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(54) Title: A METHOD OF DIAGNOSIS AND KIT THEREFOR

(57) Abstract: The invention provides kits and methods for detecting or monitoring the number of cells in sample. The cell comprises a cell surface associated protein (CSAP) comprising a cytoplasmic (cytosolic) and an extracellular (ecto) domain. The kit comprises: (i) a chromatographic device; and (ii) a CSAP-binding agent. The method comprises: (i) optionally contacting the sample with an agent capable of lysing or permeabilizing CSAP bearing cells; (ii) contacting the sample with a CSAP-binding agent that binds to the cytoplasmic domain of the CSAP; and (iii) directly or indirectly evaluating the level or presence of bound CSAP in the sample.



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A METHOD OF DIAGNOSIS AND KIT THEREFOR

FIELD

The present invention relates generally to the field of diagnosis and provides
5 methods and reagents for accurately assessing in a sample from a subject the presence or
amount of a specific cell type, its cell-associated proteins or soluble (extracellular) forms
thereof. In some embodiments, the invention relates to rapid, self contained diagnostics
which can be used in most environments such as the clinic, laboratory, in the field, at home
and in regions having poor resources. In one embodiment, a method and kit is provided
10 for evaluating CD4 T-cell numbers in a sample from a subject.

BACKGROUND

Bibliographic details of references in the subject specification are also listed at the
end of the specification.

15 Reference to any prior art in this specification is not, and should not be taken as, an
acknowledgement or any form of suggestion that this prior art forms part of the common
general knowledge in any country.

A diverse range of techniques are used in research, analysis, development and
clinically to detect cells of interest. Manual or automated techniques are available to count
20 cells in specially designed chambers that permit cell numbers in a sample to be evaluated.
Cells may be stained with particular stains in order to differentiate between cell types.
Histochemical techniques may be applied to further differentiate between cells in a sample.
The ability of cells to respond to particular antigens by proliferating or producing
cytokines, bind other cells, engulf other cells or move by chemotaxis may also be
25 diagnostic. Cell surface markers are particularly useful for differentiating between cell
types and evaluating the number of particular cells in a sample. Many techniques use
antibodies to detect the presence of the marker.

Cells of prokaryotic and eukaryotic organisms, in all their different forms, comprise
a cell membrane, which separates the contents of the cell from the extracellular
30 environment. The cell membrane is a selective barrier that determines what goes in and
out of the cell and which also undertakes complex signalling activities with other cells and

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molecules in the environment of the cell. The membrane comprises a phospholipid bilayer and most molecules that cross or interact with the bilayer do so with the assistance of proteins inserted into the bilayer that have both cytoplasmic and extracellular portions.

Frequently, a cell's initial interaction with its surroundings occurs *via* receptors or cell surface associated molecules expressed on the plasma membrane. Activation of these receptors, whether through binding endogenous ligands (such as cytokines or hormones) or exogenous ligands (such as antigens) triggers a biochemical cascade from the membrane through the cytoplasm to the nucleus. A wide range of receptors or cell surface associated proteinaceous molecules are known to mediate cell:cell interactions and cell:molecule interactions. These include, for example, MHC proteins, cytokine, hormone or neurotransmitter receptors, tethered ligands, G-protein coupled receptors, receptor protein tyrosine kinases, receptor protein tyrosine phosphatases, protein-serine/threonine kinases and receptor guanylyl cyclases.

Most of the proteins that mediate cell-cell recognition or antigen recognition in the immune system contain Ig or Ig-like domains, suggesting that they have a common evolutionary history. These include antibodies, T cell receptors, cluster of differentiation (CD) antigens such as the CD4, CD8, and CD28 proteins, the invariant polypeptide chains associated with B and T cell receptors and Fc receptors on lymphocytes and other white blood cells. About half of the proteins that have been characterized on the surface of white blood cells belong to this superfamily. Cells of the immune system thus express a range of cell surface proteins whose identity can be used to evaluate the number and status of different cells in a sample.

B and T lymphocytes, for example, may be identified using antibodies to the constant regions of the B- and T-cell antigen receptors. T-helper and cytotoxic T-cells may be identified on the basis of expression of the co-receptor proteins, CD4 and CD8, respectively.

Flow cytometry is a powerful tool for identifying and enumerating cells. The flow cytometer detects and counts individual cells passing in a stream through a laser beam. By examining large numbers of cells, flow cytometry can give quantitative data on the percentage of cells bearing different molecules, such as surface immunoglobulin, which characterizes B cells, the T-cell receptor-associated molecules known as CD3, and the

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CD4 and CD8 co-receptor proteins that distinguish the major T-cell subsets. Individual cells within a mixed population are tagged with specific antibodies labelled with fluorescent dyes, or for example, by specific antibodies followed by labelled anti-immunoglobulin antibodies. The suspended mixture of labelled cells is then forced
5 through an aperture, creating a fine stream of liquid containing cells spaced singly at intervals. As each cell passes through a laser beam it scatters the laser light, and any dye molecules bound to the cell will be excited and will fluoresce. Sensitive photomultiplier tubes detect both the scattered light, which gives information on the size and granularity of the cell, and the fluorescence emissions, which give information on the binding of the
10 labelled antibodies and hence on the expression of cell-surface proteins by each cell. If two or more antibodies are used, each coupled to a different fluorescent dye, then the data may be displayed in the form of a two-dimensional scatter diagram or as a contour diagram, where the fluorescence of one dye-labelled antibody is plotted against that of a second, with the result that a population of cells labelling with one antibody can be further
15 subdivided on the basis of its reactivity with the second antibody.

Immunoassays are another particularly useful form of assay that exploit the specificity, strength and diversity of antibody-antigen reactions to analyse samples and detect specific components therein. A wide range of immunoassay techniques are available, such as those described in Wild D. "*The Immunoassay Handbook*" Nature
20 Publishing Group, 2001.

A wide range of methods for the detection of antibody to specific antigens are also known. For example, the enzyme-linked immunosorbent assay (ELISA) and radio-immunoassay (RIA) are routinely used in laboratories. Arrays and high throughput screening methods are also employed. These methods generally require a high level of skill
25 in laboratory techniques.

A variety of methods have also been developed which require little skill and are rapid to perform, and which are therefore suitable for the detection of antibody to specific antigens, and/or the detection of specific antigens, at the point of care. In particular, lateral flow, dipstick and capillary tube kits have been developed to assay for a number of
30 infections including viral infections.

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In subjects with an immunodeficiency disease such as AIDS, the level of CD4 expressing T-cells is an indication of when to commence anti-retroviral drug treatment. The virus infects these T cells and ultimately destroys them. Low CD4⁺ T-cell levels are also an indication of the risk of clinical progression and susceptibility to opportunistic
5 infection.

In one method of detecting CD4 cells, dynabeads coated with anti-CD4 antibodies are used to bind CD4⁺ T-lymphocytes. Monocytes, that express CD14 and CD4, are excluded from fresh blood samples sample using beads coated with anti-CD14 antibodies. Thereafter, the isolated CD4 T-lymphocytes are lysed, stained with acridine orange and
10 stained nuclei are enumerated by fluorescence microscopy. A "TRAx CD4" test kit is described in Paxton *et al.*, Clin. Diagn. Lab. Immunol., 2(1):104-114, 1995. This kit is an ELISA based method to measure total CD4 in whole blood samples. The antibodies used did not distinguish between cell bound and soluble CD4 (see Lyamuya *et al.*, J. Imm. Methods., 195:103-112, 1996). International Publication No. WO 2006/115866 describe
15 an immunochromatographic device for measuring CD4 antigens. However, again there is no disclosure in this document of a capture reagent capable of distinguishing between cell bound and soluble CD4 lacking a cytoplasmic domain in sample from a subject. Further, the device described in WO 2006/115866 depends upon the flow of sample over a series of numbered capture areas to capture CD4 by saturating consecutive capture areas on a test
20 strip to subsequently provide a visual indication of the concentration of CD4 cells in the sample.

There is a need for improved methods of measuring particular cell types or their cell associated proteins, particularly methods that can be used at point of care and that provide rapid and accurate results.

25

SUMMARY

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to denote the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that no other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

It must be noted that, as used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. For example, reference to a "sample pad" includes one sample pad or more than one sample pad.

Each embodiment in this specification is to be applied *mutatis mutandis* to every other embodiment unless expressly stated otherwise.

Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc. A summary of sequence identifiers is provided in Table 1. A sequence listing is provided after the claims.

In a broad embodiment, the present specification describes method for evaluating in a sample from a subject the level or presence of a cell surface associated protein (CSAP) comprising a cytoplasmic (cytosolic) and an extracellular (ecto) domain or the level or presence of cells bearing the CSAP, the method comprising: (i) optionally contacting the sample with an agent capable of lysing or permeabilizing CSAP bearing cells; (ii) contacting the sample with a CSAP-binding agent that binds to the cytoplasmic domain of the CSAP; and (iii) directly or indirectly evaluating the level or presence of bound CSAP

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in the sample. In some embodiments the level or presence of bound CSAP comprising at least a cytoplasmic domain is indicative of the number of CSAP bearing cells in the subject.

In another embodiment, the specification describes a method for evaluating in a sample from a subject the level a cell surface associated protein (CSAP) comprising a cytoplasmic (cytosolic) and an extracellular (ecto) domain or the level of CSAP-bearing cells, the method comprising: a) applying the test sample to a sample portion of an immunochromatographic device wherein the sample portion is operably connected to a capture portion of the device and wherein components of the test sample flow from the sample portion to and through the capture portion which comprises an antibody or antigen-binding fragment thereof that binds to the cytoplasmic domain of CSAP such that only CSAP comprising a cytoplasmic domain and not soluble CSAP that does not comprise a cytoplasmic domain binds to the antibody or fragment thereof to form a captured CSAP; b) contacting the capture portion with a second binding agent that binds to CSAP including to the cytoplasmic or extracellular domain and which comprises a detection marker or which is capable of binding to a third or subsequent binding partner comprising a detection marker; c) optionally contacting the second binding agent with a third or subsequent binding agent comprising a detection marker; evaluating the presence of the detection marker.

In an illustrative embodiment, the method is used for evaluating in a blood sample from a subject the level of T-cell associated CD4 comprising a cytoplasmic (cytosolic) and an extracellular (ecto) domain or the level of CD4 T-cells, the method comprising: (i) optionally contacting the sample with an agent capable of lysing or permeabilizing CD4 T-cells; (ii) contacting the sample with an antibody or antigen-binding fragment thereof that binds to the cytoplasmic domain of CD4; and (iii) directly or indirectly evaluating the level or presence of bound CD4 in the sample.

In another embodiment of this aspect, the method is for evaluating from a subject the level of T-cell associated CD4 comprising a cytoplasmic (cytosolic) and an extracellular (ecto) domain or the level of CD4 T-cells and comprises: a) applying the test sample to a sample portion of an immunochromatographic device wherein the sample portion is operably connected to a capture portion of the device and wherein components

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of the test sample flow from the sample portion to and through the capture portion which comprises an antibody or antigen-binding fragment thereof that binds to the cytoplasmic domain of CD4 such that only CD4 comprising a cytoplasmic domain and not soluble CD4 that does not comprise a cytoplasmic domain binds to the antibody or fragment thereof to
5 form a captured CD4; b) contacting the capture portion with a second binding agent that binds to CD4 including to the cytoplasmic or extracellular domain and which comprises a detection marker or which is capable of binding to a third or subsequent binding partner comprising a detection marker; and c) optionally contacting the second binding agent with
10 a third or subsequent binding agent comprising a detection marker; evaluating the presence of the detection marker.

The agent that binds to the cytoplasmic domain of a CSAP or a sCSAP may be any form of molecule that specifically binds to a CSAP. CSAP-binding agents such as a ligand, aptamer or an antibody are particularly contemplated. Antibodies or an antigen binding fragments thereof are conveniently prepared with high specificity for a portion of a CSAP.
15 In addition, antibodies can be conveniently conjugated to or produced with detectable markers or bound by secondary or subsequent antibodies conjugated to detectable markers. Either form of antibody whose function is directly or indirectly to detect an agent-CSAP complex may be referred to as a detection antibody.

In another broad embodiment, the method comprises (i) optionally contacting the
20 sample with an agent capable of lysing or permeabilizing CSAP bearing cells, (ii) contacting the sample to be tested with an agent that binds to the extracellular domain of the CSAP such that both full length (CSAP) and a soluble form of the CSAP (sCSAP) are bound by the agent and form complexes comprising agent-CSAP and/or agent-sCSAP (iii) optionally, if the agent is not already bound to or associated with a region of a solid or
25 semi-solid support, contacting the sample with a solid or semi-solid support such that the agent and any agent-CSAP complex and/or agent-sCSAP complex is bound to the solid-support (iii) contacting the sample or appropriate region of the support with a second agent that specifically binds to the cytoplasmic domain of the CSAP and (iv) directly or indirectly determining the presence or level of the bound second agent.

30 In another broad embodiment, the present specification provides a method for determining in a sample from a subject a) the level or presence of a protein expressed on

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the surface of a cell (CSAP) comprising a cytoplasmic (cytosolic) and an extracellular (ecto) domain or cells bearing the CSAP and b) the level of presence of a soluble non-membrane bound extracellular form of the CSAP (sCSAP) in the sample to be tested. The method comprises (i) optionally contacting the sample to be tested with an agent capable
5 of lysing or permeabilizing CSAP bearing cells, (ii) contacting the sample with an agent that binds to the cytoplasmic domain of the CSAP and a second agent that binds the soluble form of CSAP lacking a cytoplasmic domain, (iii) directly or indirectly evaluating the level of bound CSAP and bound sCSAP in the sample. In embodiments where the level or presence of sCSAP is also measured, the evaluation step (iii) distinguishes between
10 agent-CSAP complexes and agent-sCSAP complexes. In this embodiment, the relative presence or amount of cell bound and soluble sCSAP in the sample from a subject may be determined.

In another aspect, the subject specification describes kits for detecting or monitoring the number of cells in sample from a subject, wherein the cell is characterised
15 by comprising a cell surface associated protein (CSAP) comprising a cytoplasmic (cytosolic) and an extracellular (ecto) domain. In a broad embodiment, the kit comprises: (i) a chromatographic device comprising a porous membrane operably connected to a sample portion, a test (capture) portion, a conjugate portion, a sucker portion, optionally a control portion and optionally a lysis portion; (ii) a CSAP-binding agent such as an
20 antibody or antigen-binding fragment thereof that binds to the cytoplasmic domain of CSAP, wherein the agent or antibody is either immobilised (bound) to one or more test portions and/or contained within a conjugate portion; and (iii) instructions for using the device to detect or monitor the level or presence of CSAP-bearing cells in the sample or subject.

25 In an illustrative embodiment, a kit for detecting or monitoring the number of CD4 T-cells in a blood sample from a subject is provided. In a broad embodiment, the kit comprises: (i) a chromatographic device comprising a porous membrane operably connected to a sample portion, a test (capture) portion, a conjugate portion, a sucker portion, optionally a control portion and optionally a lysis portion; (ii) a CD4-binding
30 agent such as an antibody or antigen-binding fragment thereof that binds to the cytoplasmic domain of CD4, wherein the agent or antibody is either immobilised (bound)

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to one or more test portions and/or contained within a conjugate portion; and (iii) instructions for using the device to detect or monitor the level or presence of CD4 cells in the sample or subject. As will be appreciated by those of skill in the art of immunochromatography devices, the subject kits may be designed in any suitable format including reverse or lateral flow immunochromatography formats.

5

The above summary is not and should not be seen in any way as an exhaustive recitation of all embodiments of the present invention.

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BRIEF DESCRIPTION OF THE FIGURES

Figure 1 (A) is a schematic representation of human CD4 associated with a T-lymphocyte cell surface membrane. The extracellular domain comprising amino acids 1-395 is shown, arranged as four immunoglobulin-like domains together with disulphide bonded regions thereof. The transmembrane domain comprising amino acids 396-420 is shown spanning the T-lymphocyte cell surface membrane. The cytoplasmic (cytosolic) domain comprising amino acids 421-458 is also illustrated. **Figure 1 (B)** represents the structure of TM^{CD4} comprising a cytoplasmic and extracellular domain and with a deletion of the transmembrane domain plus the first two amino acids of the cytoplasmic domain, such that this TM^{CD4} protein represents amino acids 1-395 fused to amino acids 423-458.

Figure 2 is a diagrammatic representation of the human CD4 cytoplasmic domain and the sequence of sub sequence peptides that were synthesised in order to generate antibodies against the cytoplasmic domain. Peptide 1 comprises 17 amino acids from amino acids 436 to 454 with amino acids 446 to 447 (QC) omitted (SEQ ID NO: 1). Peptide 2 comprises 20 amino from amino acids 422 to 441 of human CD4 (SEQ ID NO: 2). Numbering of amino acids is as described in Example 1.

Figure 3 is a graphical representation of the results of an ELISA assay conducted to measure the specificity and titre of sheep polyclonal antibodies to CD4 cytoplasmic peptides. **Figure 3A** and **3B** represent the results from sheep G-6 and sheep G-7, respectively. Blood was samples pre-bleed (PB), in two test bleeds (TB1 and TB2) and a final bleed (FB).

Figure 4 is a graphical representation of the results of an ELISA assay conducted to measure the specificity of CD4 polyclonal antibodies for peptide 1 and peptide 2. As shown therein, sheep have similar reactivity to both peptides by ELISA. TB2 is blood from a second test bleed after immunisation of G-7 or G-6 sheep.

Figure 5 is a graphical representation of the results of an ELISA assay conducted to compare the reactivity of anti-CD4_{cyto} monoclonal antibodies (MAbs) to CD4_{cyto} peptide 2. As shown therein, three of the monoclonal antibodies show binding to peptide 2.

Figure 6 is a graphical representation of the results of flow cytometry analyses conducted to assess the ability of anti CD4_{cyto} monoclonal antibodies ID2 and 4B4 to bind

to fresh (non-fixed/permeabilised) CD4⁺ T-cells (A, B, C and D). Antibodies failed to bind to fresh cells.

Figure 7 is a graphical representation of the results of flow cytometry analyses conducted to assess the ability of anti CD4_{cyto} monoclonal antibodies ID2 and 4B4 to bind
5 to fixed/permeabilised CD4⁺ T-cells (A, B, C, D). Antibodies bound to fixed/permeabilised cells and 92% of CD4⁺ cells were selected.

Figure 8 is a schematic representation of two immunoassay formats suitable for the practice of the present invention.

Figure 9 is a graphical representation of results of an ELISA conducted to measure
10 the reactivity of monoclonal antibodies directed to the cytoplasmic domain of CD4 including a commercially available anti-CD4 cyto monoclonal antibody (chemicon).

Figure 10 is a graphical representation of results of a capture ELISA conducted to measure the specificity of monoclonal antibody 1D2 directed to the cytoplasmic domain of CD4 and its ability to capture CD4 from JC53 cell lysates and detection with a monoclonal
15 antibody directed to the ectodomain of CD4 (RPAT4). 1D2 captured CD4 from JC53 cell lysates that was detectable with RPAT4, but soluble CD4 was not captured.

Figure 11 is a graphical representation of results of a capture ELISA conducted to measure the specificity of monoclonal antibody 1D2 directed to the cytoplasmic domain of CD4 and its ability to capture CD4 from JC53 cell lysates and detection with a polyclonal
20 antibody directed to the ecto domain of CD4. 1D2 captured CD4 from JC53 cell lysates that was detectable with polyclonal antibody to the CD4 ectodomain, but soluble CD4 was not captured.

Figure 12 is a graphical representation of results of capture ELISAs of cellular CD4 from donor peripheral blood mononuclear cell (PBMC). A shows a titration of
25 PBMC lysate captured by monoclonal antibody 4B4 from Donors 1 to 6, who have different proportions of CD4 T-cells in the total PBMC population. B shows the capture of cellular CD4-enriched lymphocytes.

Figure 13 is a graphical representation of results of capture ELISAs of cellular CD4 from donor peripheral blood mononuclear cell (PBMC). The results from three
30 donors are shown and a correlation between OD450-620 and the number of CD4⁺ T-cells *per* microlitre.

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Figure 14 is a graphical representation of results of capture ELISAs of cellular CD4 from donor peripheral blood mononuclear cell (PBMC). The results from 5 different HIV-infected patients are shown and demonstrate a close correlation between OD450-620 and the number of CD4⁺ T-cells *per* microlitre of whole blood.

5 **Figure 15** is a schematic representation of an immunochromatographic device for detection of full length cell-associated CD4 and estimation of CD4⁺ cell numbers thereby.

Figure 16 is a representation of the nucleic acid sequence encoding one form of TM[^]CD4. Underlined text is the His tag. Boxed text is the enterokinase recognition site. Bold text is the extracellular domain of CD4 and Italics text is the cytoplasmic tail of
10 CD4.

Figure 17 is a a representation of the amino acid sequence of one form of TM[^]CD4. Underlined text is the His tag. Boxed text is the enterokinase recognition site. Bold text is the extracellular domain of CD4 and Italics text is the cytoplasmic tail of
15 CD4.

Figure 18 is a graphical and photographic representation of data showing the detection of recombinant TM[^]CD4 by ELISA (A) and by Western immunoblotting (B). (A) Full-length (FL) CD4 or TM[^]CD4 were expressed in 293 T-cells by transfection of the respective pcDNA4/HisMax constructs, or mock-transfected. Cell lysates and cell supernatants were tested by CD4 ELISA using the method described. The results show
20 that full-length CD4 is efficiently expressed in cells but is not released into the supernatant, whereas TM[^]CD4 is efficiently expressed in cells and most of the recombinant protein is released into the supernatant, allowing easy purification if required. (B) TM[^]CD4 was detected in culture supernatant by Western immunoblotting with custom MA b 4B4 (3 µg/ml) against the cytoplasmic domain of CD4.

Figure 19 is a graphical and photographic representation of data showing detection of TM[^]CD4 by ELISA and immunochromatographic rapid, point of care assay. Supernatant from HEK293 cells stably transfected with the TM[^]CD4 plasmid were serially diluted (starting at 1:5 dilution) for assay by ELISA (A), showing a linear response in the ELISA demonstrating the utility of the TM[^]CD4 as a control reagent for estimating total
30 CD4 T-cell numbers in the CD4 ELISA assay. In this Figure, four different samples of whole blood were tested in the same assay (samples IDS72, 51, 40, 49), and the relative

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amount of CD4 compared to the standard can be estimated using the regression curve shown, or other methods. To enable conversion of the amount of CD4 to the number of CD4 T-cells per microlitre, a reference sample of whole blood was tested multiple times by flow cytometry and ELISA in comparison with the TM^{CD4} standard, allowing the
5 correct conversion factor to be determined (data not shown). (B) Supernatant from two different stable HEK293-TM^{CD4} cell line clones (H5 and F5) were detected by rapid point of care assay, showing that TM^{CD4} can be used as a control reagent or standard in such assays.

Figure 20 is a graphical representation showing the good correlation between CD4
10 cell numbers calculated by flow cytometry and ELISA. Using the recombinant soluble form of CD4 as an internal assay control for the CD4 ELISA, and conversion factor based on parallel determination of a reference sample by flow cytometry and ELISA, very close correlation is obtained for the estimated CD4 T-cell numbers using CD4 ELISA compared to CD4 flow cytometry. In particular, note that all samples are correctly identified as
15 being either greater than, or less than, 250 T-cells per microlitre, as shown in the boxed region. In this assay, monocytes were removed from whole blood using CD14 magnetic beads before assay of whole blood for CD4 T-cells by ELISA.

Figure 21 is a photographic representation of results in the rapid immunochromatographic format showing a good correlation between different amounts of
20 TM^{CD4} (as a surrogate for CD4 comprising a cytoplasmic domain) and signal strength. This demonstrates that the TM^{CD4} is useful as a control reagent for preparation, testing and quality control of both ELISA and rapid, point of care formats of CD4 assays. This Figure also demonstrates that both the Chemicon 3706 anti-cytoplasmic domain Mab, and the custom 4B4 anti-cytoplasmic domain Mab, can be used separately or as a mixture for
25 capture of CD4 in the rapid format of the test. Further, this demonstrates that the signal strength in the rapid format is proportional to the amount of CD4 in the sample, allowing the use of the rapid point of care format for quantitative or semiquantitative estimation of CD4 T-cell numbers.

Figure 22 is a photographic representation of results in the rapid immunographic
30 device. (A) provides test results for rapid point of care assay format, using purified T-cells at the indicated numbers of cells per microlitre (reacting at Test lines). (B): Control lines

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were prepared to be equivalent to 500 T-cells/microlitre and 250 T-cells/microlitre, by striping biotinylated monoclonal antibody that reacts directly with the anti-biotin gold conjugate.

Figure 23 (A) is a schematic representation of a whole-blood CD4 rapid point of care assay in accordance with the present invention. (B). A sample of whole blood containing high CD4 T-cell levels (1452/ μ l by flow cytometry) was tested in the whole blood assay as shown in A, with detergent in the lysis pad as normal (“+TX”), or with omission of the detergent from the lysis pad (“-TX”). In this example, CD4 signal is only seen in the rapid test when detergent is present to lyse the T-cells in the pad. (C).
10 Examples of representative whole-blood samples assayed in the CD4 rapid point of care assay. CD4 counts for each sample by flow cytometry are shown, and it can be seen that high CD4 T-cell counts give stronger signals while low CD4 T-cell counts give weak signals in this whole blood format.

Figure 24 is a representation of data showing the reduction of monocytes in a sample. In the ELISA format of the assay, monocytes can be removed from whole blood before assay by use of anti-CD14 magnetic beads and strong magnets designed for this purpose as described above, in methods well known in the art. This method may also be used for pre-treatment of whole blood before point of care assays, but would require sample handling and processing that is preferably avoided for point-of-care testing. No
20 methods have been described for depletion of a particular white blood cell type or other cell type using methods that are compatible with inclusion in a rapid, point of care assay. The inventors have devised a method for efficient removal of monocytes in the sample pad of the rapid point of care test as described in Example 12.

In the Figure, purified monocytes and purified peripheral blood leucocytes (PBLs, depleted of monocytes) were separately labelled with infrared fluorescent dyes - monocytes with green (Mini CellVue NIR815, PTI Research, Inc), PBLs with red (Mini CellVue Burgundy, PTI Research Inc.). The images display the same sample pads viewed through different filters on the Licor Odyssey infrared fluorescent scanner: (A) = combined filters, (B) = Em 800, (C) = Em 700. The magnet obscures scanning of the sample pad where it is used. 1.5×10^4 monocytes (attached to magnetic beads) and 2×10^4 monocyte
30 depleted PBLs were combined and spotted onto anti-glycophorin A coated sample pads

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either without (left) or with the magnet (right). Sample was flushed with 60ul PBS to allow sample to flow into the absorbent pad (used here instead of an actual lysis pad and nitrocellulose test strip for illustrative purposes). Retention of >80% monocytes (green) can be seen in panel (A) and (B) in the presence of a magnet, with no green cells entering
5 the absorbent pad, whilst flow-through of monocyte-depleted PBLs into the absorbent pad is demonstrated in (A) and (C) in both the presence or absence of a magnet. (D) shows a schematic description of the test as shown in (A)-(C).

BRIEF DESCRIPTION OF THE TABLES

Table 1 provides a description of the SEQ ID NOs provided herein.

Table 2 provides an amino acid sub-classification.

5 **Table 3** provides exemplary amino acid substitutions.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the work leading up to the present invention, the inventors have developed a strategy for accurately enumerating specific cell types in cellular samples from a subject. The invention is predicated, in part, upon the recognition that prior art methods for detecting proteinaceous markers for particular cell types employ agents that bind to the extracellular portion of a cell marker. As many cell surface associated molecules are present in both cell associated and soluble forms, these agents detect soluble forms of the marker leading to false positives or an overestimation of the number of a particular cell type. In some embodiments, the method does not detect soluble CSAP lacking a cytoplasmic domain which may be present in the sample. In other embodiments, the method distinguishes between cell associated CSAP comprising an cytoplasmic domain and soluble CSAP lacking a cytoplasmic domain.

In some embodiments, the invention provides assays such as immunoassays comprising agents that distinguish between soluble and full length forms of cell surface markers. In a particularly useful embodiment of the invention, rapid point of care (RPOC) methods and kits are provided that require no sample processing, microscopy, other instrumentation or scientific expertise.

Accordingly, in one embodiment, the present invention provides a method for evaluating in a sample from a subject the level or presence of a cell surface associated protein (CSAP) comprising a cytoplasmic (cytosolic) and an extracellular (ecto) domain or cells bearing the CSAP, the method comprising (i) optionally contacting the sample with an agent capable of lysing or permeabilizing CSAP bearing cells, (ii) contacting the sample with an agent that binds to the cytoplasmic domain of the CSAP (iii) directly or indirectly evaluating the level of bound CSAP in the sample. In accordance with this aspect of the present invention, the method permits the detection or measurement of cell-bound CSAP in a sample that also comprises soluble CSAP. The level of agent-CSAP complexes can be used to qualify the presence of a specific cell bound CSAP or quantify the amount of a specific cell bound CSAP molecule in the sample. Using information concerning how many CSAP molecules are on average present on the surface of the specific cell type it is possible to estimate the number of cells bearing CSAP that are present in the sample.

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The agent that binds to the cytoplasmic domain of a CSAP or the soluble form of CSAP may be any CSAP-binding agent such as a ligand, aptamer or antibody, however specific antibody or an antigen binding fragments thereof are conveniently prepared. In particular, antibodies can be conveniently conjugated to or produced with detectable markers or bound by secondary or subsequent antibodies conjugated to detectable markers.

Immunoassays can be done in any convenient format known in the art. These include Western blots, immunohistochemical assays and ELISA assays. The use of monoclonal antibodies in an immunoassay is particularly preferred because of the ability to produce them in large quantities and the homogeneity of the product. However, as shown herein, polyclonal antibodies may also be used. The preparation of hybridoma cell lines for monoclonal antigen production is derived by fusing an immortal cell line and lymphocytes sensitized against the antigen of interest (in a non-limiting example the antibody is in the case of CD4 polypeptides, peptides 1 or 2 or homologs, derivatives, variants thereof) or can be done by techniques which are well known to those who are skilled in the art. (See, for example, Douillard and Hoffman, Basic Facts about Hybridomas, in *Compendium of Immunology* Vol. II, ed. by Schwartz, 1981; Kohler and Milstein, *Nature* 256: 495-499, 1975; *European Journal of Immunology* 6: 511-519, 1976 or more recent references Sambrook, *Molecular Cloning: A Laboratory Manual*, 3rd Edition, CSHLP, CSH, NY, 2001).

Another aspect of the present invention contemplates a method for detecting a CSAP in a biological sample from a subject, said method comprising contacting said biological sample with an antibody specific for the cytoplasmic domain of the CSAP for a time and under conditions sufficient for an antibody-CSAP complex to form, and then detecting said complex.

The presence of CSAP may be evaluated in a number of ways such as by Western blotting and ELISA procedures. A wide range of immunoassay techniques are available as can be seen by reference to U.S. Patent Nos. 4,016,043, 4,424,279 and 4,018,653. These include both single-site and two-site or "sandwich" assays of the non-competitive types, as well as in the traditional competitive binding assays.

Sandwich assays are among the most useful and commonly used assays and are favoured for use in the present invention. A number of variations of the sandwich assay

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technique exist, and all are intended to be encompassed by the present invention. Briefly, in a typical forward assay, an antibody is immobilized on a solid or semi-solid substrate and the sample to be tested brought into contact with the bound molecule. After a suitable period of incubation, for a period of time sufficient to allow formation of an antibody-antigen complex, a second antibody specific to the antigen, labelled with a reporter molecule capable of producing a detectable signal is then added and incubated, allowing time sufficient for the formation of another complex of antibody-antigen-labelled antibody. Any unreacted material is washed away, and the presence of the CSAP is determined by observation of a signal produced by the detectable marker (reporter molecule). The results may be qualitative or quantitative, by simple observation of the visible signal, or may be quantitated by comparing with a control sample containing known amounts of CSAP. Variations on the forward assay include a simultaneous assay, in which both sample and labelled antibody are added simultaneously to the bound antibody. These techniques are well known to those skilled in the art, including any minor variations as will be readily apparent. In accordance with the present invention, the sample is one which might contain a CSAP, such as a cell extract or tissue biopsy. The sample is, therefore, generally a biological sample comprising biological fluid.

In a typical forward sandwich assay, a first antibody having specificity for the cytoplasmic domain of CSAP is either covalently or passively bound to a solid or semi-solid support. The support is typically glass or a polymer, the most commonly used polymers being nitrocellulose, cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride, polypropylene or mixture or derivatives of these. The solid supports may be in the form of tubes, beads, discs or microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well-known in the art and generally consist of cross-linking covalently binding or physically adsorbing the polymer-antibody complex to the solid surface which is then washed in preparation for the test sample. An aliquot of the sample to be tested is then added to the solid phase complex and incubated for a period of time sufficient (e.g. 2-40 minutes or overnight if more convenient) and under suitable conditions (e.g. from room temperature to about 37°C including 25°C) to allow binding of any subunit present in the antibody. Following the incubation period, the antibody subunit solid phase is washed and incubated with a second antibody specific for a portion of the

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antigen. The second antibody is linked to a detectable marker which is used to indicate the binding of the second antibody to the antigen.

An alternative method involves immobilizing the target molecules in the biological sample and then exposing the immobilized target to specific antibody which may or may not be labelled with a detectable marker. Depending on the amount of target and the strength of the signal from the detectable marker, a bound target may be detectable by direct labelling with the antibody. Alternatively, a second labelled antibody, specific to the first antibody is exposed to the target-first antibody complex to form a target-first antibody-second antibody tertiary complex. The complex is detected by the signal emitted by the reporter molecule. A significant improvement of the bead-based methods involves tagging each bead with a unique identifier tag, such as an oligonucleotide or electrophoretic tag, so as to facilitate identification of the amino acid sequence of each library member. These improved bead-based methods are described in International Publication No. WO 93/06121.

In other embodiments, the method is a liquid phase method. In one example of a liquid phase immunoassay (see for example US Patent No. 6,632,603) the sample is contacted with an agent capable of binding the cytoplasmic portion of a CSAP and a detector agent comprising a visually detectable agent such as colloidal gold or silver labelled. The test sample is applied by flowing onto a defined zone of an insoluble porous support film having a pore size impassable to a complex formed between the CSAP, if present, with the binding substance and the detector substance, but passable to the binding substance and detector substance while remaining uncomplexed in the absence of the desired CSAP. If the CSAP is present in the test specimen, the detector substance binds with the CSAP and the binding substance to form a visually inspectable complex on the surface of the porous support film. After application of the test sample to the porous support, the surface of the porous support is visually inspected for colour to determine the presence and quantity or the absence of the CSAP being assayed.

In another assay, magnetic antibodies that bind to CSAP markers are used to tag CSAP and a high T_c superconducting quantum interference device is used to measure the amount of free and bound antibody and hence the presence or level of CSAP. A liposome

immunomigration, liquid-phase competition strip immunoassay is, for example, described in Glorio-Paulet *et al J Agric Food Chem* 48 (5):1678-1682, 2000.

General formats and protocols for the conduct of various formats of ELISA are disclosed in the art and are known to those of skill in the field of diagnostics. For example, reference may be made to Chapter 11 of Ausubel (Ed) *Current Protocols in Molecular Biology*, 5th Edition, John Wiley & Sons, Inc, NY, 2002.

In a further preferred embodiment, the agent that binds to the cytoplasmic domain of the CSAP serves to attach the CSAP to a solid or semi-solid support. This allows any soluble CSAP in the sample to be physically separated from the CSAP, which may then be detected with agents that bind to the extracellular domain of the CSAP.

In another embodiment, the method comprises (i) optionally contacting the sample with an agent capable of lysing or permeabilizing CSAP bearing cells, (ii) contacting the sample to be tested with an agent that binds to the extracellular domain of the CSAP such that both CSAP and soluble forms of the CSAP are bound by the agent and form complexes comprising agent-CSAP and/or agent-sCSAP (iii) optionally, if the agent is not already bound to or otherwise associated with a region of a solid or semi-solid support, contacting the sample with a solid or semi-solid support such that the agent and any agent-CSAP complex and/or agent-sCSAP complex is bound to the solid-support (iii) contacting the sample with a second agent that specifically binds to the cytoplasmic domain of the CSAP and (iv) directly or indirectly determining the presence or level of bound second agent.

In another embodiment, the present invention provides a method for determining in a sample from a subject a) the level or presence of a protein expressed on the surface of a cell (CSAP) comprising a cytoplasmic (cytosolic) and an extracellular (ecto) domain or cells bearing the CSAP and b) the level of presence of a soluble non-membrane bound extracellular form of the CSAP in the sample. In this embodiment, the sample is further contacted with an agent that binds the soluble form of CSAP. In embodiments where the level or presence of soluble CSAP is also measured, the evaluation step (iii) distinguishes between agent-CSAP complexes and agent-sCSAP complexes. In this embodiment, the relative presence or amount of cell bound and soluble CSAP in the sample from a subject may be determined.

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In some embodiments, the method comprises (i) applying a sample from a subject to a portion of a chromatographic or immunographic device bearing an antibody that specifically binds to the cytoplasmic domain of a CSAP such that substantially only cell bound CSAP comprising a cytoplasmic portion is captured onto the test portion. In some
5 embodiments, where the presence or level of soluble CSAP is required to be detected, another portion of the chromatographic device bears an antibody that binds soluble CSAP such that any soluble CSAP is captured onto further test portions of the chromatographic device. Of course, an antibody that binds to the extracellular domain of soluble CSAP will also be capable of binding to the extracellular domain CSAP. However, if all the CSAP
10 present in the sample is bound to other portions of the device *via* antibodies to the cytoplasmic domain, the anti-soluble CSAP antibody will bind only soluble forms of the CSAP.

In some embodiments, the sample to be tested comprises cells derived from the body, such as, spinal fluid, bone or tissue samples, serum, plasma, saliva, tears and milk. In
15 some embodiments, the test sample is whole blood or diluted whole blood is a derivative of whole blood. Blood may, in some embodiments, be maintained in the presence of an anticoagulant such as heparin, sodium citrate or ethylene diamine tetra acetic acid (EDTA).

In one or more of the above described methods the detection step comprises contacting the bound CSAP with a second binding agent that binds to the CSAP and which
20 comprises a detection marker or which is capable of binding to a third binding agent comprising a detection marker and detecting the detection marker. In some especial embodiments the CSAP-binding agent is an antibody or antigen binding fragment thereof. Similarly, in some embodiments the second binding agent is an antibody or antigen-binding fragment thereof which binds to CSAP including to the cytoplasmic or
25 extracellular domain of the CSAP.

As mention above, an especial embodiment of the method comprises evaluating the number of CSAP bearing cells in the sample. In some embodiments, this is achieved by comparison of the level or presence of bound CSAP or the level or presence of bound
30 detection marker with a predetermined control. In some embodiments, the predetermined control is a predetermined amount of a control polypeptide sufficient to produce, when bound to a visual detection marker, a signal providing a internal visual reference

equivalent to the signal produced by a predetermined number of CSAP cells.

The method for evaluating cells will depend upon the format of the method used and ELISA type, flow cytometry or chromatographic methods are all contemplated. In some embodiments where the method is an ELISA or chromatographic method the CSAP-
5 binding agent that binds to the cytoplasmic domain of CSAP is immobilised on a solid or semi-solid support. In other embodiments, the method is an ELISA or chromatographic method and the second binding agent that binds to the CSAP including to the cytoplasmic or extracellular domain of CSAP is immobilised on a solid or semi-solid support.

The method is suitable for use in an immunochromatographic device. In some
10 embodiments of this aspect there is provided a method for evaluating in a sample from a subject the level a cell surface associated protein (CSAP) comprising a cytoplasmic (cytosolic) and an extracellular (ecto) domain or the level of CSAP-bearing cells. In some embodiments, the method comprises: a) applying the test sample to a sample portion of an immunochromatographic device wherein the sample portion is operably connected to a
15 capture portion of the device and wherein components of the test sample flow from the sample portion to and through the capture portion which comprises an antibody or antigen-binding fragment thereof that binds to the cytoplasmic domain of CSAP such that only CSAP comprising a cytoplasmic domain and not soluble CSAP that does not comprise a cytoplasmic domain binds to the antibody or fragment thereof to form a captured CSAP; b)
20 contacting the capture portion with a second binding agent that binds to CSAP including to the cytoplasmic or extracellular domain and which comprises a detection marker or which is capable of binding to a third or subsequent binding partner comprising a detection marker. In some embodiments, the method further comprises c) optionally contacting the second binding agent with a third binding agent comprising a detection marker; evaluating
25 the presence of the detection marker. Of course, further binding partners may be employed, if required.

In an illustrative embodiment, the specification provides a method for evaluating in a blood sample from a subject the level of T-cell associated CD4 comprising a cytoplasmic (cytosolic) and an extracellular (ecto) domain or the level of CD4 T-cells, the method
30 comprising: (i) optionally contacting the sample with an agent capable of lysing or permeabilizing CD4 T-cells; (ii) contacting the sample with an antibody or antigen-binding

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fragment thereof that binds to the cytoplasmic domain of CD4; and (iii) directly or indirectly evaluating the level or presence of bound CD4 in the sample.

In some embodiments, step (iii) comprises contacting the CD4-antibody complex with a second antibody or antigen-binding fragment thereof that binds to CD4 including to the cytoplasmic or extracellular domain and which comprises a detection marker or which is capable of binding to a third binding partner comprising a detection marker, and detecting the detection marker. In some embodiments, the method comprises determining the number of CD4 bearing cells in the sample by comparison of the level or presence of bound CD4 or bound detection marker with a predetermined control. In some embodiments, the predetermined control is a predetermined amount of a control polypeptide sufficient to produce, when bound to a visual detection marker, a signal providing an internal visual reference equivalent to the signal produced by a predetermined number of CD4 cells. As above however, in some embodiments, the method is an ELISA, flow cytometry or chromatographic method.

In some embodiments, the method is an ELISA or chromatographic method and the antibody or antigen-binding fragment thereof that binds to the cytoplasmic domain of CD4 (anti-cytoCD4 antibody) is immobilised on a solid or semi-solid support.

In relation to chromatographic devices, many different formats are available. however, in one embodiment a method for evaluating in a blood sample from a subject the level of T-cell associated CD4 comprising a cytoplasmic (cytosolic) and an extracellular (ecto) domain or the level of CD4 T-cells is provided. In some embodiments, the method comprising: a) applying the test sample to a sample portion of an immunochromatographic device wherein the sample portion is operably connected to a capture portion of the device and wherein components of the test sample flow from the sample portion to and through the capture portion which comprises an antibody or antigen-binding fragment thereof that binds to the cytoplasmic domain of CD4 such that only CD4 comprising a cytoplasmic domain and not soluble CD4 that does not comprise a cytoplasmic domain binds to the antibody or fragment thereof to form a captured CD4; b) contacting the capture portion with a second binding agent that binds to CD4 including to the cytoplasmic or extracellular domain and which comprises a detection marker or which is capable of binding to a third or subsequent binding partner comprising a detection marker; c) optionally contacting the

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second binding agent with a third or subsequent binding agent comprising a detection marker; evaluating the presence of the detection marker.

For the control polypeptide, in some embodiments the control polypeptide is CD4 polypeptide comprising at least cytoplasmic domain. Regarding the number of CD4 cells, 5 in some embodiments this is one or more of less than 200, less than 250, more than 250, 250 to 350, 350 to 500, 250 to 500 and more than 500 cells/ μ l of blood. In other embodiments, the number of CD4 T-cells is one or more of less than 200, less than 500, 500 to 1,000, 1,000 to 2,000, and more than 2,000 cells/ μ l of blood.

Various methods are available for depleting monocytes or red blood cells from 10 blood cells, if required. In some embodiments, monocytes in the sample are depleted by contacting the sample with anti-CD14 antibodies bound to a solid or semi-solid support. In another embodiment, red blood cells are depleted in the sample by contacting the sample with anti-glycophorin antibodies bound to a solid or semi-solid support.

In another aspect, the present invention provides a device or kit suitable for the 15 practice of the subject methods.

In some embodiments, a chromatographic device is provided comprising material which has a pore size which allows or facilitates capillary flow of the components of the method. In some embodiments, the device comprises portions comprising material of different pore size, or non-porous material, the material being contiguous with the first 20 material and designed to receive a sample or receive or store components of the method. In some embodiments, the portions of the chromatographic device are separate, contiguous or overlapping or designed to come together in use.

In some embodiments, the sample pad is chromatographically connected to a test portion of the device, the test portion comprising an antibody or an antibody binding 25 fragment thereof. In an illustrative embodiment, the subject is a mammal and the test portion comprises an antibody which, under the appropriate conditions, recognizes and binds the cytoplasmic domain of a CSAP polypeptide.

In some embodiments, the sample pad is chromatographically connected to a test portion of the device, the test portion comprising an antibody or an antibody binding 30 fragment thereof. In an illustrative embodiment, the subject is a mammal and the test

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portion comprises an antibody which, under the appropriate conditions, recognizes and binds the cytoplasmic domain of a protein comprising Ig or Ig-like domains.

In other embodiments, the CSAP is a receptor such as without limitation a cytokine, hormone or neurotransmitter receptors, a tethered ligand, G-protein coupled
5 receptors, receptor protein tyrosine kinases, receptor protein tyrosine phosphatases, protein-serine/threonine kinases and receptor guanylyl cyclases.

In some embodiments, when chromatographically active portions of the sample to be tested move from the sample pad towards and through the test portion, CSAP comprising a cytoplasmic domain is captured onto test or control portions of the device
10 and the remainder of the sample flowing from the sample pad is uncaptured. This arrangement ensures that essentially only the non-soluble CSAP to be detected in this embodiment is allowed to interact with detection antibodies, which may therefore be directed to soluble or non-soluble CSAP. In a preferred embodiment, the CSAP is detected with an antibody that binds to the extracellular domain of CSAP to avoid binding to sites
15 used by the antibodies that bind to the cytoplasmic domain of the CSAP. In some embodiments, the uncaptured components of the test sample are collected chromatographically into an absorbent pad, which is positioned in any orientation with respect to the test portion. Components of the subject sample, such as red blood cells or particular white blood cells may be retained in the sample pad, for example, by selecting a
20 pad of suitable mesh or pore size and/or by the inclusion of specific reagents such as antibodies or lectins to bind and retain these components. For example monocytes may be retained using anti-CD14 antibodies. Anti-glycophorin antibodies may be used to retain/remove red blood cells.

In some embodiments, once the test portion of the immunochromatographic device
25 has been exposed to CSAP in the subject sample, the method proceeds by allowing contact between a detection marker and the test portion. In some embodiments, the detection marker is stored in a separate detection marker pad.

In some embodiments, the detection marker comprises a visually detectable reporter molecule and a positive result may be essentially immediately observed in the test
30 and/or control portions of the immunochromatographic device. In other embodiments, the detection marker may be detected using further detection protocols and devices such as

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will be well known to those of ordinary skill in the art. For example, in addition to colloidal gold, as exemplified herein, other colloidal metal or metal oxide particles or colloidal non-metal particles or dyes or coloured latex are conveniently used.

In an illustrative embodiment, the specification describes kits for detecting or
5 monitoring the number of cells in sample from a subject, wherein the cell is characterised by comprising a cell surface associated protein (CSAP) comprising a cytoplasmic (cytosolic) and an extracellular (ecto) domain. In some embodiments, the kit comprises:
10 (i) a chromatographic device comprising a porous membrane operably connected to a sample portion, a test (capture) portion, a conjugate portion, a sucker portion, optionally a control portion and optionally a lysis portion; (ii) a CSAP-binding agent such as an antibody or antigen-binding fragment thereof that binds to the cytoplasmic domain of CSAP, wherein the agent or antibody is either immobilised (bound) to one or more test
15 portions and/or contained within a conjugate portion; and (iii) instructions for using the device to detect or monitor the level or presence of CSAP-bearing cells in the sample or subject.

In a further non-limiting illustrative embodiment, the kit comprises: (i) a chromatographic device comprising a porous membrane operably connected to a sample portion, a test (capture) portion, a conjugate portion, a sucker portion, optionally a control portion and optionally a lysis portion; (ii) a CD4-binding agent such as an antibody or
20 antigen-binding fragment thereof that binds to the cytoplasmic domain of CD4, wherein the agent or antibody is either immobilised (bound) to one or more test portions and/or contained within a conjugate portion; and (iii) instructions for using the device to detect or monitor the level or presence of CD4 cells in the sample or subject. Such kits may of course be presented any suitable format such as reverse or lateral flow
25 immunochromatography formats. In preferred embodiments the conjugate portion comprises a visual detection marker. In other embodiments, the control portion comprises a pre-determined amount of a control polypeptide sufficient to produce, when bound to a visual detection marker, a signal providing an internal reference equivalent to the signal produced by a pre-determined number of cells. In some embodiments, the control portion
30 comprises a pre-determined amount of a control CD4 polypeptide comprising at least a cytoplasmic domain and sufficient to produce, when bound to a visual detection marker, a

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signal providing a visual standard of a pre-determined number of CD4 cells. In some embodiments, the CD4 polypeptide is recombinant CD4 lacking a transmembrane domain.

For CD4 T cell evaluations, in some embodiments, the kits advantageously provide for a pre-determined number of one or more of: less than 200, less than 250, more than 5 250, 250 to 350, 350 to 500, 250 to 500 and more than 500 cells/ μ l of blood. Alternatively, the pre-determined number is one or more of: less than 200, less than 500, 500 to 1,000, 1,000 to 2,000, and more than 2,000 cells/ μ l of blood. Red blood cells and/or monocytes are deleted in some embodiments and kits employ reagents to effect red blood cell and/or monocyte depletion. In some embodiments, the sample pad comprises a red 10 blood cell capture agent such as glycophorin A and/or anti-CD14 magnetic beads other monocyte specific reagents.

In accordance with the present invention it has been determined that an inexpensive or disposable magnetic is effective in the present immunochromatographic strips to effect cell separation. In some embodiments, a magnet is used having a field strength extending 15 less than about 5mm through a sample. In some embodiments, a magnet is included in the kit. In some embodiments, the magnetic material is attached or attachable to the sample pad or to a portion of the immunochromatographic device capable of coming into contact with the sample pad.

In another embodiment, the number of CSAP-bearing cell is evaluated in a kit by: 20 (i) applying the test sample from the subject to the sample portion under conditions in which the CSAP bearing cells will flow over the capture portion towards the sucker portion; (ii) directly or indirectly detecting the presence of captured CSAP in the capture portion using a visual detection marker; and (iii) comparing the intensity of the signal from the detection marker in (ii) with the intensity of the signal from the control portion comprising 25 a control polypeptide providing an internal reference equivalent to the signal produced by a pre-determined number of CSAP bearing cells.

In some embodiments, the test sample is contacted with a lysis buffer or passes through a lysis portion capable of or for lysing or permeabilising CSAP bearing cells.

Recombinant CD4 lacking a transmembrane domain for use or suitable for use as a 30 control in methods or kits to determine CD4 T-cell numbers. Accordingly, the present invention contemplates the use of recombinant CD4 lacking a transmembrane domain in

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the manufacture of a solid or semi-solid support or a kit for quantifying the number of CD4 T-cells in a sample from a subject.

In some embodiments, the subject methods and/or kits are for use in evaluating AIDS or other immunodeficiency disease patients.

5 In some embodiments, the subject is a human. The present invention extends, however, to primates, livestock animals, laboratory test animals, companion animals and avian species as well as non-mammalian animals such as reptiles. The method has applications, therefore in human, livestock, veterinary and wild-life therapy and diagnosis.

The present invention further provides a kit in compartmental form comprising
10 reagents and a chromatographic device required to perform the subject method. Generally the kit further comprises a set of instructions.

Accordingly, in another aspect, the present invention provides a kit for evaluating in a sample from a subject the level or presence of a cell surface associated protein (CSAP) comprising a cytoplasmic (cytosolic) and an extracellular (ecto) domain or the level or
15 presence of cells bearing the CSAP, the kit in compartmental form comprising an immunochromatographic device comprising portions for receiving the sample, portions for receiving or comprising a detection marker, together with test and control portions of the device comprising an agent such as an antibody or antigen binding fragment thereof which binds to the cytoplasmic domain of a protein expressed on the surface of cell (CSAP). In
20 some embodiments, the portions of the device are separate, contiguous or overlapping. In some embodiments, the kit uses reverse flow immunochromatography. In other embodiments, the kit uses lateral flow immunochromatography. In some embodiments, the CSAP is a marker for a particular cell type, such as an Ig or Ig-like domain comprising protein. In one embodiment, the cell marker is selected from the group comprising cluster
25 of differentiation (CD) antigens. In an exemplified embodiment the CSAP is CD4. In this aspect of the invention, the level of CD4 or CD4 bearing cells is indicative of a subject's need for antiretroviral therapy. Specifically, in the case of subjects with HIV infections a level of less than about 200 cells/ μ l of blood or of less than about 250 cells/ μ l of blood is indicative that anti-retroviral therapy is required. In accordance with this aspect of the
30 invention, an immunochromatographic device is provided that allows a quantitative estimate of CD4 T-cells in clinically useful ranges such as less than 250, more than 250,

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250 to 350, 350 to 500, 250 to 500 and more than 500 cells/ μ l of blood. In some embodiments these ranges or amended versions thereof are useful monitoring HIV patients. In another embodiment, the device allows a quantitative estimate of CD4 T-cells of less than 500, 500 to 1,000, 1,000 to 2,000, and more than 2,000 cells/ μ l of blood suitable for monitoring pediatric HIV patients.

In another embodiment the CSAP is a receptor such as a receptor for a cytokine, hormone or neurotransmitter. In one preferred embodiment, the receptor is a TNF receptor.

Reference herein to the term "hematopoietic cell" encompasses undifferentiated hematopoietic stem cell (HSC) and any one or more of the blood cell types which arise from HSC. The term refers to multipotent cells as well as the various different forms of myeloid- or lymphoid-restricted cells that ultimately give rise to fully differentiated mature blood cells. In adults, HSC reside in the bone marrow, peripheral blood, lung, liver, spleen and other organs. HSC are the first in a hierarchy of progenitor cells. They are capable of long-term self renewal (long term (LT)-HSCs). LT-HSCs differentiate into short-term multipotent HSCs, (ST-HSCs) that retain the ability to produce all blood types but only proliferate for a relatively short time. Next, lymphoid progenitors arise that ultimately produce immune cells, and myeloid progenitors arise that ultimately produce mainly red blood cells and platelets and some innate immune cells. These progenitor cells have various abilities to proliferate and differentiate and from these cells ultimately arise terminally differentiated cells. Accordingly, reference to HSC and hematopoietic progenitors include all the above mentioned progenitor cells and reference to hematopoietic or blood cells include any of their terminally differentiated descendants. These include without limitation: HSC, hematopoietic stem cell; CLP, common lymphoid precursor; CMP, common myeloid precursor; GMP, granulocyte-macrophage precursor; MEP, megakaryocyte-erythroid precursor; CFU-GM, colony forming unit-granulocytic/macrophage; CFU-G, colony forming unit-granulocytic; CFU-M, colony forming unit-macrophage; CFU-Mk, colony forming unit-megakaryocytic; BFU-e, Burst-forming unit erythroid; and CFU-E, colony forming unit-erythroid cells and their progeny.

Reference herein to a "CD" protein or polypeptide includes one or more CD antigens selected from the group comprising CD1a,b,c,d, CD2, CD3, CD4, CD5, CD6, CD7, CD8, CD9, CD10, CD11a, CD11b, CD11c, CD11d, CDw12, CD13, CD14, CD15,

CD15s, CD15u, CD16, CDw17, CD18, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD26, CD28, CD29, CD30, CD31, CD32, CD33, CD34, CD35, CD36, CD37, CD38, CD39, CD40, CD41, CD42a,b,c,d, CD43, CD44, CD45, CD45RO, CD45RA, CD45RB, CD46, CD47, CD48, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD50, CD51, 5 CD52, CD53, CD54, CD55, CD56, CD57, CD58, CD59, CD60a, CD60b, CD60c, CD61, CD62E, CD62L, CD62P, CD63, CD64, CD65, CD66a, CD66b, CD66c, CD66d, CD66e, CD66f, CD68, CD69, CD70, CD71, CD72, CD73, CD74, CD75, CD75s, CD77, CD79 α , β , CD80, CD81, CD82, CD83, CDw84, CD85, CD86, CD87, CD88, CD89, CD90, CD91, CD92, CD93, CD94, CD95, CD96, CD97, CD98, CD97, CD100, CD101, CD102, CD103, 10 CD104, CD105, CD106, CD107a, CD107b, CD108, CD109, CD110, CD111, CD112, CD114, CD115, CD116, CD117, CD118, CD119, CD120a, CD120b, CD121a, CDw121b, CD122, CD123, CD124, CD125, CD126, CD127, CDw128, CD129, CD130, CDw131, CD132, CD133, CD134, CD135, CDw136, CDw137, CD138, CD139, CD140a,b, CD141, CD142, CD143, CD144, CD145, CD146, CD147, CD148, CD150, CD151, CD152, 15 CD153, CD154, CD155, CD156a, CD156b, CD157, CD158, CD158a, CD158b, CD159a, CD160, CD161, CD162, CD162R, CD163, CD164, CD165, CD166, CD167a, CD168, CD169, CD170, CD171, CD172a, CD173, CD174, CD175, CD175s, CD176, CD177, CD178, CD179a, CD179b, CD180, CD183, CD184, CD195, CDw197, CD200, CD201, CD202b, CD203c, CD204, CD205, CD206, CD207, CD208, CD209, CDw210, CD212, 20 CD213a1, CD213a2, CDw217, CD220, CD221, CD222, CD223, CD224, CD225, CD226, CD227, CD228, CD229, CD230, CD231, CD232, CD233, CD234, CD235a, CD235b, CD236, CD236R, CD238, CD239, CD240CE, CD240D, CD241, CD242, CD243, CD244, CD245, CD246 and CD247. Such molecules and the cells upon which they are found are described in Janeway *et al.*, *Appendix II. CD Antigens*, Immunology, Garland Publishing, 25 New York, 5th edition, 2001, that is incorporated herein in its entirety by reference.

Reference herein to a "chromatographic device" includes a device of any solid, semi-solid, matrix or gel material which is known in the art for facilitating or supporting chromatographic flow or separation. As used herein, the components of the sample including antibodies, and the detection marker-antigen complex and components thereof 30 are moved relative to each other by capillary flow or diffusion through chromatographic material. The material may be functionalized or coated to permit for example cross-

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linking of reagents. Methods for immobilizing antibodies to solid supports are well known in the art and are described for example in US Patent No. 4,168,146, Cautrecases *J. Biol. Chem.* 245: 3059, 1970. Materials contemplated for use herein include inorganic materials such as silica, glass, polymeric material such as cellulose, starch, dextrose, agarose, special
5 fibrous paper (filter/chromatography paper) nitrocellulose, cellulose acetate, PVC, polyacrylamide, polysaccharide, polyacrylate, polyethylsulphonate, polyethylene and the like.

"Chromatographically active" components are simply those capable of flow through all or part of the immunochromatographic device.

10 Reference herein to "derived from" means that the sample is obtained from a particular source but not necessarily directly from that source.

"Antibodies" include immunoglobulin gene products that interact with an antigen i.e., an antigen-binding agent, fragments thereof and non-immunoglobulin gene derived proteinaceous molecules that are capable of serving as an antigen binding agent. The term
15 antibody therefore, includes polyclonal and monoclonal antibodies and parts thereof including Fab portions and antigen-binding determinants. Immunoglobulin genes include κ , λ , α , γ , (IgG₁, IgG₂, IgG₃, IgG₄), δ , ϵ , and μ constant regions and multiple variable region genes.

Typically an immunoglobulin comprises two identical pairs of immunoglobulin
20 chains each pair comprising light chain (V_L) and heavy chain (V_H) variable portions which have antigen-binding regions. Each pair also comprises a constant region which provides generic antibody functions. Other forms of immunoglobulin are F_v, scF_v, Fab, Fab¹, and (Fab¹)₂ forms. Reference a "class" of an antibody includes reference to any class such as IgM, IgG, IgA etc.

25 The ability to rapidly and accurately determine CD4⁺ cell levels is important for assessing the ability of a subject to respond to antiviral agents or to mount an immune response.

As used herein reference to "detecting" "evaluating" "enumerating" is meant in its broadest sense to include assays which qualitatively or quantitatively or semi-
30 quantitatively test for the presence or level of a CSAP in the presence of sCSAP and hence the number of CSAP-positive cells, or, assays which qualitatively or quantitatively test for

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the presence or level of CSAP and sCSAP using reagents capable of distinguishing between the two forms.

Chromatographic assays are particularly sophisticated and a large number of different formats are available which are tailored to the particular reagents and instruments and the outcomes required in any particular investigation. "Rapid" assays, using chromatographic principles, are tailored for accuracy, speed and ease of use. Immunoassay or enzyme-based chromatographic assays are particularly preferred and these are described in Wild D "The Immunoassay Handbook", Nature Publishing Group, 2001 and by reference to U.S. Patent Nos. 4,016,043; 4,590,159; 5,266,497; 4,962,023; 5,714,389; 5,877,028, 5,922,537, 6,168,956 and 6,548,309, 6,180,417, and 5,266,497 incorporated herein and information disclosed by references cited therein. Various modifications of immunochromatographic methods are described in Published US Patent Application Nos. 20010006821, 20040087036 and 20040214347 which are incorporated herein in their entirety. Immunogold filtration methods for multiple analyte analyses are described in Published US Patent Application No. 20030165970 incorporated herein.

By "detection marker" or "reporter molecule" is meant a molecule or particle which, by its chemical nature, provides an analytically identifiable signal which allows the detection of antigen bound antibody. As will be well recognised, a wide variety of different reporter systems are available and those allowing rapid visual detection are clearly the most useful in the context of point of care diagnostics.

In some embodiments, the detection marker is a colloidal particle or microparticle. Colloidal metal and metalloid particles include those comprising gold, silver, platinum, iron, copper, selenium; metal complexes such as cyclopentadienylmanganese(I) tricarbonyl, gold cluster; and microparticles such as latex and dyed latex particles.

The present invention extends to qualitative or quantitative detection using any of the commonly used reporter molecules in this type of immunoassay such as enzymes, fluorophores or radionuclide containing molecules and chemiluminescent molecules. In the case of an enzyme immunoassay, an enzyme is conjugated to a second antibody generally by means of glutaraldehyde or periodate. Commonly used enzymes include horseradish peroxidase, glucose oxidase, beta galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen

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for the production, upon hydrolysis by the corresponding enzyme, of a detectable colour change. Examples of suitable enzymes include alkaline phosphatase and peroxidase. It is also possible to employ fluorogenic substrates which yield a fluorescent product rather than the chromogenic substrates listed above. In all cases, the enzyme labelled antibody is added to the first antibody antigen complex, allowed to bind, and the excess reagent is washed away. A solution containing the appropriate substrate is then added to the complex of antibody-antigen antibody. The substrate will react with the enzyme linked to the second antibody, giving a qualitative visual signal, which may be further quantified, usually spectrophotometrically, to give an indication of the amount of antigen which is present in the sample. Alternatively, fluorescent compounds, such as fluorescein and rhodamine are chemically coupled to antibodies without altering their binding capacity. When activated by a illumination with light of a particular wave length, the fluorochrome labelled antibody absorbs the light energy inducing a state of excitability in the molecule followed by emission of the light at a characteristic wavelength visually detectable with a microscope.

Reference to "antibodies" includes humanised, recombinant, synthetic, hybrid and single chain antibodies. Antibodies may be conveniently prepared and used as described, for example, in Harlow and Lane, "*Antibodies: A Laboratory Manual*" (Cold Spring Harbor Laboratory, 1988). Monoclonal antibodies are conveniently prepared in pure form and in large quantities. The preparation of hybridoma cell lines for monoclonal antibody production by fusing sensitized lymphocytes with an immortal cell line and selecting specific antibody producers is routine in that art as described in Harlow and Lane (*supra*); and Kohler and Milstein, *European Journal of Immunology*, 6:511-519, 1976.

Methods for the construction of bacteriophage antibody display libraries and lambda phage expression libraries are well known in the art (Kang *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 88:4363, 1991; Clackson *et al.*, *Nature*, 352:624, 1991; Lowman *et al.*, *Biochemistry*, 30:10832, 1991; Burton *et al.*, *Proc. Natl. Acad. Sci U.S.A.*, 88:10134, 1991; Hoogenboom *et al.*, *Nucleic Acids Res.*, 19:4133, 1991, incorporated herein by reference in their entirety). One particularly advantageous approach has been the use of scFv phage-libraries (Huston *et al.*, *Proc. Natl. Acad. Sci U.S.A.*, 85:5879-5883, 1988; Chaudhary *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 87:1066-1070, 1990; Clackson *et al.*, 1991, (*supra*)).

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Various embodiments of scFv libraries displayed on bacteriophage coat proteins have been described. Further phage display approaches are also known, for example as described in International Publication Nos. WO 96/06213 and WO 92/01047 (Medical Research Council *et al.*) and International Publication No. WO 97/08320 (Morphosys) which are
5 incorporated herein by reference.

Antibody single chain Fv fragments may be cloned and expressed on the surface of, for example, yeast. High affinity scFvs are selected by screening and sorting. High affinity antibodies are developed using chain shuffling strategies, that is by sequentially replacing the heavy and light chain variable (v) region genes with repertoires of v-genes
10 from unimmunized donors. Antibodies comprising csFvs from any species may be employed.

Of particular use are display systems, which enable a nucleic acid to be linked to the polypeptide it expresses. Selection protocols for isolating desired members of large libraries are known in the art, as typified by phage display techniques. Such systems, in
15 which diverse peptide sequences are displayed on the surface of filamentous bacteriophage, are useful for creating libraries of antibody fragments (and the nucleotide sequences that encode them) for the *in vitro* selection and amplification of specific antibody fragments that bind a target antigen. The nucleotide sequences encoding the V_H and V_L regions are linked to gene fragments which encode leader signals that direct them
20 to the periplasmic space of *E. coli* and the resultant antibody fragments are displayed on the surface of the bacteriophage, typically as fusions to bacteriophage coat proteins (e.g., pIII or pVIII). Alternatively, antibody fragments are displayed externally on lambda phage capsids (phage bodies). An advantage of phage-based display systems is that selected library members can be amplified simply by growing the phage containing the selected
25 library member in bacterial cells. Furthermore, since the nucleotide sequence that encode the polypeptide library member is contained on a phage or phagemid vector, sequencing, expression and subsequent genetic manipulation is relatively straightforward.

The present invention is further described by the following non-limiting Examples.

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EXAMPLE 1***Polyclonal and monoclonal antibodies to the cytoplasmic or extracellular domain of CD4***

Anti-CD4 polyclonal antibodies were generated in sheep against two human CD4
5 ectodomain subsequences P1 and P2 (SEQ ID NO: 1 and SEQ ID NO: 2). The peptide
sequences and the position of the peptide in the cytoplasmic CD4 sequence are set out in
Figure 2. Two sheep, G-6 and G-7, were injected with P1 and P2 using standard
procedures. The specificity and titre of polyclonal antibodies to CD4_{cyto} were determined
by ELISA using immobilised biotinylated peptides and detected using Streptavidin
10 conjugated to horseradish peroxidase. The results are represented graphically in Figure
3(A) sheep G-6 and (B) sheep G-7. Sera from either sheep recognised peptide 1 and
peptide 2 equally well (see Figure 4). Monoclonal antibodies were also generated to
peptide 2 and tested by ELISA against Peptide 2 of CD4_{cyto} (see Figure 5).

The reactivity of monoclonal antibodies 1D2 and 4B4 were tested in
15 immunofluorescence assays on HeLa cells and JC53 T-cells. Both monoclonal antibodies
(at 10 micrograms per ml) showed no reactivity against HeLa cells. However, the
antibodies recognised intracellular components on fixed T-cells. SIM2 monoclonal
antibody recognising the ectodomain of CD4 did not recognise HeLa cells but showed
binding to the surface of JC53 cells at 10 micrograms per ml. As expected, monoclonal
20 antibodies 1D2 and 4B4 failed to bind to fresh CD4 positive T-cells (Figure 6). In
contrast, permeabilised cells were detected by both monoclonal antibody 1D2 and 4B4
(See Figure 7C and D).

Antibodies or antigen binding fragments may be generated against CD4 molecules
from different species. In an illustrative example the sequences of cytoplasmic domains
25 from mouse, rabbit, chicken, sheep are provided below.

human CD4: 421rcrhrrrrqae rmsqikrlls ekktcqcphr fqkctcspi458
 mouse CD4: 420rcrh**qqr**qaa rmsqikrlls ekktcqcphr **m**qk**shnli**457
 5 rabbit CD4: 422**k**crhrr**h**gaq rmsqik**k**lls ekktcqcphr **l**qkt**ynll**459
 chicken CD4: 455r**wqr**rr**kr**ar r**ma**q**ak**q**yl**l ekktcqc**qrr** **my**k487
 10 sheep CD4: 418**k**c**w**hrrrrqae rmsqikrlls ekktcqcphr **l**qkt**h**s**lt**455

Boxed residues highlight the differences between human CD4 and the species CD4.

- key: human & mouse (are ~79% conserved sequences 30/38)
- 15 human & rabbit (are ~76% conserved sequences 29/38)
- human & chicken (are ~55% conserved sequences 18/33)
- human & sheep (are ~84% conserved sequences 32/38)

NB.

- 20 The sequence 'ekktcqc' is the putative p56^{lck} binding site, conserved in all species.

EXAMPLE 2

CD4 ELISA

A CD4 capture ELISA was developed to allow quantification of cell associated
 25 CD4 without interference from soluble CD4, lacking a cytoplasmic domain. Antibodies
 directed against the cytoplasmic domain of CD4 are used as "capture" antibodies in
 combination with antibodies directed against the extracellular domain of CD4 as
 "detection" antibodies. Only cell-associated CD4 is measured while soluble CD4 lacking
 the cytoplasmic domain or the epitope recognised by anti-CD4 cyto antibodies is excluded.
 30 The CD4 capture ELISA shows close correlation with flow cytometry and has been
 adapted to provide a rapid point of care diagnostic test as shown herein.

Figure 9 shows the results of an ELISA conducted to measure the reactivity of
 monoclonal antibodies directed to the cytoplasmic domain of CD4 including a
 commercially available anti-CD4 monoclonal antibody (Chemicon). Antibodies were

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generated against Peptide 1 (SEQ ID NO: 1) and Peptide 2 (SEQ ID NO: 2) as shown in Figure 2.

Figure 10 shows the results of a capture ELISA conducted to measure the specificity of monoclonal antibody ID2 directed to the cytoplasmic domain of CD4 and ability to capture CD4 from JC53 cell lysates and detection with a monoclonal antibody directed to the ecto domain of CD4 (RPAT4).

Figure 11 shows the results of a capture ELISA conducted to measure the specificity of monoclonal antibody ID2 directed to the cytoplasmic domain of CD4 and ability to capture CD4 from JC53 cell lysates and detection with a polyclonal antibody directed to the ecto domain of CD4.

Figure 12 shows the results of capture ELISAs of cellular CD4 from donor peripheral blood mononuclear cell (PBMC). A) shows a titration of PBMC lysate captured by monoclonal antibody 4B4 from Donors 1 to 6. B) shows the capture of cellular CD4-enriched lymphocytes.

In an illustrative embodiment, the ELISA protocol a standard amount of CD4 comprising cytoplasmic and extracellular domains is used as a control diluted in PBS+0.5%Tween20. Whole blood or diluted blood is added to an equal volume of lysis buffer comprising 10% Triton-X100 lysis buffer and dispensed into designated wells of a standard ELISA plate coated with anti-CD4 cyto antibody. The ELISA plate is sealed and incubated at room temperature (18 to 23°C) for 60 minutes. Biotinylated anti-CD4 monoclonal antibody directed to the ectodomain of CD4 is diluted in 0.05% Tween20 to give a final concentration of 0.25 micrograms *per* ml. ELISA plates are washed with standard ELISA wash buffer and drained and 100 microlitres of diluted anti-CD4 biotinylated monoclonal antibody is added to each well. The plates are sealed and incubated at room temperature for 60 minutes. Thereafter, the plates are washed (times 6) using ELISA wash buffer and drained. Streptavidin-horseradish peroxidase conjugate is diluted in 1:1000 in PBS 0.05% Tween20 and 100 microlitre added to each well. ELISA plates are sealed and incubated at room temperature for 60 minutes. Thereafter the plates are washed and drained as before prior to addition of enzyme chromogene substrate tetramethylbenzidine (TMB) diluted in substrate buffer according to the manufacturer's instructions. The enzyme reaction is allowed to proceed for approximately 15 minutes in

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the dark at room temperature and at the end of the substrate incubation period 100 microlitres of stock solution is added to each well prior to reading plates for optical density using a dual λ ELISA reader with a 450 millimetre filter and a 620 millimetre filter as a reference. The mean standard deviation and %CV is calculated and a mean OD readings
5 generated using a scatter plot.

EXAMPLE 3

Capture ELISA Assays show correlation between OD and number of CD4⁺ T-cells

ELISAs of cellular CD4 from donor peripheral blood mononuclear cell (PBMC)
10 were conducted and the results show in Figures 13 and Figure 14. The results from three donors are shown on Figure 13 and a correlation between OD450-620 and the number of CD4⁺ T-cells *per* microlitre. The results from clinical samples from 5 separate HIV-infected patients are shown in Figure 14 and a positive correlation between OD450-620 and the number of CD4⁺ T-cells *per* microlitre of whole blood is demonstrated.

15

EXAMPLE 4

Immunochromatographic devices employing antibodies to the cytoplasmic domain of a CSAP in accordance with the present invention

Immunochromatography or immunographic devices provide a robust technology
20 that is used in a wide variety of rapid, point of care diagnostic tests manufactured by many different companies throughout the world. This provides redundancy in manufacturing capability and ready acceptance of the technology in target countries. Immunochromatographic devices have been used having as a test platform for measurement of cell-associated CD4 as a correlate of CD4 T-cell numbers. Soluble CD4
25 (comprising an extracellular domain but no cytoplasmic domain (present in blood)) is excluded in these assays. In addition, in some embodiments, CD4 expressed on monocytes is excluded in subject assays. In some embodiments, an immunochromatographic device provides a quantitative estimate of CD4 T-cell numbers, (less than 250, more than 250, 250 to 350, 350 to 500, 250 to 500 and more than 500 cells per microlitre) by visual
30 comparison with standards in each test strip. This fulfils the current criteria for access of adult patients to anti-retroviral drugs. A further test, having appropriate means for

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detecting appropriate cells numbers, is made using the same methods for monitoring paediatric HIV patients who have, on average, higher levels of CD4 T-cell counts (i.e., less than 500, 500 to 1000 and 1000 to 2000 cells *per* microlitre).

In Figure 15 an immunochromatographic device for detection of full length cell-associated CD4 and estimation of CD4⁺ cell numbers thereby is schematically described. A porous membrane (such as nitrocellulose) is prepared by striping a test line consisting of monoclonal antibody to the cytoplasmic domain of human CD4, a control line consisting of anti-mouse IgG (or other controls), and an inert limit line to provide a guide during assay performance. The nitrocellulose is assembled together with a “sample pad” (sample portion) at one end, consisting of porous material to which has been applied a defined quantity of blood and to which reagents may have been added to trap red blood cells or monocytes and other extraneous material in the sample, a second sample pad (lysis portion) comprising lysis buffer, and a “conjugate pad” (conjugate portion) at the other end, consisting of porous material, to which is applied the conjugate (detection marker) consisting of a detection reagent (such as colloidal gold) conjugated to anti-CD4 antibody” and an absorbent material (sucker portion).

In some embodiments, the lysis step is optional in certain circumstances such as when, for example, an antibody or other agent capable is employed that is capable of penetrating a cell or where a subject is ill and the majority of a particular cell type are, in any event, permeable to passage of anti-CSAP agents.

EXAMPLE 5

Cloning and expression of full-length CD4 and recombinant soluble full-length CD4 lacking a transmembrane domain(TM^{CD4}).

The ELISA and rapid point of care tests for CD4 are required to give a quantitative or semi-quantitative measure of the total amount of the cell associated and/or soluble/extracellular CD4 present in a sample, providing an estimate of the number of CD4 T-cells. For this purpose, it is useful to have a defined source of CD4 that can be used in quality control and as an assay standard. Human T-cells or cells transformed with full-length human CD4 can be used as a source of CD4, but may give inconsistent yields and

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require extensive purification of the membrane-bound CD4 molecule. The inventors reasoned that a recombinant form of CD4 that was released from cells as a soluble form, while retaining the cytoplasmic domain that is required for reactivity in the CD4 capture ELISA, would be more suitable for this purpose. It is known in the art that deletion of the transmembrane domain of a protein can result in secretion of the fused ectodomain-cytoplasmic domain protein. A version of CD4 with deletion of the transmembrane domain, designated TM^ΔCD4, was therefore produced. The structure of one embodiment of the protein is shown schematically in Figure 1.

The CD4 sequence was obtained from the T4pmV7 plasmid obtained from NIH AIDS research and reference program Cat No 158. In order to make the soluble TM^ΔCD4 form of CD4 the primers were designed so they produced two individual PCR products. The first product used primers RL1 and RL3 resulting in a 1.2 kb band which has an EcoR1 restriction site at the 5' end of the extracellular domain and some overlapping sequence corresponding to the cytoplasmic tail at the 3' end. The second PCR product used primers RL2 and RL4 resulting in a 120 bp band that has some overlapping extracellular domain sequence at the 5' end of the cytoplasmic tail and a Xba1 restriction site at the 3' end. To make the final PCR product the 2 PCR products were then used as the template for a third PCR reaction and primers RL1 and RL2 are used. A full length CD4 construct was generated using primers RL1 and RL2 with plasmid T4pmV7 in a standard PCR reaction.

Primers:

RL1 5' CGG **GAA TTC** ACA ATG AAC CGG GGA GTC CC (sense) **SEQ ID NO:3**
 RL2 5' GGC **TCT AGA** TCA AAT GGG GCT ACA TGT CTT C (antisense) **SEQ ID NO:4**
 RL3 5' G CCT TCG GTG CCG GCA CCT ***CTG CAC CGG GGT GGA CC*** (antisense) **SEQ ID NO:5**
 RL4 5' ***GG TCC ACC CCG GTG CAG*** AGG TGC CGG CAC CGA AGG C (sense) **SEQ ID NO:6**

The restriction site EcoR1 in primer RL1 and the restriction site Xba1 in primer RL2 are highlighted in bold and underlined. The extracellular region in both the primers RL3 and RL4 are highlighted in bold and italics and the cytoplasmic tail is normal text.

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Both the full length CD4 construct (FLCD4) and the soluble CD4 construct with a cytoplasmic domain without the TM region (CD4TM) were cloned into the EcoR1 and Xba1 restriction sites of pcDNA4/HisMax version C (Invitrogen).

Clones were identified by restriction digests and sequencing. The full nucleotide and amino acid sequence of the insert and encoded protein for TM^{CD4} is shown in Figure 16 (SEQ ID NO: 7) and Figure 17 (SEQ ID NO:8) respectively. Variants of the recited sequences are contemplated, such as, for example those derived from different naturally occurring sequences or from modification of one or up to 20 nucleotide or amino acid modifications. Modifications that result in conservative amino acid changes are preferred in some embodiments and these are as set out in Tables 2 and 3.

EXAMPLE 6

Detection of TM^{CD4} in CD4 Capture ELISA and Western immunoblotting

Recombinant TM^{CD4} was detected by ELISA and by Western immunoblotting (see Figure 18). Full-length CD4 or TM^{CD4} were expressed in 293 T-cells by transfection of the respective pcDNA4/HisMax constructs, or mock-transfected, and cell lysates and cell supernatants were tested by CD4 ELISA, using the method described. The results (see Figure 18A) show that full-length CD4 is efficiently expressed in cells but is not released into the supernatant, whereas TM^{CD4} is efficiently expressed in cells and most of the recombinant protein is released into the supernatant, allowing easy purification if required. TM^{CD4} was detected in culture supernatant by Western immunoblotting with custom MAb 4B4 (3 µg/ml) against the cytoplasmic domain of CD4 (see Figure 18B).

EXAMPLE 7

TM^{CD4} as control reagent in ELISA and rapid, point of care assays

Supernatant from HEK293 cells stably transfected with the TM^{CD4} plasmid were serially diluted (starting at 1:5 dilution) for assay by ELISA (A) (see Figure 19), showing a linear response in the ELISA demonstrating the utility of the TM^{CD4} as a control reagent for estimating total CD4 T-cell numbers in the CD4 ELISA assay. For example, four different samples of whole blood were tested in the same assay (samples IDS72, 51, 40, 49), and the relative amount of CD4 compared to the standard can be estimated using the

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regression curve shown (in red), or other methods. To enable conversion of the amount of CD4 to the number of CD4 T-cells per microlitre, a reference sample of whole blood was tested multiple times by Flow cytometry and ELISA in comparison with the TM^{CD4} standard, allowing the correct conversion factor to be determined (data not shown). As shown in Figure 19B, supernatant from two different stable HEK293-TM^{CD4} cell line clones (H5 and F5) were detected by immunochromatographic rapid point of care assay, showing that TM^{CD4} can also be used as a positive control reagent or standard in such assays. In some embodiments, TM^{CD4} is used as an antigen for the production of Ab.

10

EXAMPLE 8***Strong correlation in estimates of CD4 T-cell counts between CD4 Capture ELISA using TM^{CD4} as standard, and Flow cytometry.***

Using the recombinant TM^{CD4} as an internal assay control for the CD4 ELISA, and a conversion factor based on parallel determination of a reference sample by Flow cytometry and ELISA, a very close correlation is obtained for the estimated CD4 T-cell numbers using CD4 ELISA compared to CD4 flow cytometry. As shown in Figure 20, all samples are correctly identified as being either greater than, or less than, 250 T-cells per microlitre, as shown in the boxed region. In this assay, monocytes were removed from whole blood using CD14 magnetic beads (Dynabeads CD14 (DynaL Biotech Cat No 111.49)) before assay of whole blood for CD4 T-cells by ELISA. Accurate counts can generally be obtained without monocyte removal.

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EXAMPLE 9***Correlation between amount of CD4 (TM^{CD4}) and signal strength in rapid, point of care assay***

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Using the recombinant TM^{CD4} a clear dose-response curve can be seen (see Figure 21) for signal intensity in the rapid format compared to the amount of TM^{CD4} in the assay. This demonstrates that the TM^{CD4} is useful as a control reagent for preparation, testing and quality control of both ELISA and rapid, point of care formats of CD4 assays. This Example also demonstrates that both the Chemicon 3706 anti-cytoplasmic domain Mab, and the custom 4B4 anti-cytoplasmic domain Mab, can be used

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separately or as a mixture for capture of cyto CD4 in the rapid format of the test. Further, this demonstrates that the signal strength in the rapid format is proportional to the amount of cell-associated CD4 in the sample, allowing the use of the rapid point of care format for quantitative or semiquantitative estimation of CD4 T-cell numbers.

5

EXAMPLE 10

Correlation between number of CD4 T-cells and signal strength in rapid, point of care assay

Actual test results for rapid point of care assay format, using purified T-cells at the indicated numbers of cells per microlitre (reacting at Test lines) (see Figure 22A). Control lines were prepared to be equivalent to 500 T-cells/microlitre and 250 T-cells/microlitre, by striping biotinylated monoclonal antibody that reacts directly with the anti-biotin gold conjugate. It can be seen (see Figure 22B) that visual discrimination allows the level of CD4 T-cells in the sample to be correctly identified as more than or less than 250 cells *per* microlitre or more than or less than 500 cells *per* microlitre. Test was performed on Millipore HFP90 nitrocellulose membrane with Chemicon MAb 3706 (test line capture) striped at 0.5 mg/mL; SAMPLE: 50 μ L CD4 +ve T cells in 1%Tx100/PBS; DETECTION: 13B8.2-biotin / anti-biotin gold. It is obvious that any reactive protein could be used as the control lines, such as but not limited to the TM[^]CD4 protein, or biotinylated proteins or antibodies or other molecules reactive with the detection system of choice. It is also obvious that the control lines and other assay parameters could be adjusted to allow detection of any desired level of cells per microlitre, such as the higher levels (around 2,000 cells *per* microlitre or even higher) that may be useful in monitoring of pediatric samples.

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EXAMPLE 11

Rapid, point of care assay detection of CD4 T-cells in whole blood.

Figure 23A provides a schematic representation of one embodiment the whole-blood CD4 rapid point of care assay. Red blood cells are captured by anti-glycophorin A (Epiclon anti-N with 0.1% azard Cat No 00992311 (HM1) Commonwealth Serum Laboratories (CSL)) in the RBC retention pad using methods well known in the art, while

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plasma and white blood cells (including CD4 T-cells) flow into the detergent-impregnated lysis pad, where cells are lysed to release CD4 that can then flow into the test strip to react with the anti-CD4 Cyto Mab for subsequent detection with the biotin anti-CD4 ecto Mab and anti-biotin colloidal gold (see Figure 23) (B). A sample of whole blood containing
5 high CD4 T-cell levels (1452/ μ l by flow cytometry) was tested in the whole blood assay as shown in A, with detergent in the lysis pad as normal (“+TX”), or with omission of the detergent from the lysis pad (“-TX”). In some embodiments, CD4 signal is only seen in the rapid test when detergent such as Triton -X100 is present to lyse the T-cells in the pad (see Figure 23). This depends upon the nature of the anti-CD4 binding agent and agents
10 that are cell membrane permeable together with detection agents that are cell membrane permeable do not require cell lysis. (C). Examples of representative whole-blood samples assayed in the CD4 rapid point of care assay. CD4 counts for each sample by flow cytometry are shown, and it can be seen that high CD4 T-cell counts give stronger signals while low CD4 T-cell counts give weak signals in this whole blood format.

15

EXAMPLE 12

Removal of specific cell population (monocytes) to prevent their detection in rapid, point of care assays

Monocytes also contain CD4, at a quantity of around 10% that found in CD4 T-
20 cells, and the presence of monocytes in a CD4 protein-based assay could in some circumstances lead to false estimates of CD4 T-cells due to the contribution of monocyte CD4. In the ELISA format of the assay, monocytes can be removed from whole blood before assay by use of anti-CD14 magnetic beads and strong magnets designed for this purpose as described above, in methods well known in the art. This method may also be
25 used for pre-treatment of whole blood before point of care assays, but would require sample handling and processing that is preferably avoided for point-of-care testing. No methods have been described for depletion of a particular white blood cell type or other cell type using methods that are compatible with inclusion in a rapid, point of care assay. The inventors have devised a method for efficient removal of monocytes in the sample pad
30 of the rapid point of care test.

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This method takes advantage of the surprising observation that by performing the magnetic separation in the thin sample pad (by placement of a magnet above or below the sample pad), the use of a much weaker magnetic field (an inexpensive “fridge magnet”) is sufficient to give retention of cells that have reacted with the magnetic beads, rather than
5 an expensive, powerful magnet as used widely in the art when retention is performed in a sample tube. This would allow, for example, the inclusion of a suitable magnet in an inexpensive, single-use disposable device for each individual assay, or an inexpensive multiple-use device or housing for performing multiple assays. In some embodiments, the magnet has a magnetic field of less than about 5mm such that the field would extend
10 through a sample pad of an immunochromatographic device but not, for example, through all of a suspension of cells in a 1cm or greater diameter test tube or vessel. One example of a suitable magnet is a ferromagnet which retains its magnetization in the absence of a field. In Figure 24, purified monocytes and purified peripheral blood leucocytes (PBLs, depleted of monocytes) were separately labelled with infrared fluorescent dyes - monocytes with
15 green (Mini CellVue NIR815, PTI Research, Inc), PBLs with red (Mini CellVue Burgundy, PTI Research Inc.). The images display the same sample pads viewed through different filters on the Licor Odyssey infrared fluorescent scanner: (A) = combined filters, (B) = Em 800, (C) = Em 700. The magnet obscures scanning of the sample pad where it is used. 1.5×10^4 monocytes (attached to magnetic beads) and 2×10^4 monocyte depleted
20 PBLs were combined and spotted onto anti-glycophorin A coated sample pads either without (left) or with the magnet (right). Sample was flushed with 60ul PBS to allow sample to flow into the absorbent pad (used here instead of an actual lysis pad and nitrocellulose test strip for illustrative purposes). Retention of >80% monocytes (green) can be seen in panel (A) and (B) in the presence of a magnet, with no green cells entering
25 the absorbent pad, whilst flow-through of monocyte-depleted PBLs into the absorbent pad is demonstrated in (A) and (C) in both the presence or absence of a magnet. (D) shows a schematic description of the test as shown in (A)-(C). It is obvious to one skilled in the art that a similar method could be used for selective retention or enrichment of any cell population in a filter or sample pad format, prior to the lysis or assay of the desired cells.

30 Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to

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be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Table 1
Summary of sequence identifiers

SEQUENCE ID NO:	DESCRIPTION
1	Peptide 1 of human CD4 cytoplasmic domain
2	Peptide 2 of human CD4 cytoplasmic domain
3	Primer 1 for generating construct encoding TM [^] CD4
4	Primer 2 for generating construct encoding TM [^] CD4
5	Primer 3 for generating construct encoding TM [^] CD4
6	Primer 4 for generating construct encoding TM [^] CD4
7	Nucleotide sequence encoding TM [^] CD4
8	Amino acid sequence of TM [^] CD4

Table 2
Amino acid sub-classification

5

Sub-classes	Amino acids
Acidic	Aspartic acid, Glutamic acid
Basic	Noncyclic: Arginine, Lysine; Cyclic: Histidine
Charged	Aspartic acid, Glutamic acid, Arginine, Lysine, Histidine
Small	Glycine, Serine, Alanine, Threonine, Proline
Polar/neutral	Asparagine, Histidine, Glutamine, Cysteine, Serine, Threonine
<i>Polar/large</i>	<i>Asparagine, Glutamine</i>
<i>Hydrophobic</i>	<i>Tyrosine, Valine, Isoleucine, Leucine, Methionine, Phenylalanine, Tryptophan</i>
<i>Aromatic</i>	<i>Tryptophan, Tyrosine, Phenylalanine</i>
<i>Residues that influence chain orientation</i>	<i>Glycine and Proline</i>

Table 3
Exemplary and Preferred Amino Acid Substitutions

Original Residue	EXEMPLARY SUBSTITUTIONS	PREFERRED SUBSTITUTIONS
Ala	Val, Leu, Ile	Val
Arg	Lys, Gln, Asn	Lys
Asn	Gln, His, Lys, Arg	Gln
Asp	Glu	Glu
Cys	Ser	Ser
Gln	Asn, His, Lys,	Asn
Glu	Asp, Lys	Asp
Gly	Pro	Pro
His	Asn, Gln, Lys, Arg	Arg
Ile	Leu, Val, Met, Ala, Phe, Norleu	Leu
Leu	Norleu, Ile, Val, Met, Ala, Phe	Ile
Lys	Arg, Gln, Asn	Arg
Met	Leu, Ile, Phe	Leu
Phe	Leu, Val, Ile, Ala	Leu
Pro	Gly	Gly
Ser	Thr	Thr
Thr	Ser	Ser
Trp	Tyr	Tyr
Tyr	Trp, Phe, Thr, Ser	Phe
Val	Ile, Leu, Met, Phe, Ala, Norleu	Leu

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CLAIMS:

1. A method for evaluating in a sample from a subject the level or presence of a cell surface associated protein (CSAP) comprising a cytoplasmic (cytosolic) and an extracellular (ecto) domain or the level or presence of cells bearing the CSAP, the method comprising:
 - (i) optionally contacting the sample with an agent capable of lysing or permeabilizing CSAP bearing cells;
 - (ii) contacting the sample with a CSAP-binding agent that binds to the cytoplasmic domain of the CSAP; and
 - (iii) directly or indirectly evaluating the level or presence of bound CSAP in the sample; andwherein the level or presence of bound CSAP comprising at least a cytoplasmic domain is indicative of the number of CSAP bearing cells in the subject.
2. The method of claim 1 wherein step (iii) comprises contacting the bound CSAP with a second binding agent that binds to the CSAP and which comprises a detection marker or which is capable of binding to a third binding agent comprising a detection marker and detecting the detection marker.
3. The method of claim 1 or 2 wherein the method comprises evaluating the number of CSAP bearing cells in the sample by comparison of the level or presence of bound CSAP or the level or presence of bound detection marker with a predetermined control.
4. The method of claim 1 wherein the CSAP-binding agent is an antibody or antigen binding fragment thereof.
5. The method of claim 2 wherein the second binding agent is an antibody or antigen-binding fragment thereof which binds to CSAP including to the cytoplasmic or extracellular domain of the CSAP.

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6. The method of claim 1 or 2 wherein the method is an ELISA type, flow cytometry or chromatographic method.
7. The method of claim 6, wherein the method is an ELISA or chromatographic method and the CSAP-binding agent that binds to the cytoplasmic domain of CSAP is immobilised on a solid or semi-solid support.
8. The method of claim 6 or 7 wherein the method is an ELISA or chromatographic method and wherein the second binding agent that binds to the CSAP including to the cytoplasmic or extracellular domain of CSAP is immobilised on a solid or semi-solid support.
9. The method of claim 1 wherein the sample to be tested is selected from the group comprising spinal fluid, a bone or tissue sample, blood, serum, plasma, saliva, tears and milk.
10. The method of claim 9 wherein the blood is whole blood or diluted whole blood or a blood derivative.
11. The method of claim 1 wherein the CSAP is a cluster of differentiation (CD) protein.
12. The method of claim 11 wherein the CSAP is CD4.
13. The method of claim 1 wherein the cell is a haematopoietic cell.
14. The method of claim 1 wherein the method does not detect soluble CSAP lacking a cytoplasmic domain which may be present in the sample.
15. The method of claim 1 wherein the method is capable of distinguishing between

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cell associated CSAP comprising an cytoplasmic domain and soluble CSAP lacking a cytoplasmic domain.

16. A method for evaluating in a sample from a subject the level of a cell surface associated protein (CSAP) comprising a cytoplasmic (cytosolic) and an extracellular (ecto) domain or the level of CSAP-bearing cells, the method comprising: a) applying the test sample to a sample portion of an immunochromatographic device wherein the sample portion is operably connected to a capture portion of the device and wherein components of the test sample flow from the sample portion to and through the capture portion which comprises an antibody or antigen-binding fragment thereof that binds to the cytoplasmic domain of CSAP such that only CSAP comprising a cytoplasmic domain and not soluble CSAP that does not comprise a cytoplasmic domain binds to the antibody or fragment thereof to form a captured CSAP; b) contacting the capture portion with a second binding agent that binds to CSAP including to the cytoplasmic or extracellular domain and which comprises a detection marker or which is capable of binding to a third binding partner comprising a detection marker; c) optionally contacting the second binding agent with a third binding agent comprising a detection marker; evaluating the presence of the detection marker.
17. The method of claim 3 wherein the predetermined control is a predetermined amount of a control polypeptide sufficient to produce, when bound to a visual detection marker, a signal providing a internal visual reference equivalent to the signal produced by a predetermined number of CSAP cells.
18. A method for evaluating in a blood sample from a subject the level of T-cell associated CD4 comprising a cytoplasmic (cytosolic) and an extracellular (ecto) domain or the level of CD4 T-cells, the method comprising:
 - (i) optionally contacting the sample with an agent capable of lysing or permeabilizing CD4 T-cells;
 - (ii) contacting the sample with an antibody or antigen-binding fragment thereof

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that binds to the cytoplasmic domain of CD4; and

(iii) directly or indirectly evaluating the level or presence of bound CD4 in the sample.

19. The method of claim 18 wherein step (iii) comprises contacting the CD4-antibody complex with a second antibody or antigen-binding fragment thereof that binds to CD4 including to the cytoplasmic or extracellular domain and which comprises a detection marker or which is capable of binding to a third binding partner comprising a detection marker, and detecting the detection marker.
20. The method of claim 18 or 19 wherein the method comprises determining the number of CD4 bearing cells in the sample by comparison of the level or presence of bound CD4 or bound detection marker with a predetermined control.
21. The method of claim 18 or 19 wherein the method is an ELISA, flow cytometry or chromatographic method.
22. The method of claim 21, wherein the method is an ELISA or chromatographic method and the antibody or antigen-binding fragment thereof that binds to the cytoplasmic domain of CD4 (anti-cytoCD4 antibody) is immobilised on a solid or semi-solid support.
23. A method for evaluating in a blood sample from a subject the level of T-cell associated CD4 comprising a cytoplasmic (cytosolic) and an extracellular (ecto) domain or the level of CD4 T-cells, the method comprising: a) applying the test sample to a sample portion of an immunochromatographic device wherein the sample portion is operably connected to a capture portion of the device and wherein components of the test sample flow from the sample portion to and through the capture portion which comprises an antibody or antigen-binding fragment thereof that binds to the cytoplasmic domain of CD4 such that only CD4 comprising a cytoplasmic domain and not soluble CD4 that does not comprise a cytoplasmic

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domain binds to the antibody or fragment thereof to form a captured CD4; b) contacting the capture portion with a second binding agent that binds to CD4 including to the cytoplasmic or extracellular domain and which comprises a detection marker or which is capable of binding to a third or subsequent binding partner comprising a detection marker; c) optionally contacting the second binding agent with a third or subsequent binding agent comprising a detection marker; evaluating the presence of the detection marker.

24. The method of claim 20 wherein the predetermined control is a predetermined amount of a control polypeptide sufficient to produce, when bound to a visual detection marker, a signal providing a internal visual reference equivalent to the signal produced by a predetermined number of CD4 T-cells.
25. The method of claim 24 wherein the control polypeptide is CD4 polypeptide comprising at least cytoplasmic domain.
26. The method of claim 20 wherein the number of CD4 T-cells is one or more of less than 200, less than 250, more than 250, 250 to 350, 350 to 500, 250 to 500 and more than 500 cells/ μ l of blood.
27. The method of claim 20 wherein the number of CD4 T-cells is one or more of less than 200, less than 500, 500 to 1,000, 1,000 to 2,000, and more than 2,000 cells/ μ l of blood.
28. The method of claim 18 further comprising depleting monocytes in the sample by contacting the sample with anti-CD14 antibodies bound to a solid or semi-solid support.
29. The method of claim 18 further comprising depleting red blood cells in the sample by contacting the sample with anti-glycophorin antibodies bound to a solid or semi-solid support.

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30. A kit for detecting or monitoring the number of cells in sample from a subject, wherein the cell is characterised by comprising a cell surface associated protein (CSAP) comprising a cytoplasmic (cytosolic) and an extracellular (ecto) domain wherein the kit comprises:
- (i) a chromatographic device comprising a porous membrane operably connected to a sample portion, a test (capture) portion, a conjugate portion, a sucker portion, optionally a control portion and optionally a lysis portion;
 - (ii) a CSAP-binding agent such as an antibody or antigen-binding fragment thereof that binds to the cytoplasmic domain of CSAP, wherein the agent or antibody is either immobilised (bound) to one or more test portions and/or contained within a conjugate portion;
 - (iii) instructions for using the device to detect or monitor the level or presence of CSAP-bearing cells in the sample or subject.
31. A kit for detecting or monitoring the number of CD4 T-cells in a blood sample from a subject, wherein the kit comprises:
- (i) a chromatographic device comprising a porous membrane operably connected to a sample portion, a test (capture) portion, a conjugate portion, a sucker portion, optionally a control portion and optionally a lysis portion;
 - (ii) a CD4-binding agent such as an antibody or antigen-binding fragment thereof that binds to the cytoplasmic domain of CD4, wherein the agent or antibody is either immobilised (bound) to one or more test portions and/or contained within a conjugate portion;
 - (iii) instructions for using the device to detect or monitor the level or presence of CD4 cells in the sample or subject.
32. A kit of claim 30 or claim 31 wherein the kit uses reverse or lateral flow immunochromatography.
33. The kit of claim 32 wherein the conjugate portion comprises a visual detection

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marker.

34. The kit of claim 30 or 31 comprising a control portion comprising a pre-determined amount of a control polypeptide sufficient to produce, when bound to a visual detection marker, a signal providing an internal reference equivalent to the signal produced by a pre-determined number of cells.
35. The kit of claim 31 comprising a control portion comprising a pre-determined amount of a control CD4 polypeptide comprising at least a cytoplasmic domain and sufficient to produce, when bound to a visual detection marker, a signal providing a visual standard of a pre-determined number of CD4 cells.
36. The kit of claim 35 wherein the CD4 polypeptide is recombinant CD4 lacking a transmembrane domain.
37. The kit of claim 34 or 35 wherein the pre-determined number is one or more of: less than 200, less than 250, more than 250, 250 to 350, 350 to 500, 250 to 500 and more than 500 cells/ μ l of blood.
38. The kit of claim 34 or 35 wherein the pre-determined number is one or more of: less than 200, less than 500, 500 to 1,000, 1,000 to 2,000, and more than 2,000 cells/ μ l of blood.
39. The kit of claim 30 or 31 wherein the sample pad comprises a red blood cell capture agent such as glycophorin A.
40. The kit of claim 30 or 31 wherein the sample pad comprises anti-CD14 magnetic beads.
41. The kit of claim 40 further comprising an inexpensive or disposable magnetic material.

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42. The kit of claim 41 wherein the magnet is attached or attachable to the sample pad or to a portion of the immunochromatographic device capable of coming into contact with the sample pad.
43. A kit of claim 30 or 31 wherein the number of CSAP-bearing cell is evaluated by:
 - (i) applying the test sample from the subject to the sample portion under conditions in which the CSAP bearing cells will flow over the capture portion towards the sucker portion;
 - (ii) directly or indirectly detecting the presence of captured CSAP in the capture portion using a visual detection marker;
 - (iii) comparing the intensity of the signal from the detection marker in (ii) with the intensity of the signal from the control portion comprising a control polypeptide providing an internal reference equivalent to the signal produced by a pre-determined number of CSAP bearing cells.
44. The kit of claim 33 wherein the test sample is contacted with a lysis buffer capable of lysing or permeabilising CSAP bearing cells.
45. Recombinant CD4 lacking a transmembrane domain for use or suitable for use as a control in methods or kits to determine CD4 T-cell numbers.
46. Use of recombinant CD4 lacking a transmembrane domain in the manufacture of a solid or semi-solid support or a kit for quantifying the number of CD4 T-cells in a sample from a subject.
47. The method of claim 23 or the kit of claim 31 for use in evaluating AIDS or other immunodeficiency disease patients.

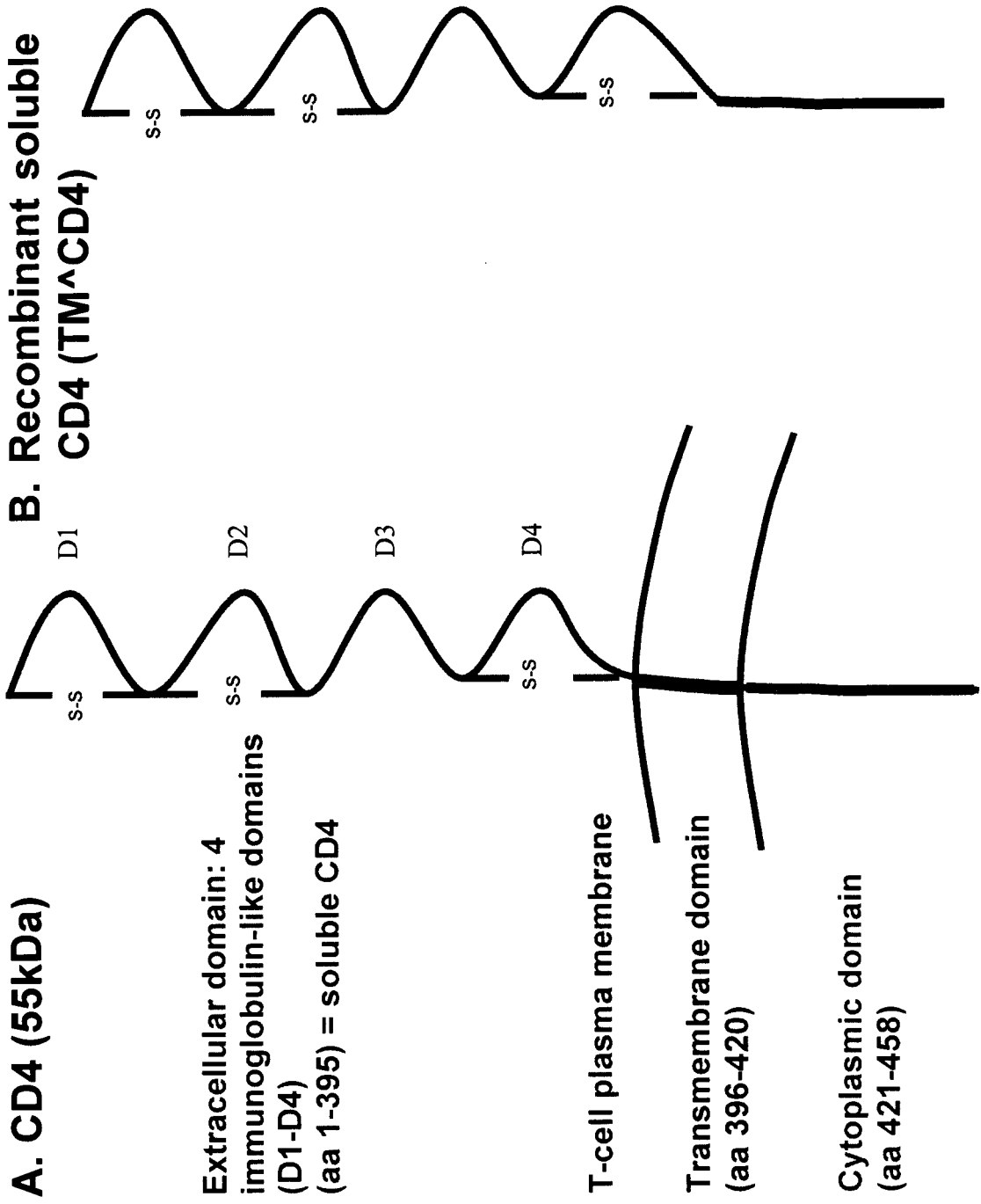


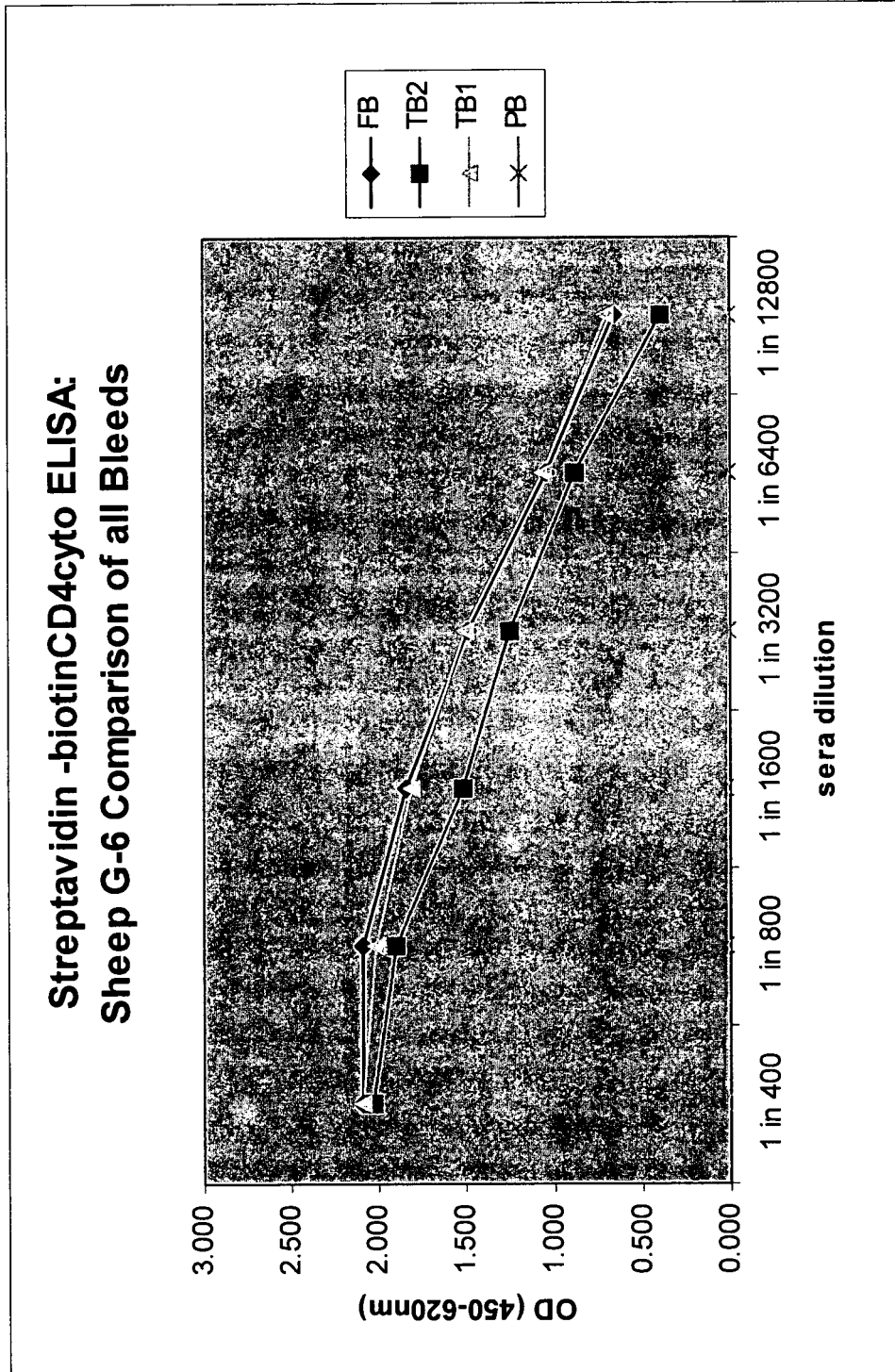
Figure 1

Two peptides based on human CD4cyto were synthesised:

- Peptide 1 (17 amino acids):
 - H-KRLLSEKKTCPHRFQKT-NH2
(₄₄₆QC₄₄₇ & ₄₅₅CSP₄₅₈ omitted*)

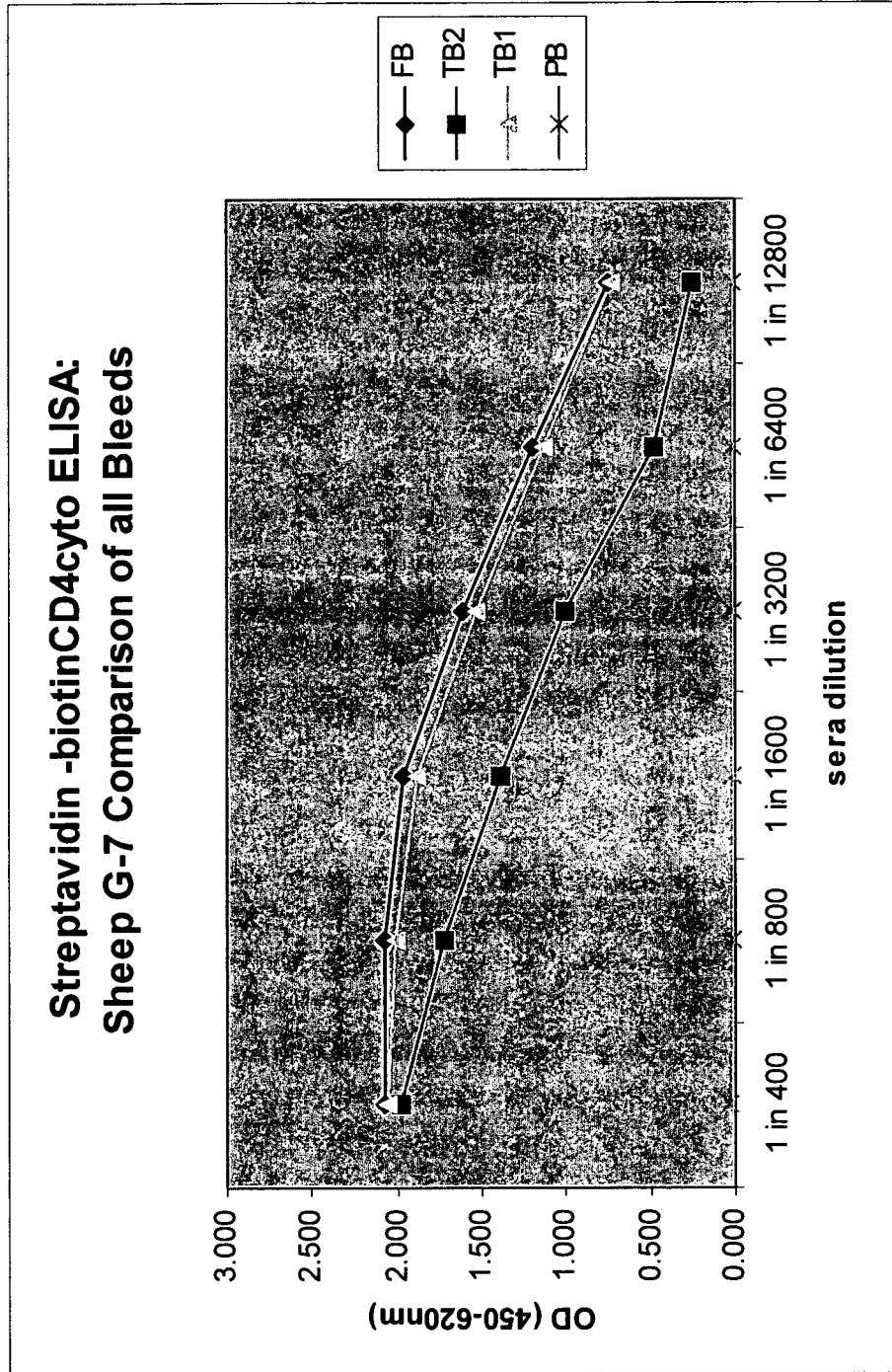
 - Peptide 2 (20 amino acids):
 - H-CRHRRQAERM₄₂₁SQIKRLLSE-NH2
(₄₂₁R₄₂₁ omitted*)
- human CD4cyto: ₄₂₁rcrhrrrqae rmsqikrlls ekkteqcphr fqkcspi ₄₅₈
 Peptide 1
 Peptide 2

Figure 2



A

Figure 3



B

Figure 3 continued

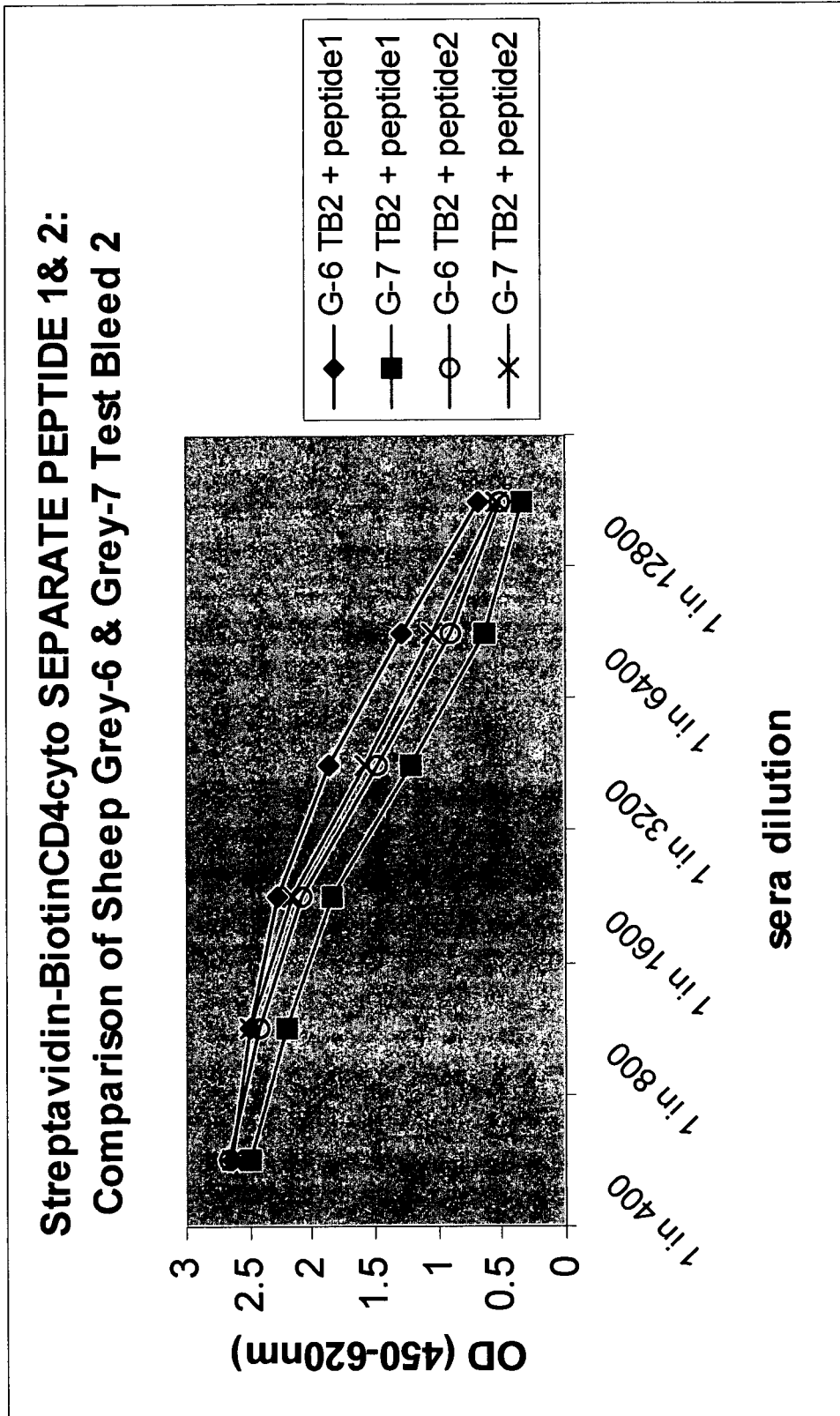


Figure 4

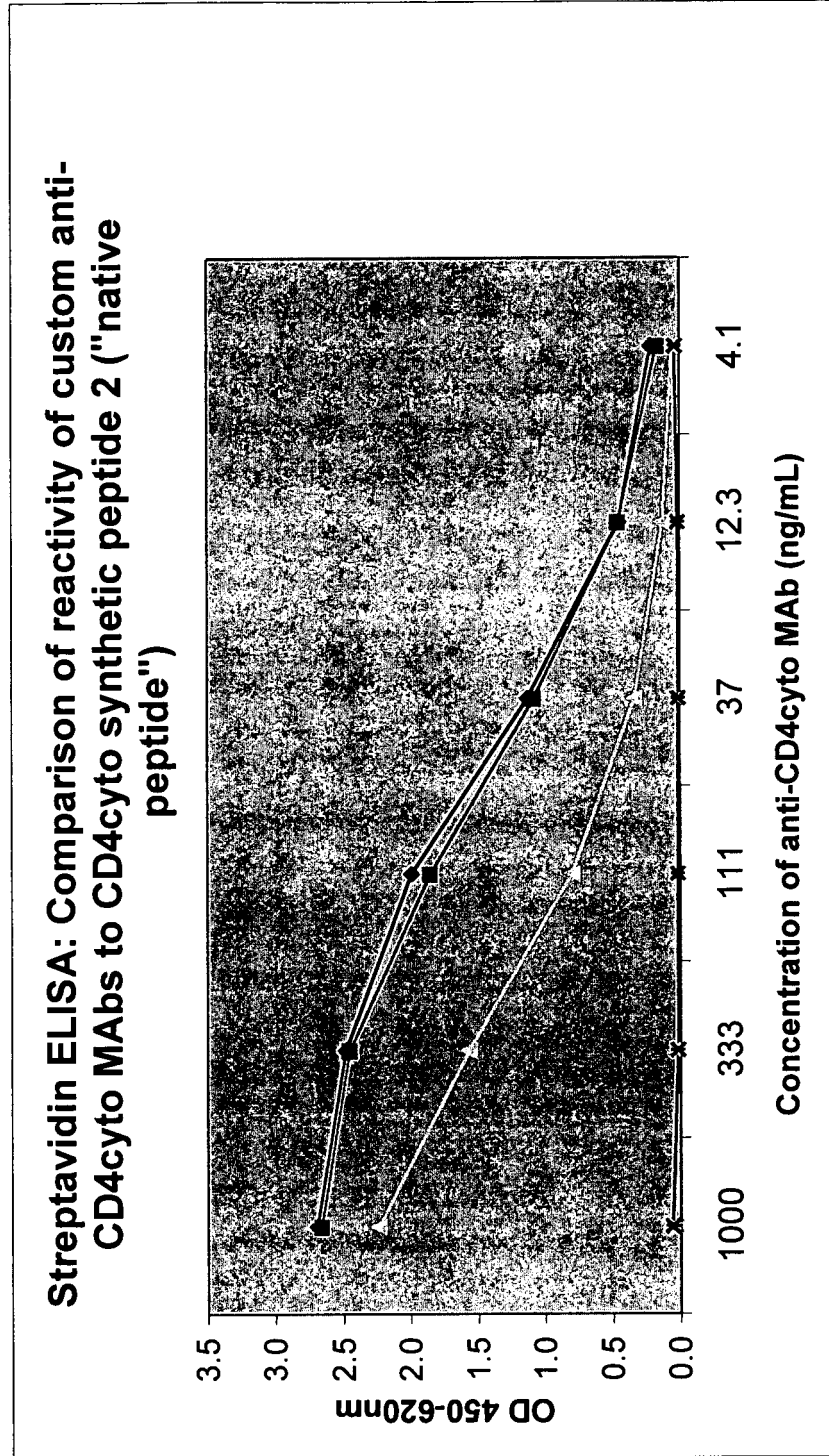


Figure 5

7/32

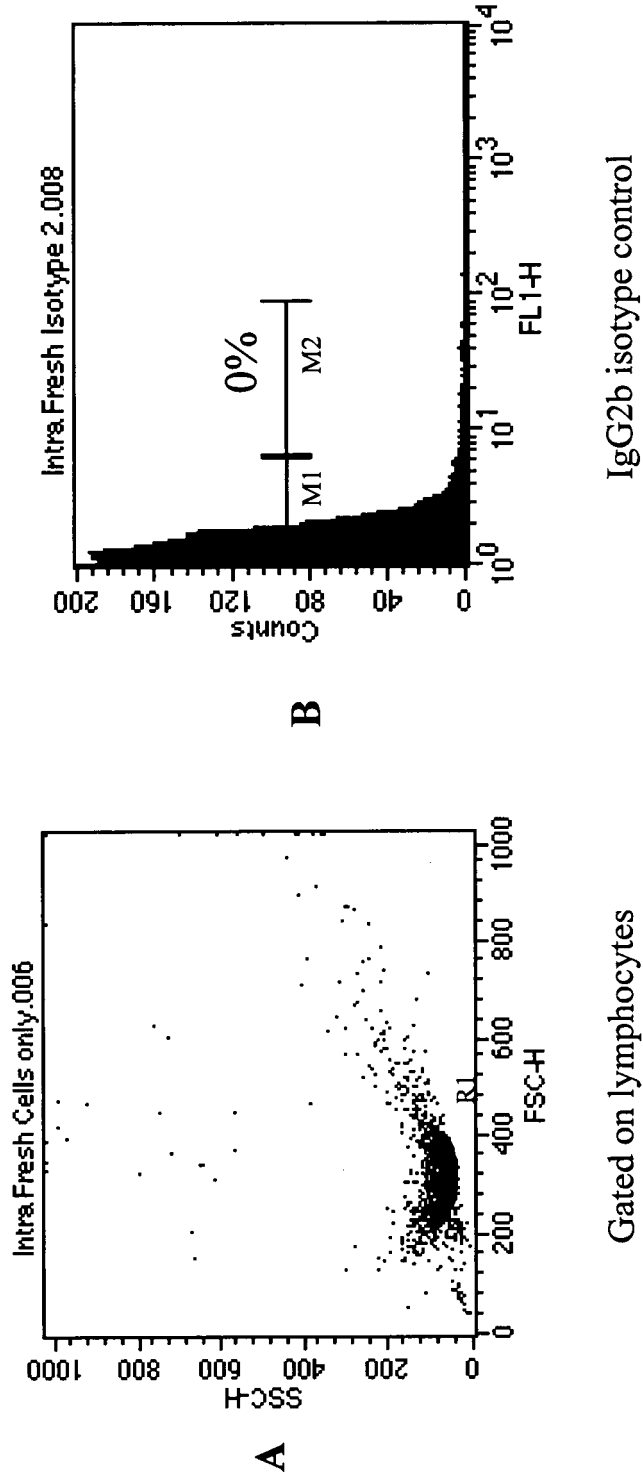
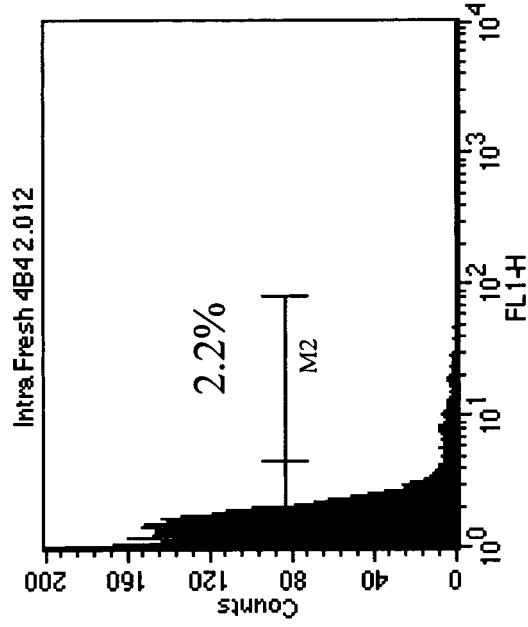


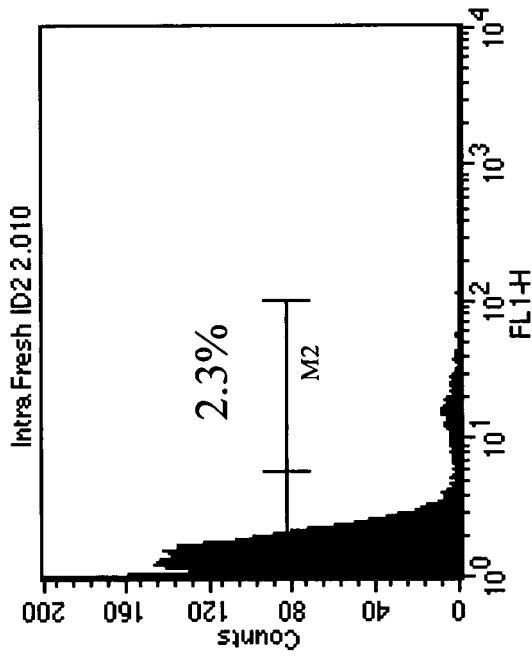
Figure 6

8/32



anti-CD4cyto MAb 4B4

D

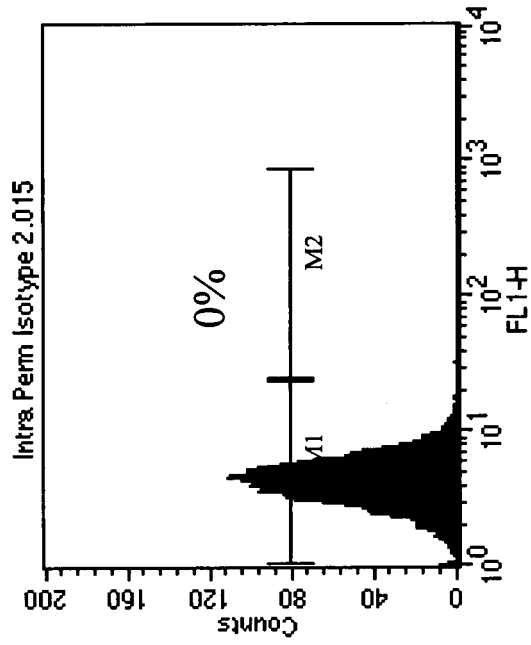


anti-CD4cyto MAb 1D2

C

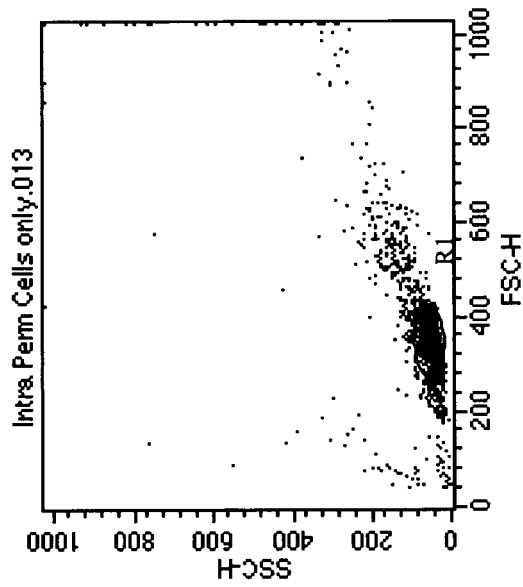
Figure 6 continued

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IgG2b isotype control

B

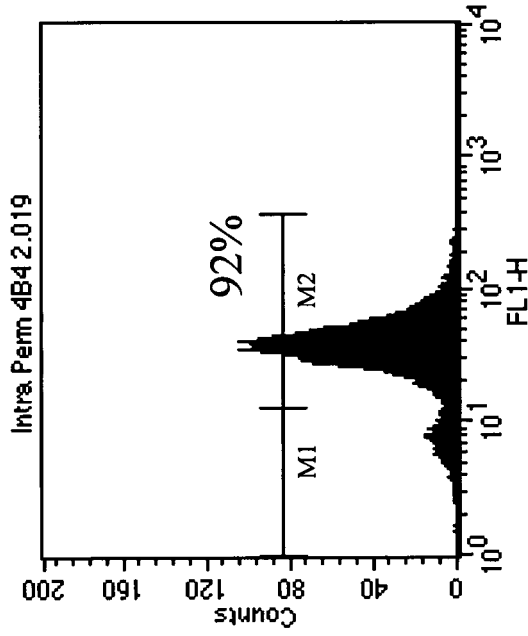


Gated on lymphocytes

A

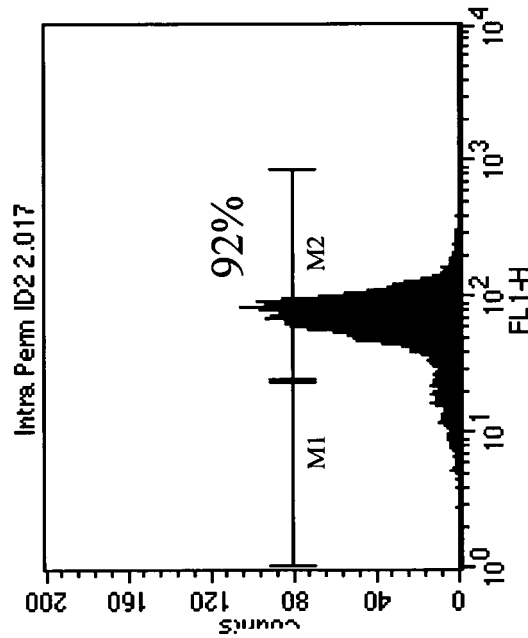
Figure 7

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anti-CD4cyto MAb 4B4

D



anti-CD4cyto MAb 1D2

C

Figure 7 continued

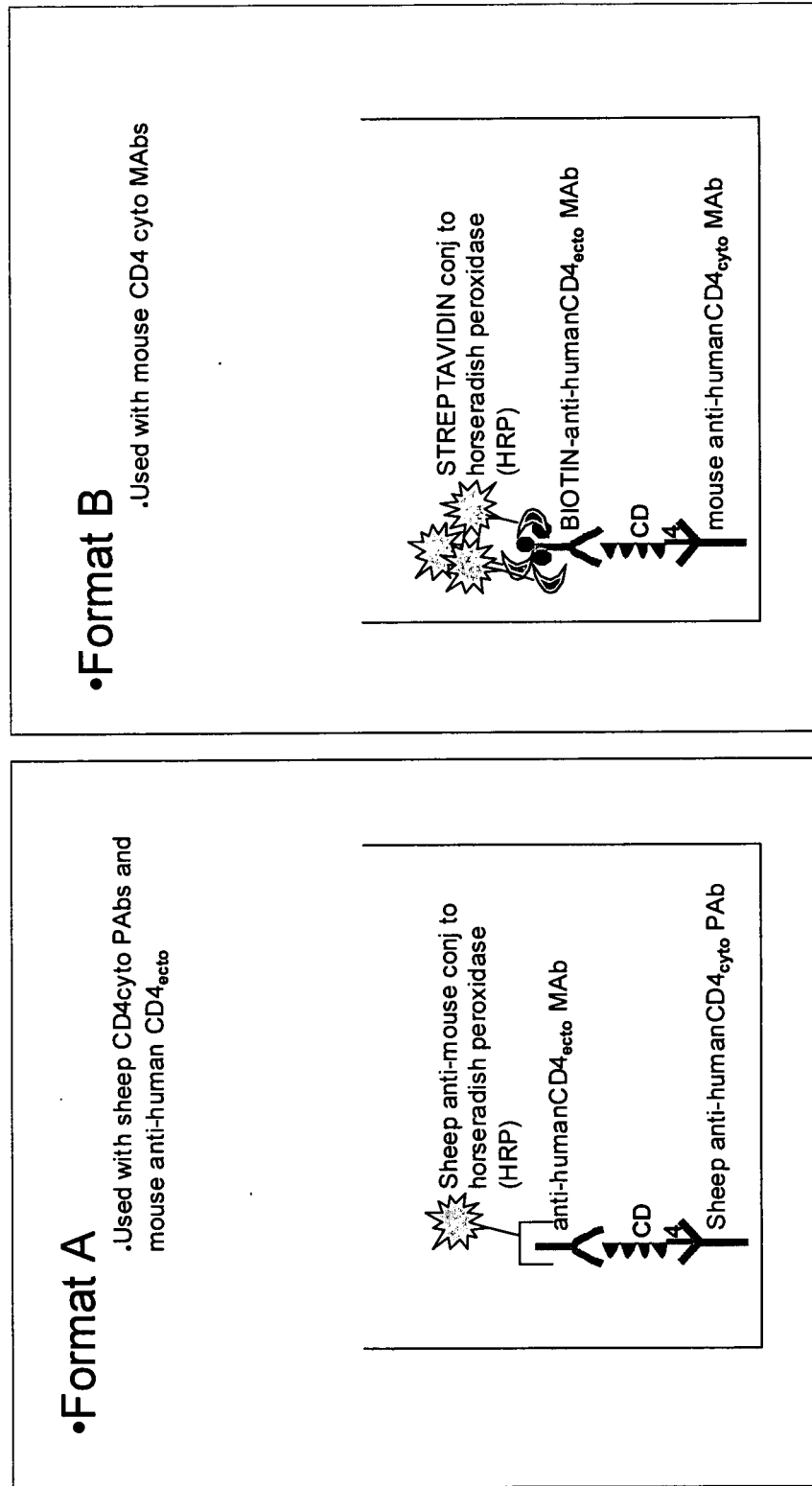


Figure 8

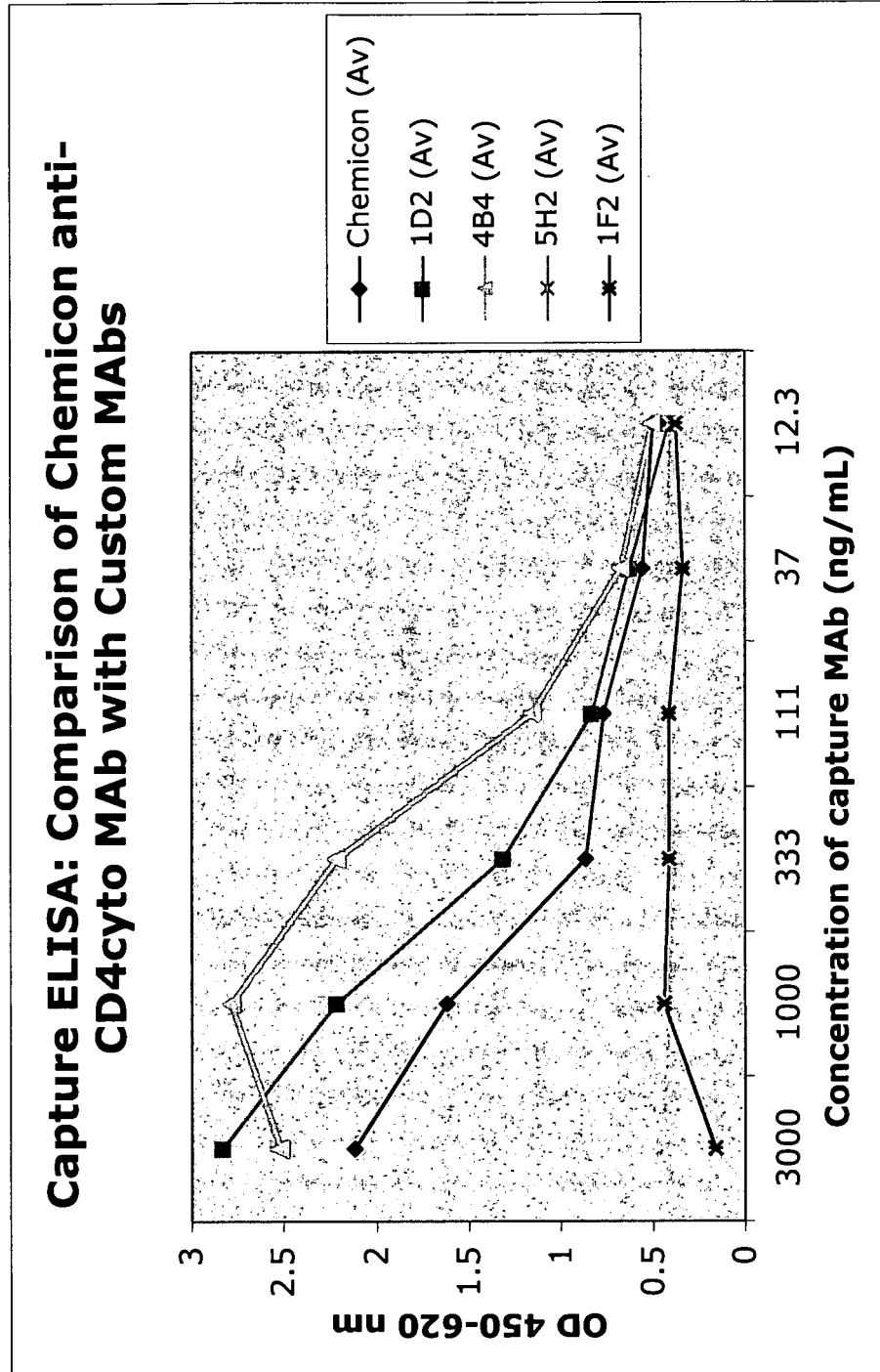


Figure 9

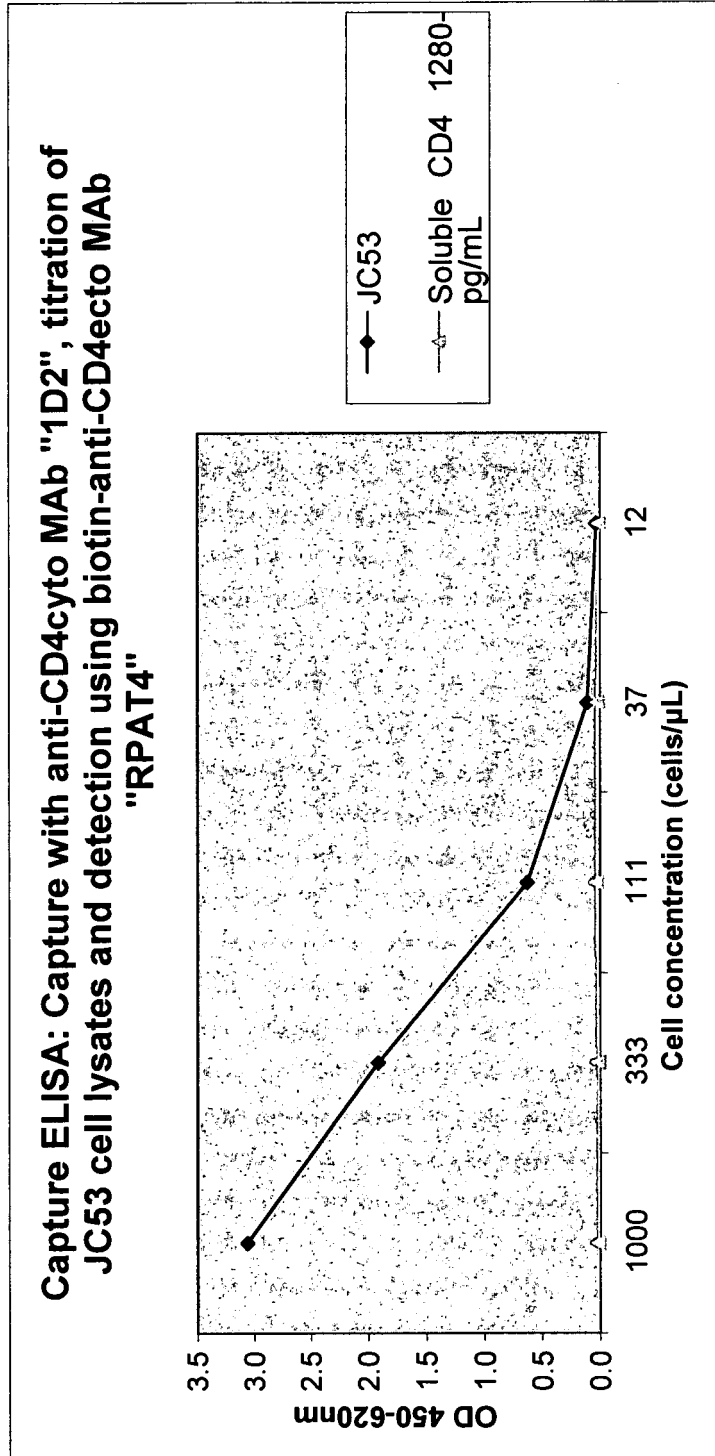


Figure 10

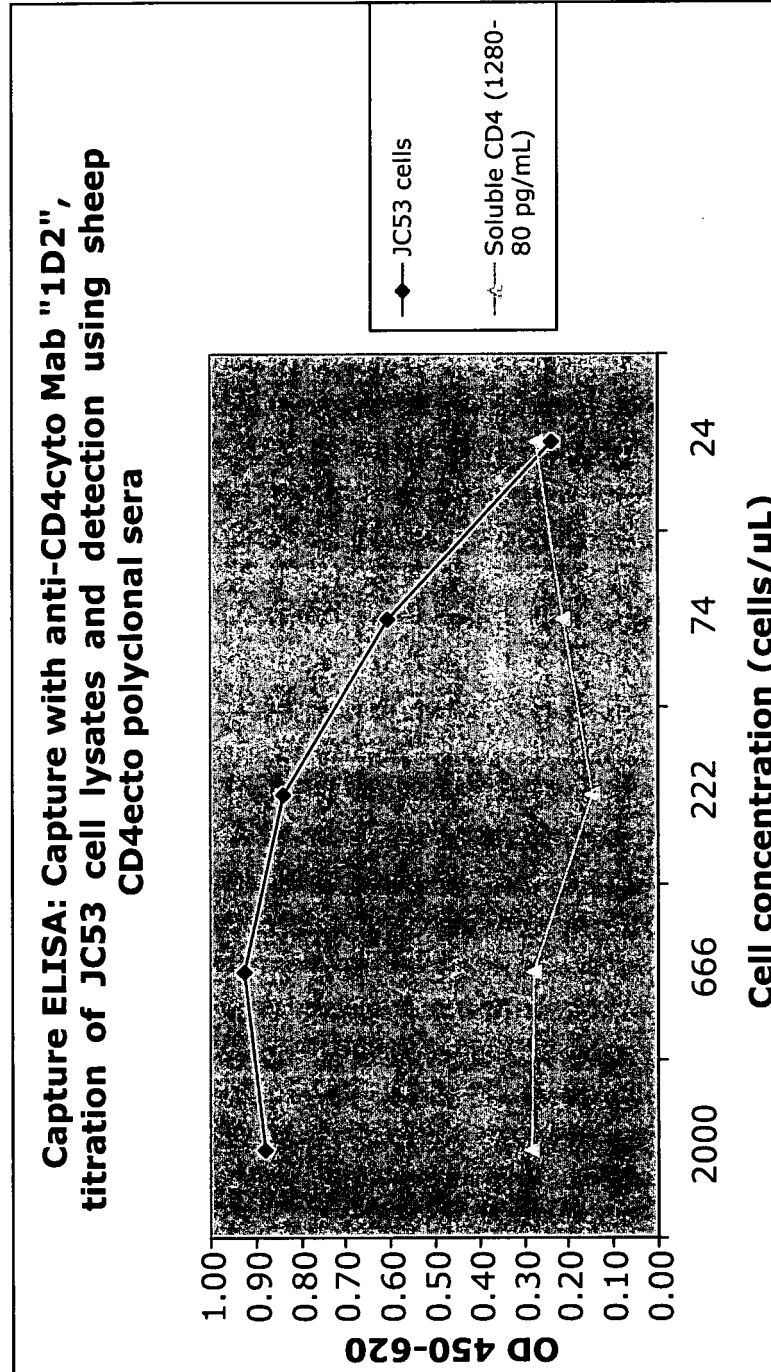


Figure 11

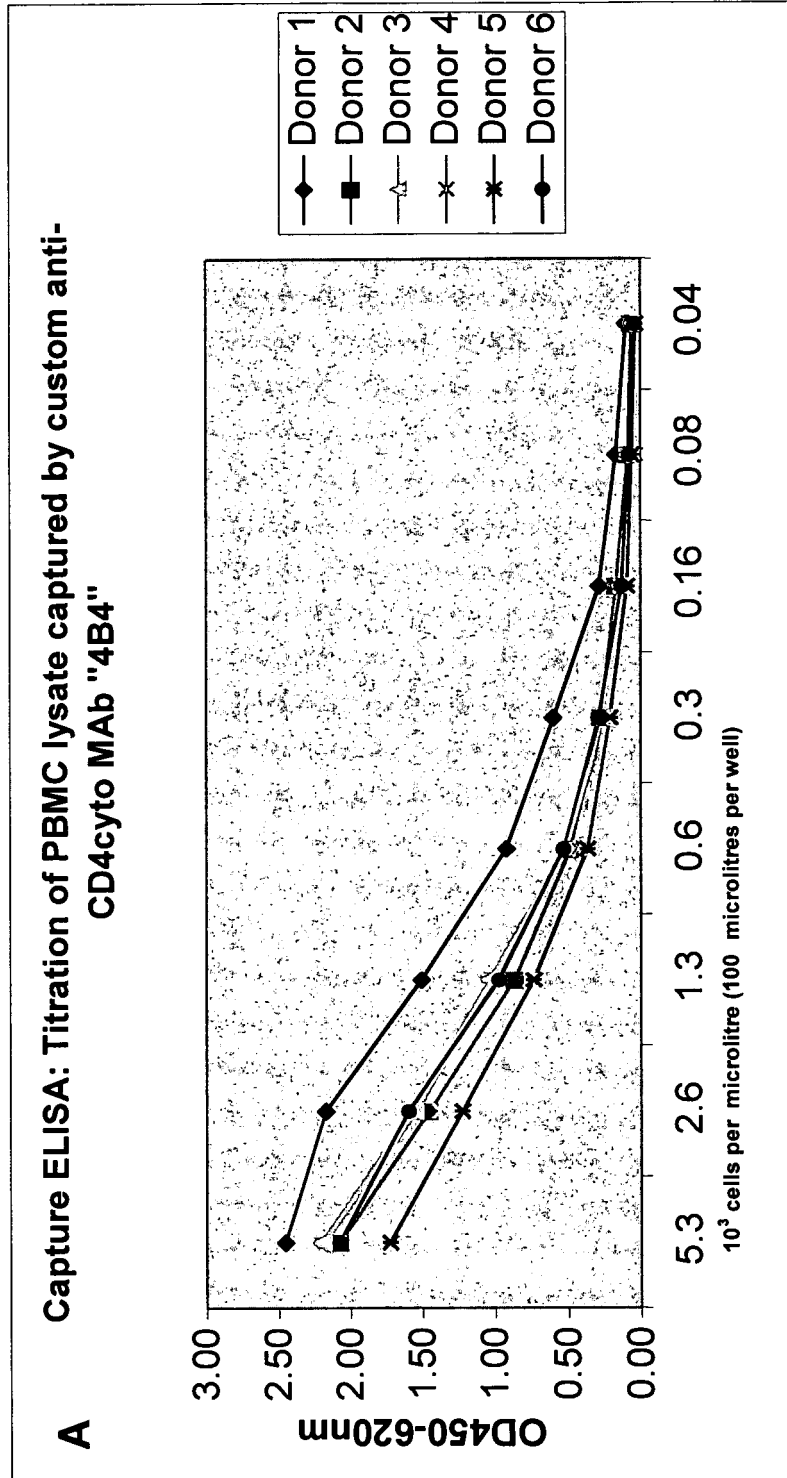


Figure 12

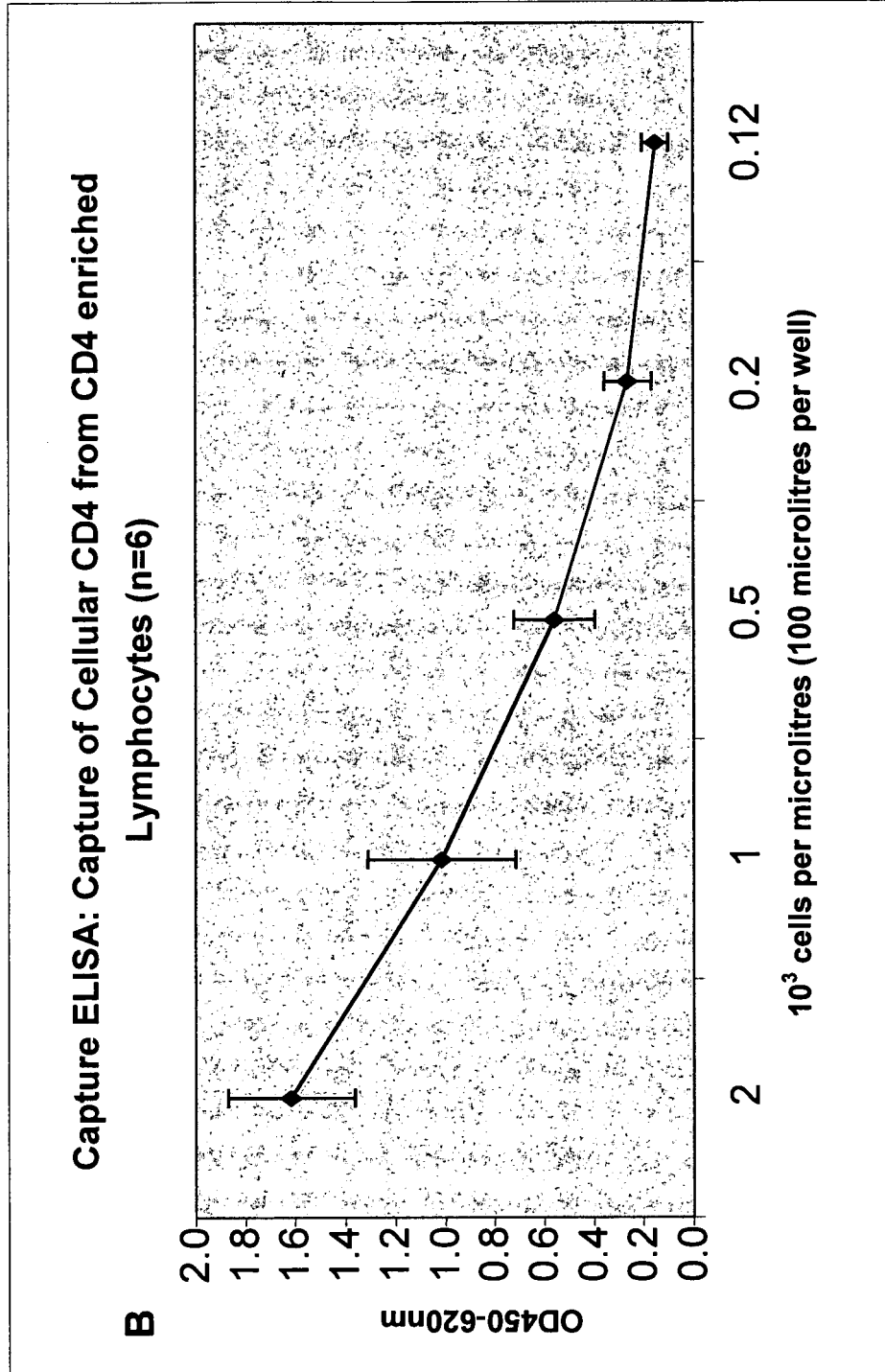


Figure 12 continued

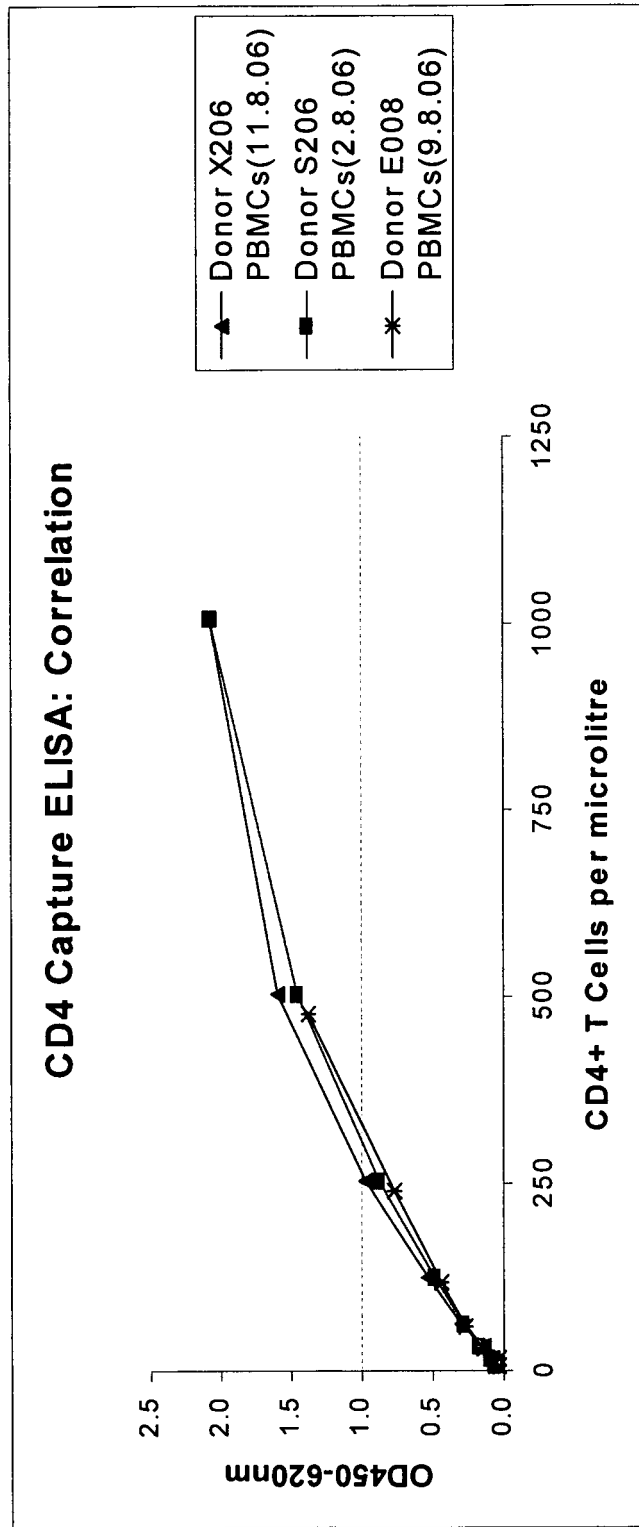


Figure 13

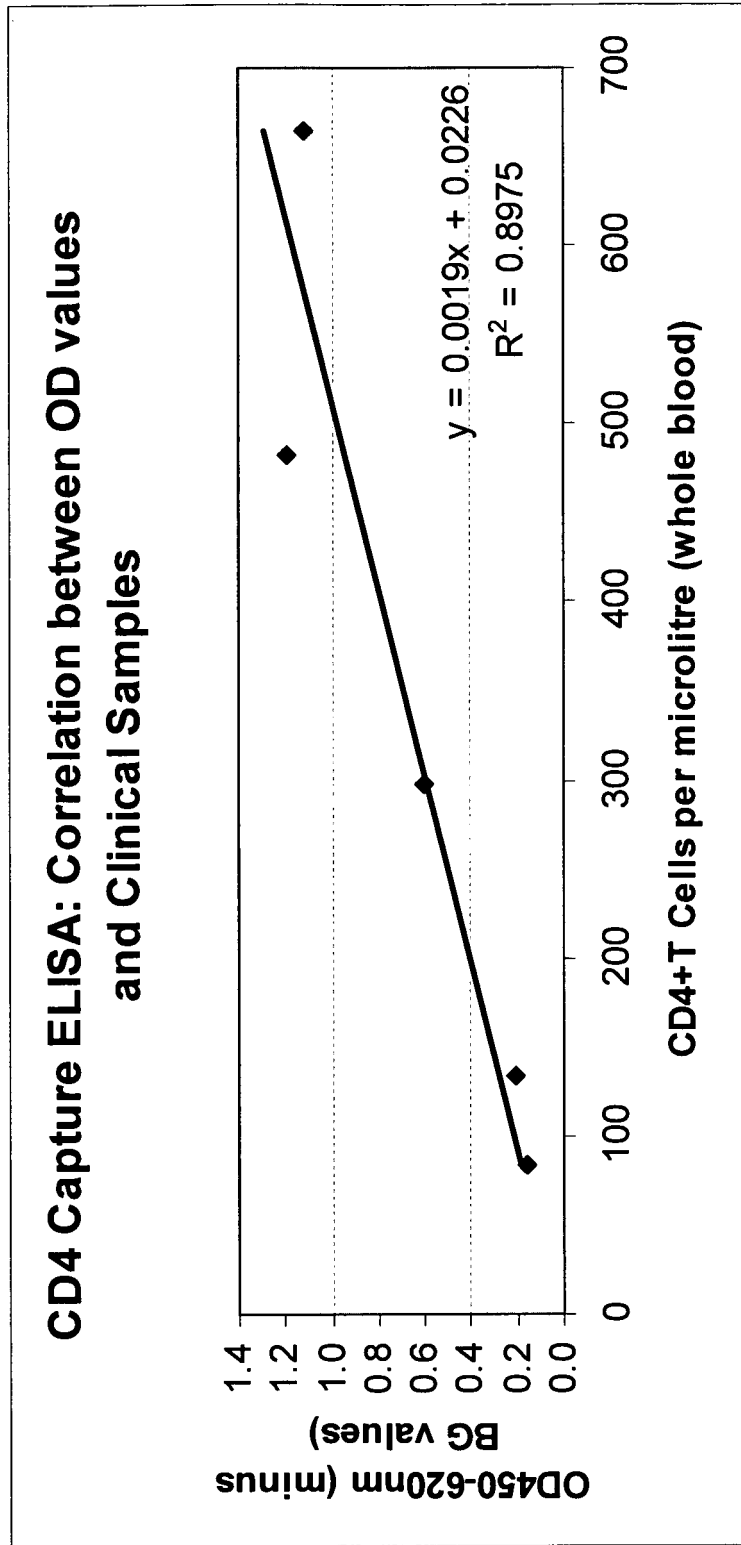


Figure 14

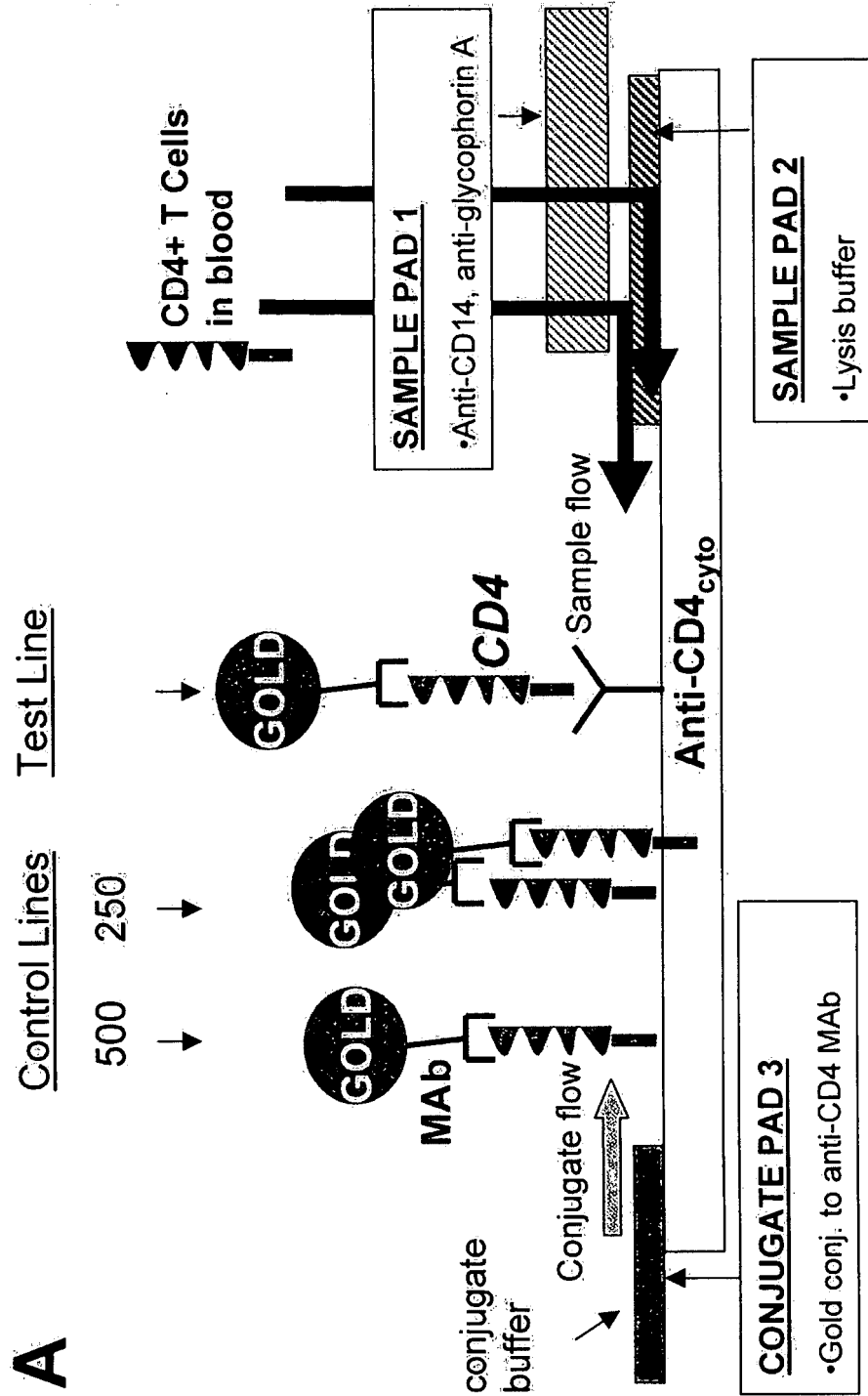


Figure 15

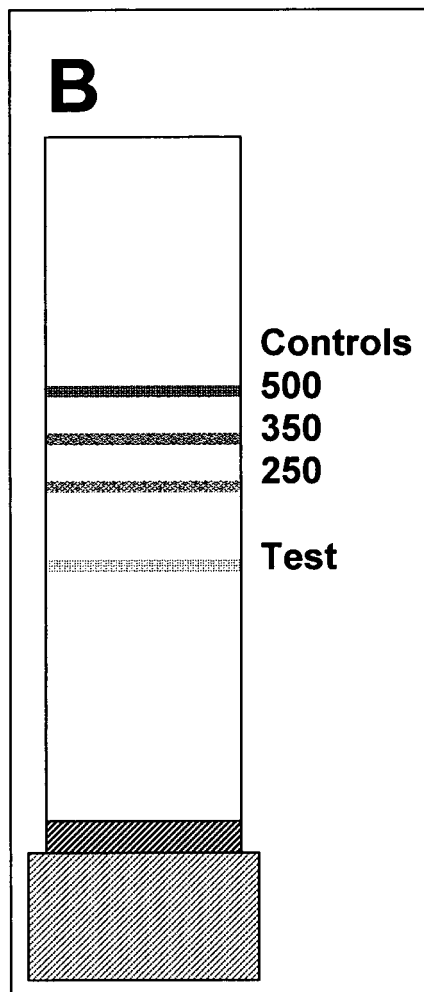


Figure 15 continued

TGG CAG GCG GAG AGG GCT TCC TCC TCC AAG TCT TGG ATC ACC
 TTT GAC CTG AAG AAC AAG GAA GTG TCT GTA AAA CGG GTT ACC
 CAG GAC CCT AAG CTC CAG ATG GGC AAG AAG CTC CCG CTC CAC
 CTC ACC CTG CCC CAG GCC TTG CCT CAG TAT GCT GGC TCT
 GGA AAC CTC ACC CTG GCC CTT GAA GCG AAA ACA GGA AAG TTG
 CAT CAG GAA GTG AAC CTG GTG ATG AGA GCC ACT CAG CTC
 CAG AAA AAT TTG ACC TGT GAG GTG TGG GGA CCC ACC TCC CCT
 AAG CTG ATG CTG AGT TTG AAA CTG GAG AAC AAG GAG GCA AAG
 GTC TCG AAG CCG GAG AAG GCG GTG TGG GTG CTG AAC CCT GAG
 GCG GGG ATG TGG CAG TGT CTG CTG AGT GAC TCG GGA CAG GTC
 CTG CTG GAA TCC AAC ATC AAG GTT CTG CCC ACA TGG TCC ACC
 CCG GTG CAG AGG TGC CCG CAC CGA AGG CCG CAA GCA GAG CGG
 ATG TCT CAG ATC AAG AGA CTC CTC AGT GAG AAG ACC TGC
 CAG TGT CCT CAC CCG TTT CAG AAG ACA TGT AGC CCC ATT TGA

Figure 16 continued

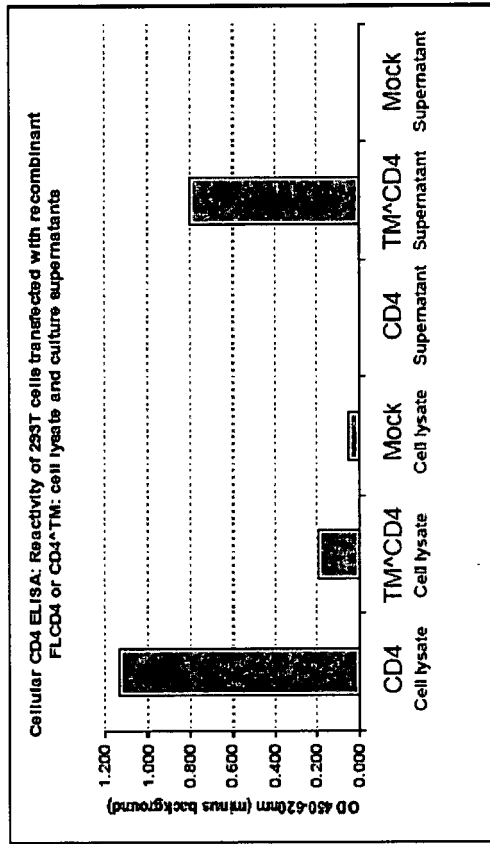
TM[^]CD4 amino acid sequence

Met	Gly	Gly	Ser	His	His	His	His	His	His	Gly	Met	Ala	Ser
Met	Thr	Gly	Gly	Gln	Met	Gly	Arg	Asp	Leu	Tyr	Asp	Asp	
Asp	Asp	Lys	Val	Pro	Gly	Ser	Val	Val	Glu	Phe	Thr	Met	
Asn	Arg	Gly	Val	Pro	Phe	Arg	His	Leu	Leu	Val	Leu	Gln	
Leu	Ala	Leu	Leu	Pro	Ala	Ala	Thr	Gln	Gly	Lys	Val	Val	
Leu	Gly	Lys	Lys	Gly	Asp	Thr	Val	Glu	Leu	Thr	Cys	Thr	Ala
Ser	Gln	Lys	Lys	Ser	Ile	Gln	Phe	His	Trp	Lys	Asn	Ser	Asn
Gln	Ile	Lys	Ile	Leu	Gly	Asn	Gln	Gly	Ser	Phe	Leu	Thr	Lys
Gly	Pro	Ser	Lys	Leu	Asn	Asp	Arg	Ala	Asp	Ser	Arg	Arg	Ser
Leu	Trp	Asp	Gln	Gly	Asn	Phe	Pro	Leu	Ile	Lys	Asn	Leu	
Lys	Ile	Glu	Asp	Ser	Asp	Thr	Tyr	Ile	Cys	Glu	Val	Glu	Asp
Gln	Lys	Glu	Glu	Val	Gln	Leu	Leu	Val	Phe	Gly	Leu	Thr	Ala
Asn	Ser	Asp	Thr	His	Leu	Leu	Gln	Gly	Gln	Ser	Leu	Thr	Leu
Thr	Leu	Glu	Ser	Pro	Pro	Gly	Ser	Ser	Pro	Ser	Val	Gln	Cys
Arg	Ser	Pro	Arg	Gly	Lys	Asn	Ile	Gln	Gly	Lys	Lys	Thr	Leu
Ser	Val	Ser	Gln	Leu	Glu	Leu	Gln	Asp	Ser	Gly	Thr	Trp	Thr
Cys	Thr	Val	Leu	Gln	Asn	Gln	Lys	Lys	Val	Glu	Phe	Lys	Ile
Asp	Ile	Val	Val	Leu	Ala	Phe	Gln	Lys	Ala	Ser	Ser	Ile	Val
Tyr	Lys	Lys	Glu	Gly	Glu	Gln	Val	Glu	Phe	Ser	Phe	Pro	Leu
Ala	Phe	Thr	Val	Glu	Lys	Leu	Thr	Gly	Ser	Gly	Glu	Leu	Trp

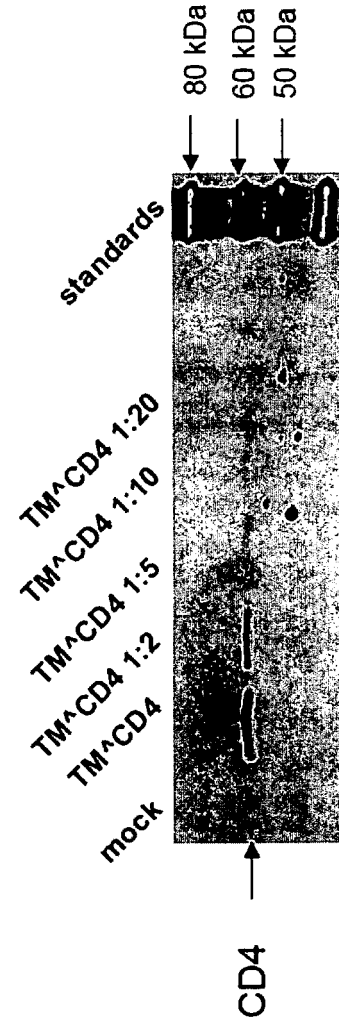
Figure 17

Trp Gln Ala Glu Lys Arg Ala Ser Ser Ser Ser Lys Ser Trp Ile Thr
 Phe Asp Leu Lys Asn Lys Glu Val Ser Ser Val Lys Arg Val Thr
 Gln Asp Pro Lys Leu Gln Met Gly Val Lys Lys Leu Pro Leu His
 Leu Thr Leu Pro Gln Ala Leu Pro Gln Tyr Ala Gly Ser Gly
 Asn Leu Thr Leu Ala Leu Glu Ala Lys Thr Gly Lys Leu His
 Gln Glu Val Asn Leu Val Val Met Arg Ala Thr Gln Leu Gln
 Lys Asn Leu Thr Cys Glu Val Trp Gly Pro Thr Ser Pro Lys
 Leu Met Leu Ser Leu Lys Leu Glu Asn Lys Glu Ala Lys Val
 Ser Lys Arg Glu Lys Ala Val Trp Val Leu Asn Pro Glu Ala
 Gly Met Trp Gln Cys Ile Lys Leu Ser Asp Ser Gly Gln Val Leu
 Leu Glu Ser Asn Ile Lys Val Val Leu Pro Thr Trp Ser Thr Pro
 Val Gln Arg His Arg Arg Gln Ala Glu Arg Met Ser Gln
 Ile Lys Arg Leu Leu Ser Glu Lys Thr Cys Gln Cys Pro
 His Arg Phe Gln Lys Thr Cys Ser Pro Ile ***

Figure 17 continued



A



B

Figure 18

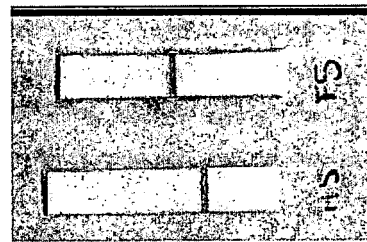
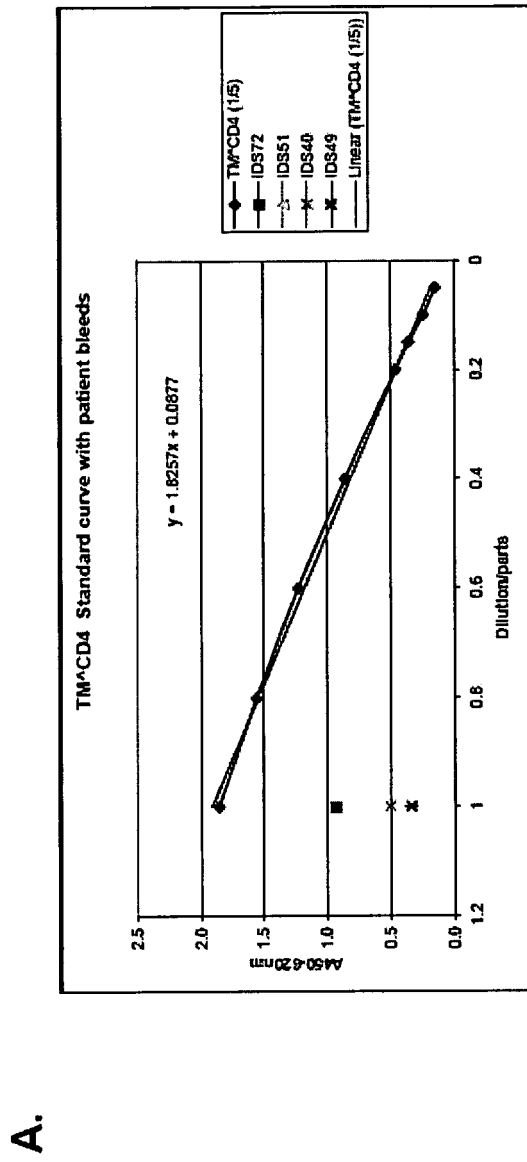


Figure 19

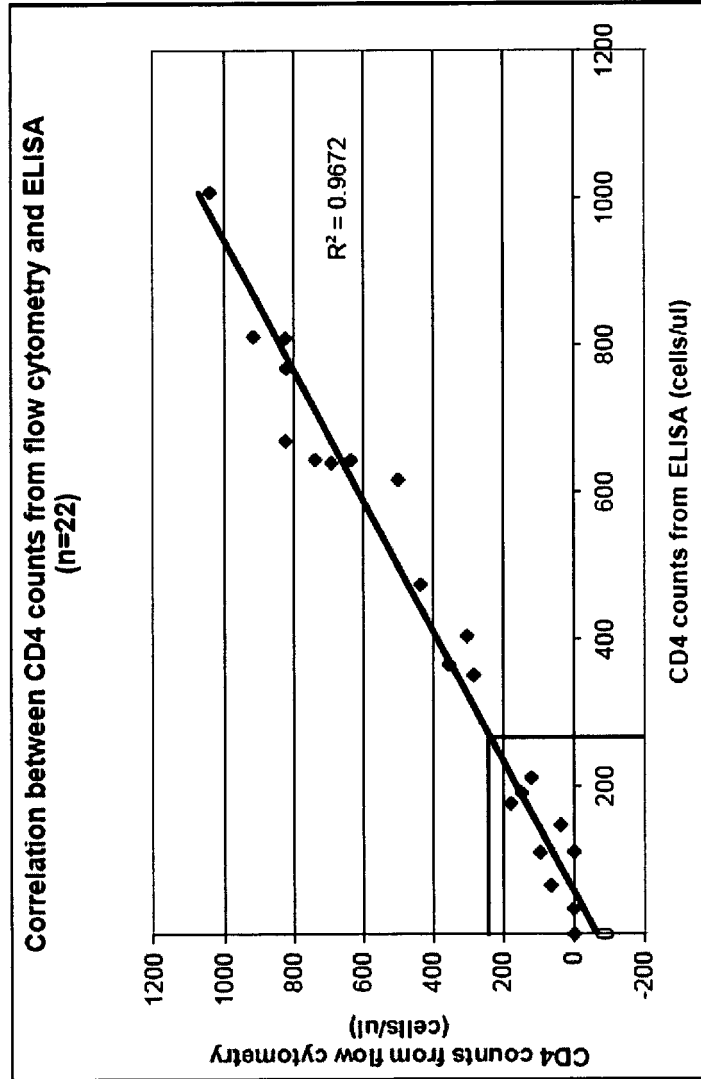


Figure 20

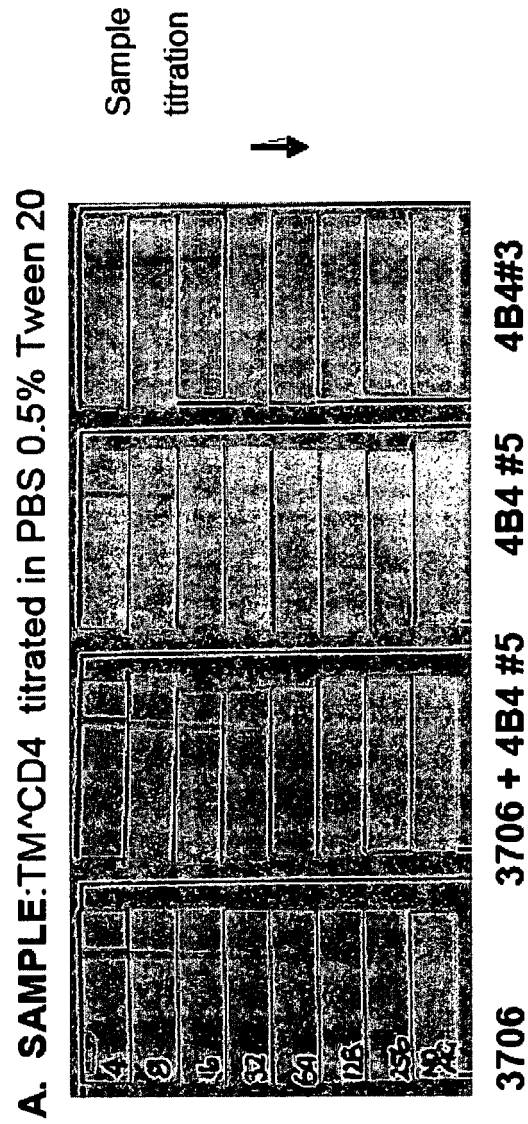


Figure 21

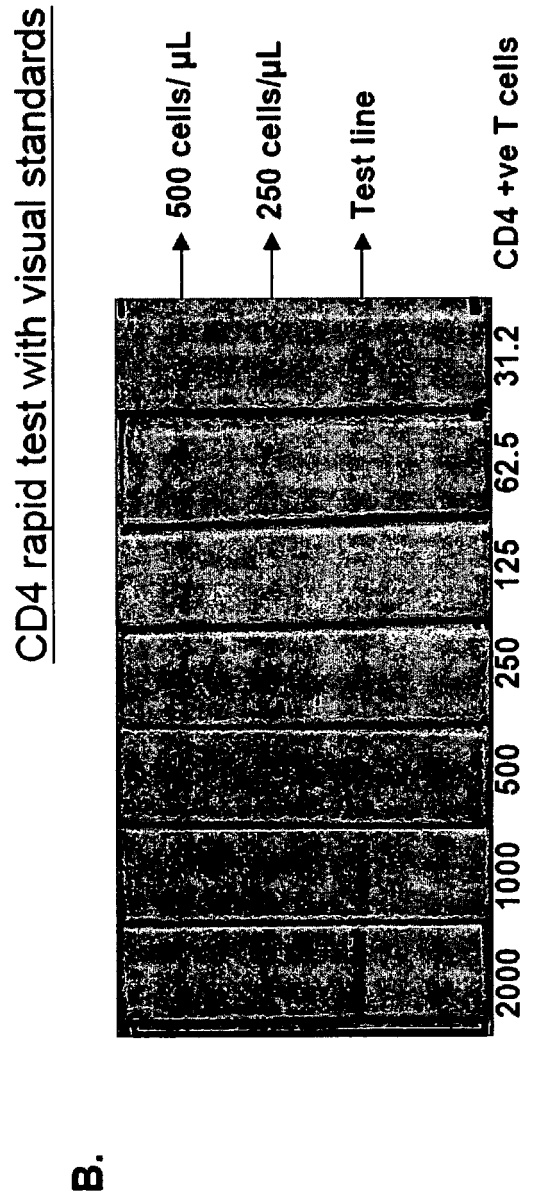
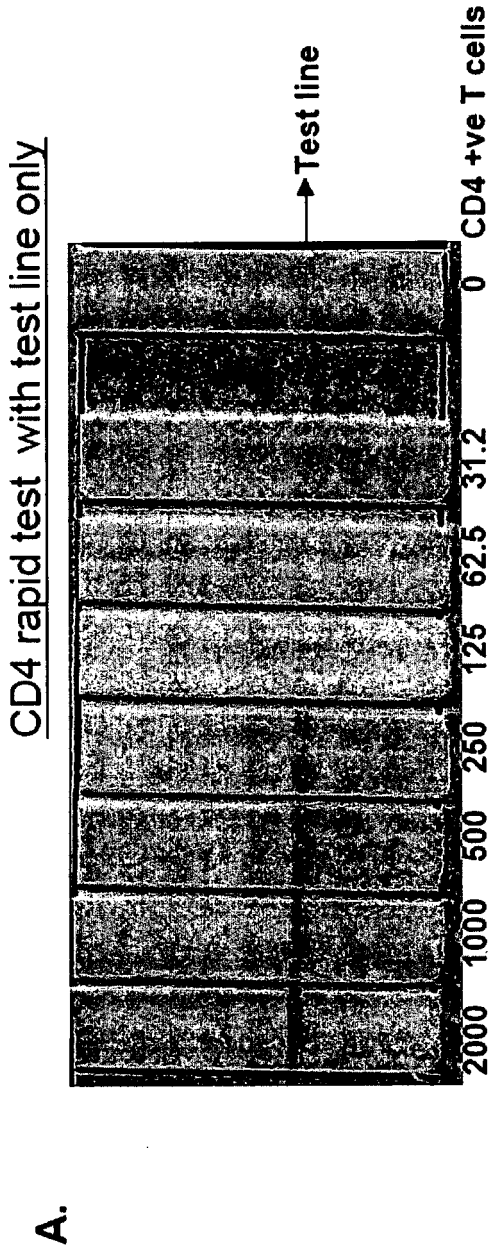


Figure 22

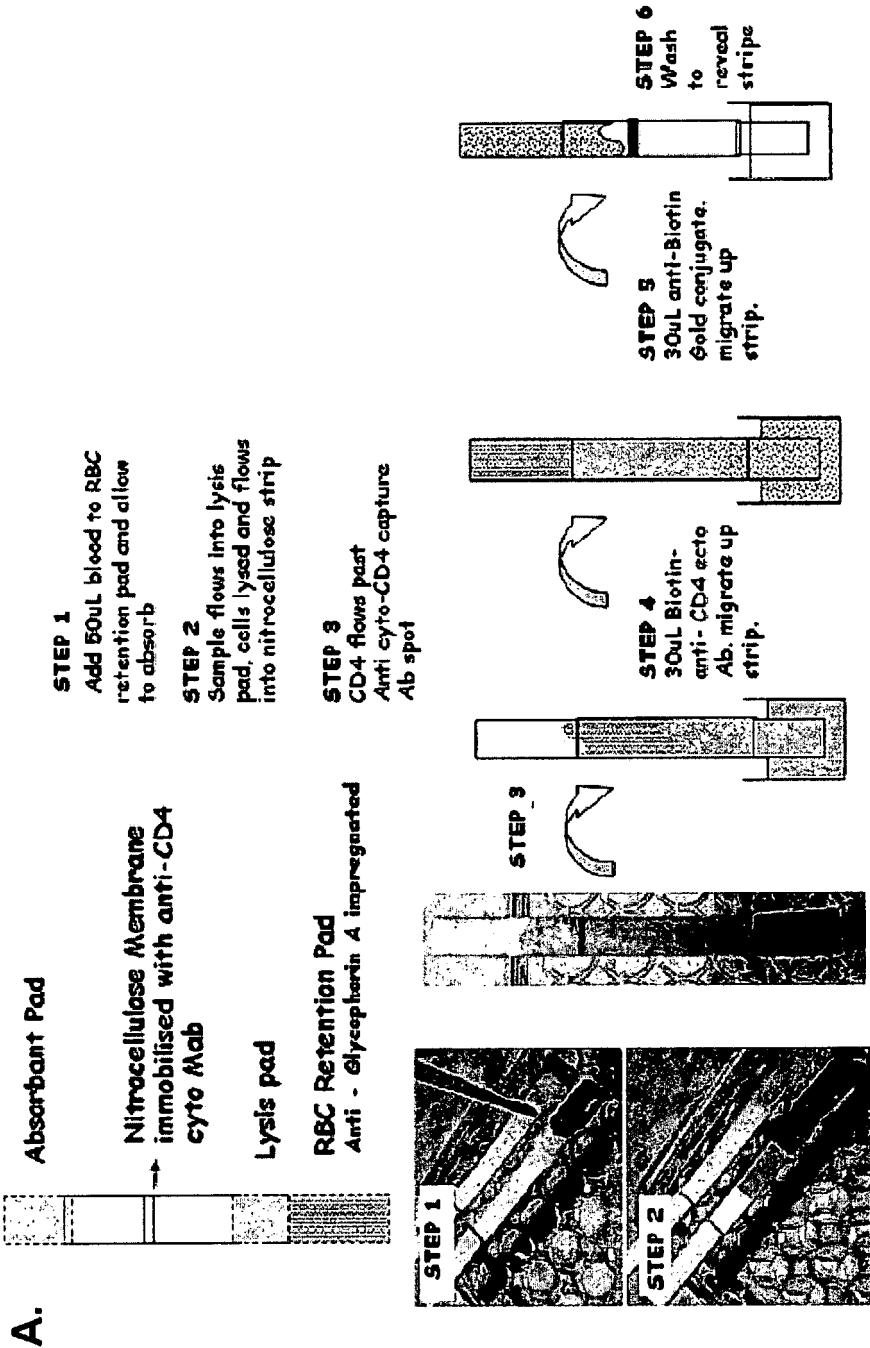


Figure 23

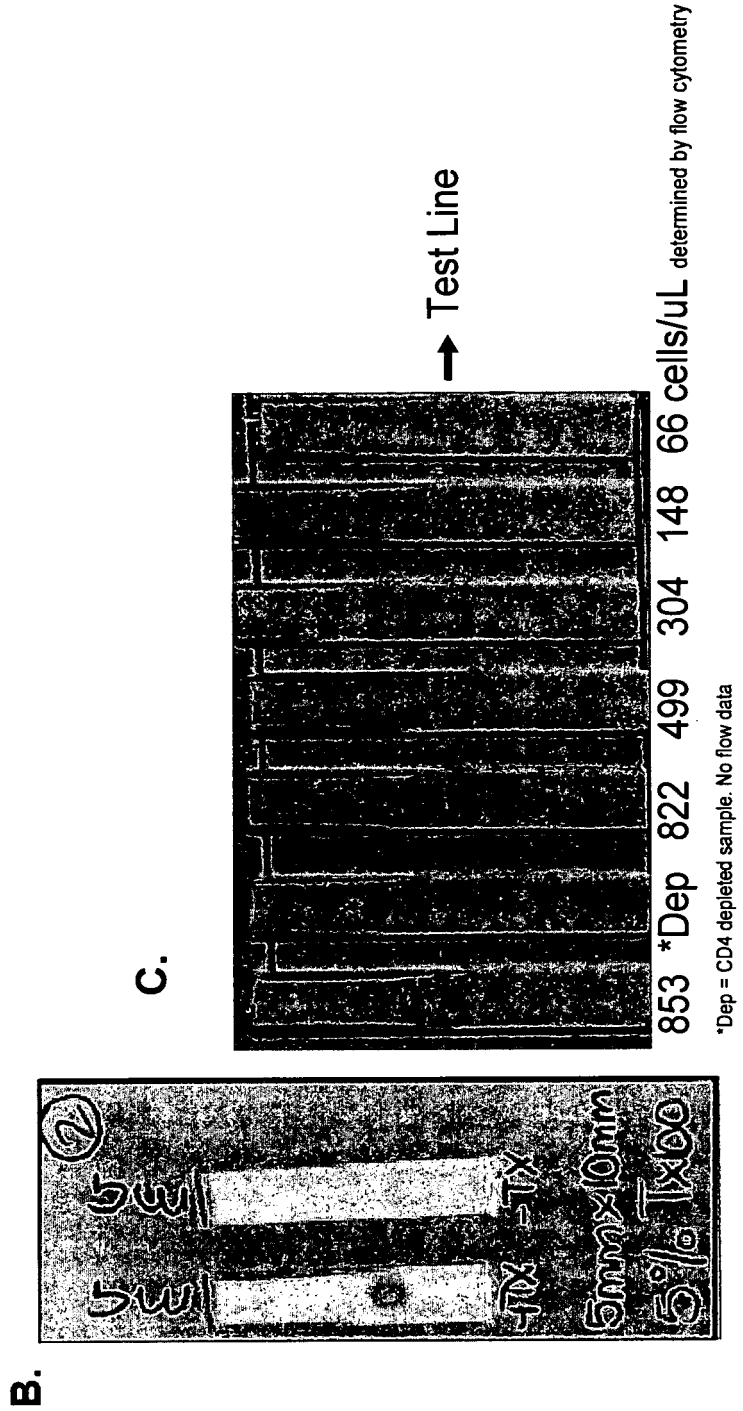
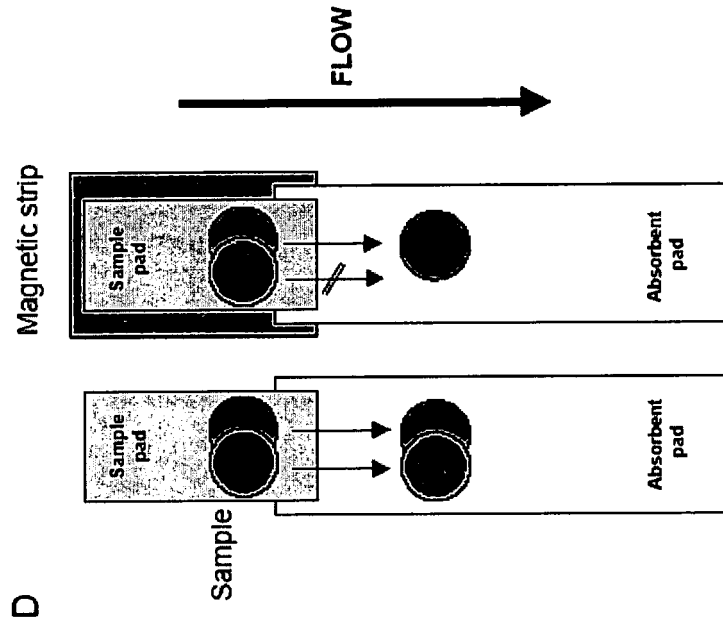


Figure 23 continued

32/32



D

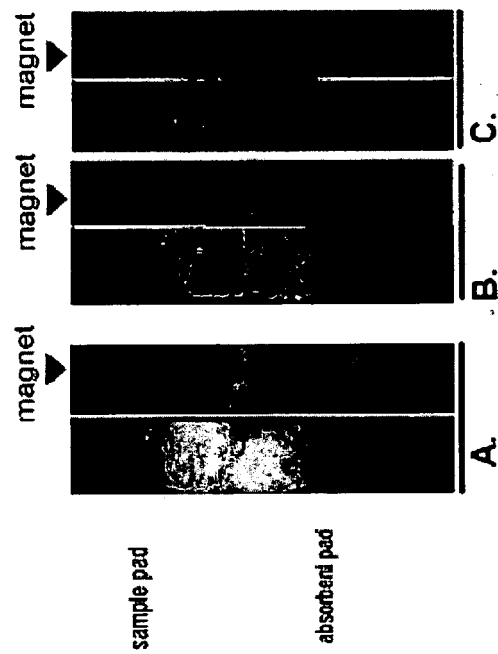


Figure 24

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2007/001449

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

G01N 33/68 (2006.01) **G01N 33/53** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN: Medline, CAlplus, Biosis, WPIDS. Key words: (surface or cell) (3a) (bound or associated); (cytosol? or cytoplas?) (2a) domain; receptor; (bind or detect? or identify? or captur?); antibody?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PAXTON, H et al., "Comparison of CD4 Cell Count by a Simple Enzyme-Linked Immunosorbent Assay Using the TRAx CD4 Test Kit and by Flow Cytometry and Hematology", <i>Clinical and Diagnostic Laboratory Immunology</i> (1995), Vol 2(1): 104-114. Whole document	45, 46
X	CARELLA, AV et al., "A Manual Bead Assay for the Determination of Absolute CD4 ⁺ and CD8 ⁺ Lymphocyte Counts in Human Immunodeficiency Virus-Infected Individuals", <i>Clinical and Diagnostic Laboratory Immunology</i> (1995), Vol 2(5): 623-625. Whole document	45, 46

 Further documents are listed in the continuation of Box C See patent family annex

* Special categories of cited documents:		
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family	
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
16 November 2007

Date of mailing of the international search report

10 DEC 2007

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2007/001449

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOHNSON, D et al., "Evaluation of Four Alternative Methodologies for Determination of Absolute CD4 ⁺ Lymphocyte Counts", Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology (1995), Vol 10(5): 522-530. Whole document	45, 46

专利名称(译)	诊断方法及其试剂盒		
公开(公告)号	EP2067045A4	公开(公告)日	2010-09-08
申请号	EP2007815265	申请日	2007-09-28
[标]申请(专利权)人(译)	麥克法蘭博尼特醫學健康研究公司		
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当前申请(专利权)人(译)	有限公司的MACFARLANE BURNET机构		
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发明人	ANDERSON, DAVID, ANDREW LLOYD, ROBYN, ELIZABETH CROWE, SUZANNE, MARY GARCIA, MARY, LOUISE LANDAY, ALAN, LEE		
IPC分类号	G01N33/68 G01N33/53 C07K14/705 G01N33/558		
CPC分类号	G01N33/56972 C07K14/70514 G01N2333/70514 Y10T436/101666 Y10T436/25125 Y10T436/25375		
代理机构(译)	DZIEGLEWSKA , HANNA EVA		
优先权	2006905393 2006-09-28 AU		
其他公开文献	EP2067045A1		
外部链接	Espacenet		

摘要(译)

本发明提供了用于检测或监测样品中细胞数量的试剂盒和方法。该细胞包含细胞表面相关蛋白 (CSAP) ， 其包含细胞质 (细胞质) 和细胞外 (胞外) 结构域。该试剂盒包括： (i) 色谱装置; (ii) CSAP结合剂。该方法包括： (i) 任选地使样品与能够裂解或透化带有CSAP的细胞的试剂接触; (ii) 使样品与CSAP结合剂接触，所述CSAP结合剂结合CSAP的细胞质结构域; (iii) 直接或间接评估样品中结合的CSAP的水平或存在。