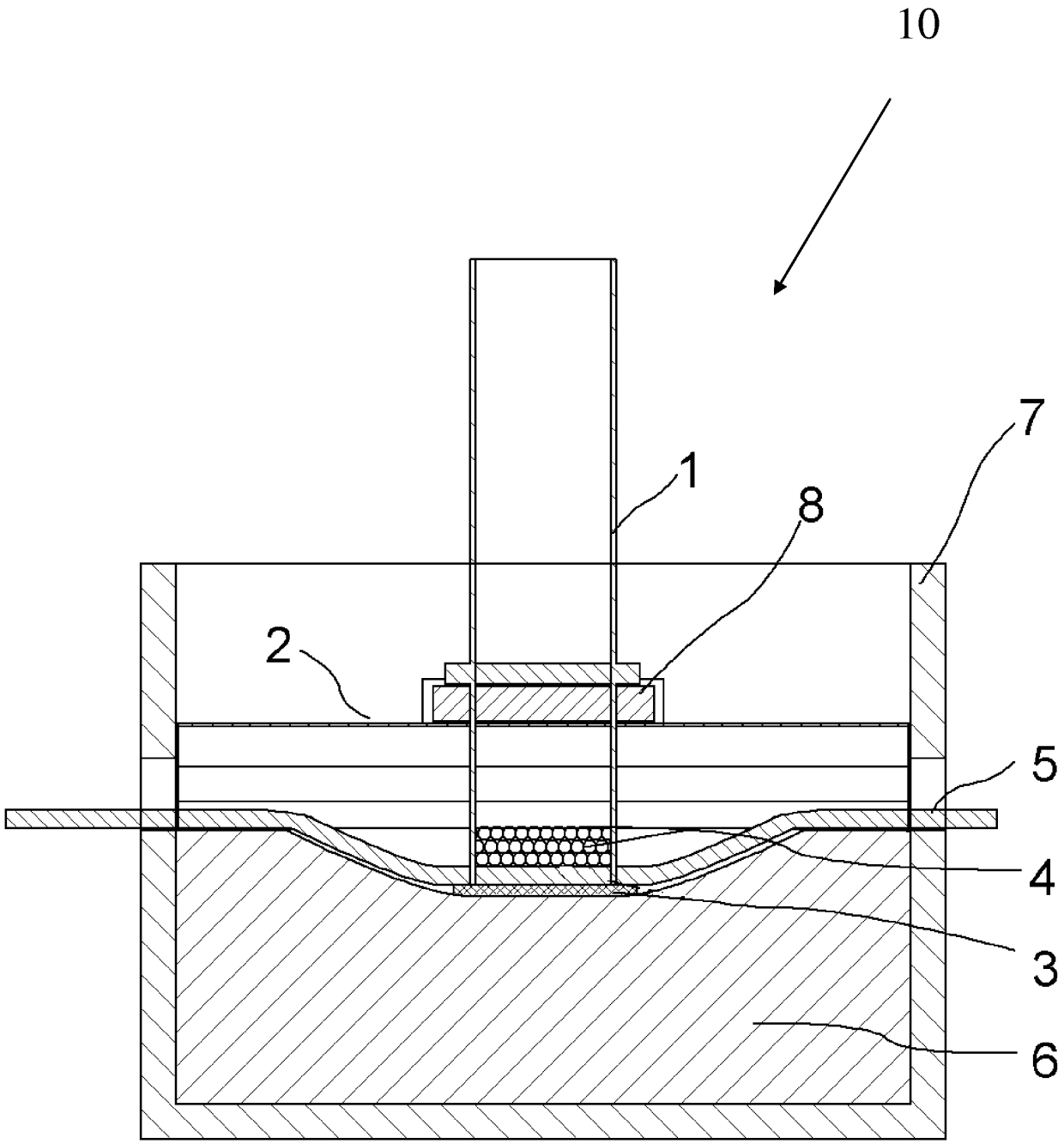

Figure 3

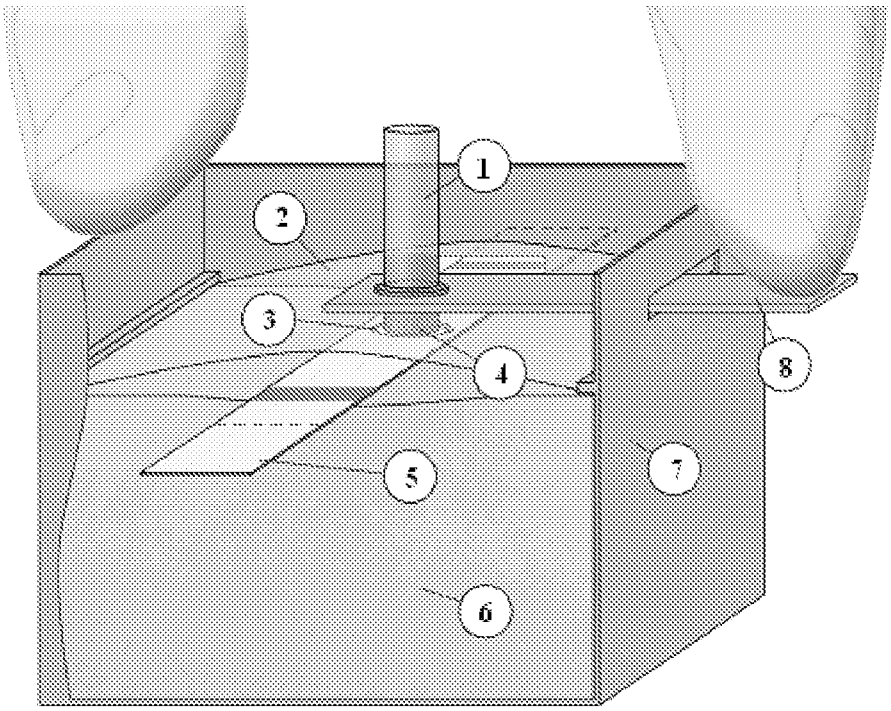

Figure 4

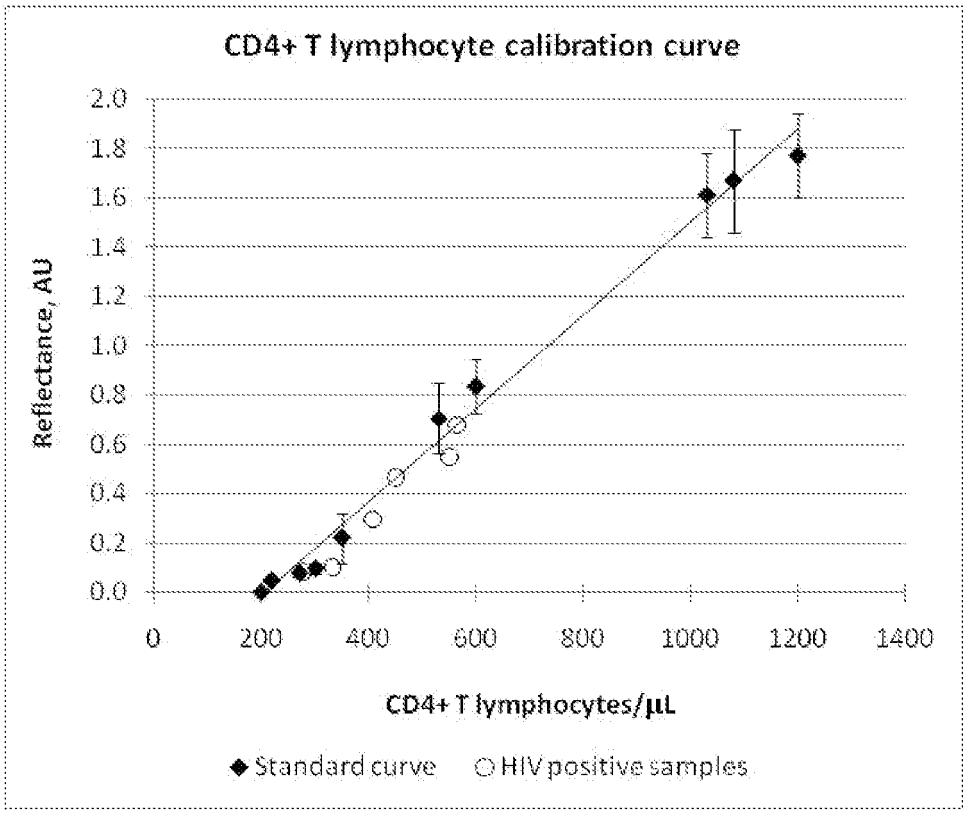
CD4+ T lymphocyte calibration curve
Reflectance, AU
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
1000
1200
1400
CD4+ T lymphocytes/µL
Standard curve
HIV positive samples

Figure 5

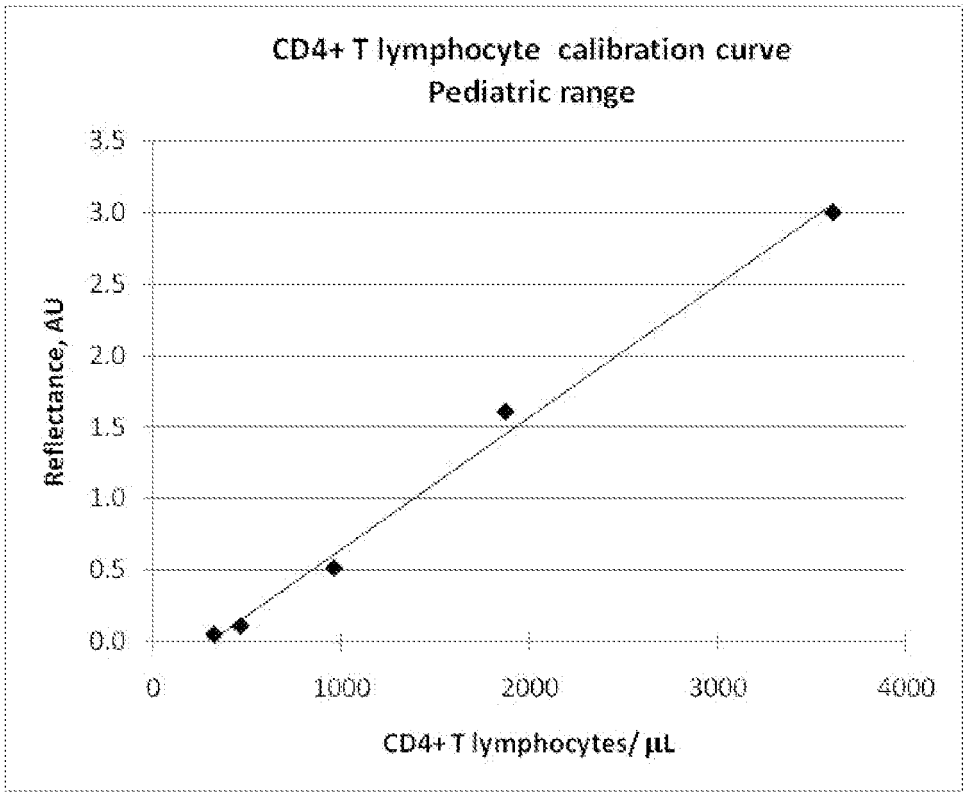
CD4+ T lymphocyte calibration curve
Pediatric range
Reflectance, AU
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
1000
2000
3000
4000
CD4+ T lymphocytes/ µL

Figure 6

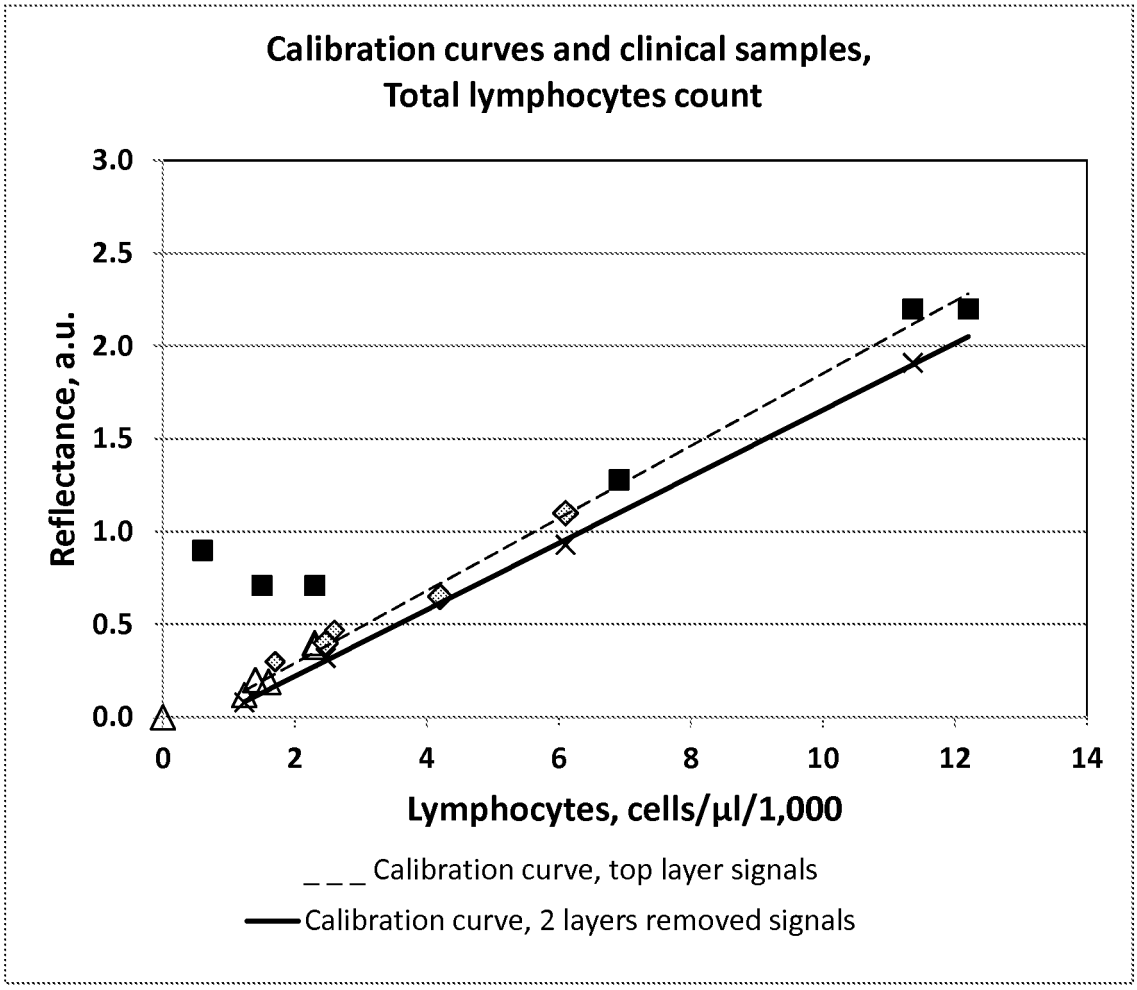
Calibration curves and clinical samples,
Total lymphocytes count
Reflectance, a.u.
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
14
Lymphocytes, cells/µl/1,000
Calibration curve, top layer signals
Calibration curve, 2 layers removed signals

Figure 7

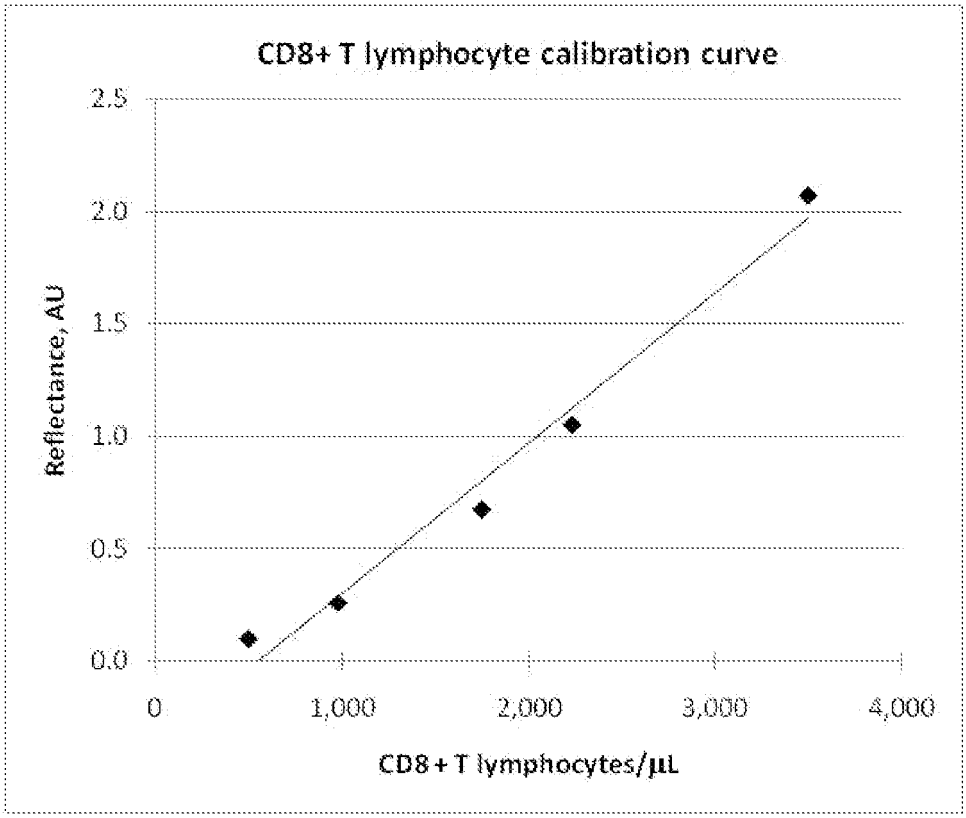
CD8+ T lymphocyte calibration curve
Reflectance, AU
2.5
2.0
1.5
1.0
0.5
0.0
0
1,000
2,000
3,000
4,000
CD8+ T lymphocytes/µL

Figure 8

REFERENCES CITED IN THE DESCRIPTION

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专利名称(译)	流通细胞计数测定		
公开(公告)号	EP2972345A1	公开(公告)日	2016-01-20
申请号	EP2014769059	申请日	2014-02-18
[标]申请(专利权)人(译)	ALLIED创新SYST		
申请(专利权)人(译)	ALLIED创新系统，LLC		
当前申请(专利权)人(译)	ALLIED创新系统，LLC		
[标]发明人	BYSTRYAK SIMON SANTOCKYTE RASA		
发明人	BYSTRYAK, SIMON SANTOCKYTE, RASA		
IPC分类号	G01N33/53 G06M11/02		
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代理机构(译)	VINAZZER，EDITH		
优先权	61/793037 2013-03-15 US 13/863490 2013-04-16 US		
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摘要(译)

用于定量测量白细胞（白细胞）计数和/或白细胞（白细胞）亚组计数的方法涉及将用标记物标记的特异性抗体添加到生物流体样品中，通过保留器从流体样品中捕获白细胞，通过洗涤或使用特定的磁珠去除除白细胞和其他干扰物质以外的其他物质，并读取结果。用于本方法的装置包括用于白细胞的保持器和用于吸收流过样品的所有过量洗涤溶液的吸收垫。