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<b>(54) Title:</b> METHOD AND DEVICE FOR DETECTING OR MEASURING THE AMOUNT OF A CELL-ASSOCIATED MOLECULE			
<b>(57) Abstract</b> <p>The invention is directed to methods and a device for detecting or measuring the amount of a cell-associated molecule in a biological fluid sample. Cell-associated molecules which can be detected or measured include, but are not limited to, cell surface antigens, intracellular cytokines, microbial antigens, pharmacological agents or their metabolites, and nucleic acids. In the preferred embodiment, a sample of whole blood is collected on filter paper and dried. A dot is punched from the dried blood spot and the analyte of interest eluted from the paper with a buffered, relatively highly concentrated detergent solution. The eluate is then assayed in a standard assay, such as an immunoassay.</p>			

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**METHOD AND DEVICE FOR DETECTING OR MEASURING  
THE AMOUNT OF A CELL-ASSOCIATED MOLECULE**

**INTRODUCTION**

This application is a continuation-in-part of  
5 Serial No. 968,793, filed October 30, 1992.

**BACKGROUND OF THE INVENTION**

There are an enormous number of cell-associated  
molecules including cell surface antigens, intracellular  
cytokines, microbial antigens, pharmacological agents and  
10 their metabolites, and nucleic acids. Many of these cell-  
associated antigens are involved in the varied cell to  
cell interaction and communications that are important to  
immune function. Still others are important markers for  
immune dysfunction and other disease states. Detection of  
15 specific molecules can be a valuable aid in analyzing  
normal as well as abnormal situations.

**Cytokines**

Cytokines are a large, diverse group of  
bioactive proteins and peptides generally having  
20 relatively low molecular weights which regulate a large  
number of cellular activities. For example, cytokines  
regulate immunoglobulin production by B lymphocytes and  
the biosynthetic activities of various cell types.  
Interleukin-2 (IL-2), originally discovered as a T-cell  
25 growth factor, and other immunologically active cytokines  
are associated with T-cell function and with the growth  
and differentiation of a variety of hematopoietic cell  
types.

Cytokines are generally produced in a non-  
30 constitutive manner, usually requiring activation or  
stimulation of the producing cell, presumably through a

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cell-surface receptor. While many of the better characterized cytokines are produced by the cells of the immune system, cytokines are also produced by a wide variety of cell types. Typically, a given cell type produces a number of different cytokines. Cytokines have been implicated in a wide variety of immune and inflammatory responses. Their actions are organized in complex interacting networks, wherein the release of one cytokine may lead to a diverse array of physiological responses, some due to the induction of yet other cytokines.

The vast majority of research on cytokine production and action was performed using *in vitro* culture systems. In fact, most cytokines are not detectable in the blood stream, even during an immune response. Because cytokines active in immune responses appear to have been designed to act over a short range and a short time interval, they are generally active at very low concentrations. Most cytokine research is based on measurement of the biological activity of cytokines in various bioassays. Cytokine levels in blood have been measured in immunoassays, such as enzyme-linked immunosorbent assay (ELISA). This approach, however, suffers from several limitations when used to detect cell derived cytokines in serum because of their low concentrations and extremely short half lives *in vivo*. The detectability of cytokines in serum by ELISA with monoclonal antibodies is affected by several factors such as: 1) locally produced cytokine *in vivo* is quickly absorbed by target cell receptors and may never reach the plasma; 2) a cytokine's short half-life makes the sampling time critical; and 3) cytokine proteins are usually unstable during sample storage. In general, immunoassays of molecules present in serum are not useful for detecting proteins that do not survive storage. For all the

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foregoing reasons, there is a need for improved methods to detect and measure cytokines in blood samples.

### Microbial Antigens

Some of the most successful microorganisms multiply in, or on the surface of, epithelial cells at the site of entry into the body, resulting in a spreading infection in the epithelium, followed by shedding to the exterior. For example, viral infections are generally confined to epithelial surfaces including Influenza, Parainfluenza, Rhinoviruses, Coronaviruses and Papillomas.

There is a need in the art for a simple, direct and sensitive method for detecting the presence of a microorganism invading the epithelium by detecting or measuring the amount of a molecule or antigen associated with the virus in an epithelial cell, tissue or fluid sample.

Some of the more important viruses regularly establish systemic infections after traversing epithelial surfaces. These include intracellular microorganisms. For obligate intracellular microbes to spread systemically, they must first enter the blood or lymph. This means they must gain access to the lumen of a subepithelial lymphatic or blood vessel as a free organism, or first enter a mobile cell (such as a leukocyte) which is carried through the blood or lymph. Examples of organisms which infect leukocytes include measles and small pox viruses. Such organisms multiply inside the cell while avoiding the intracellular defense mechanisms, such as lysosomal degradation.

It would be of great benefit if it were possible to detect and quantify various pathogenic microorganisms in a simple, direct and sensitive assay of a body fluid containing the organism or host cells harboring the

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organism with minimal handling and processing of the sample. The present invention is intended to provide such assays.

#### **Nucleic Acids**

5           The amount of DNA (deoxyribonucleic acid) or RNA (ribonucleic acid) in a cell is a useful marker for the presence of certain cell-associated molecules. Additionally, detection of DNA or RNA may be useful for determining the presence of malignant or pre-malignant  
10 cells. Normal cells have only a single copy of cellular DNA whereas malignant cells are often polyploid. Conventionally, polyploidy is assessed by flow cytometric analysis of total cellular DNA using DNA binding dyes. The present invention allows a laboratory lacking a flow  
15 cytometer to detect polyploidy or measure the amount of DNA or RNA in a cell by direct assay, thus providing a way of detecting various components, and even tumor cells in a cellular sample.

#### **Cell Surface Antigens**

20           A multitude of molecules, including proteins, glycoproteins, glycolipids, oligosaccharides, and the like are expressed on cell surface membranes. Also detectable, either on the surface, or in the intracellular compartment of a cell, are molecules produced by intracellular  
25 parasites, such as viruses. Cell surface molecules are also present in the cytoplasm. Conventional flow cytometry assays detect or enumerate cells expressing a given molecule on their surface by visualizing or measuring the binding of an antibody to an epitope of that  
30 molecule on the cell. Well known cell surface molecules with clinical significance include T-cell receptor

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molecules. Many such molecules are defined by their reactivities with particular monoclonal antibodies (Mabs). In particular, identification and enumeration of T-cells expressing CD4 and CD8 molecules has been of great clinical utility. Indeed, following the circulating levels of CD4+ cells is a key prognostic indicator for evaluating progression of Acquired Immunodeficiency Syndrome (AIDS).

The two main subsets of T cells are known as CD4 and CD8 and are distinguished by their regulatory function, helper/inducer and suppressor/cytotoxic, respectively. The CD4 molecule is a glycoprotein monomer of approximately 55 kilodaltons with extracellular, transmembrane and cytoplasmic regions. The CD4 molecule can function to transduce intracellular signals and to modulate T cell activation which results in signaling of B cells and induction of CD8+ T cells to become cytotoxic by cytokine release. Also, CD4 may serve as an adhesion molecule in support of anchoring the MHC class II molecule, or antigen presenting cells, with the T cell receptor. As such, the CD4 molecule serves as the phenotypic marker for helper T lymphocytes.

In general, the identification of human B and T lymphocyte subsets by cell surface markers has enabled profiles of immune status to be developed for normal as well as pathological conditions. For instance, the cell surface antigens CD19, CD20 and CD21 are found in greater than 90% of blood B lymphocytes, while CD2 and CD3 are expressed on nearly all T lymphocytes in normal peripheral blood. T lymphocytes, which are responsible for cell mediated immunity, can be further differentiated into two subsets depending on their phenotype, receptor specificity for MHC and cellular function. Generally, the CD4+ CD8- helper T lymphocytes comprise approximately 65% of peripheral T cells and are able to secrete cytokines upon

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antigen activation in association with MHC class II molecules. Another subset, the CD4-CD8+ cytotoxic lymphocytes (about 35% of peripheral T cells) are able to lyse target cells after activation by MHC class I 5 presented antigens.

By using monoclonal antibody reagents to these various cell surface markers in conjugation with flow cytometric methods it has been possible to monitor the changing composition of lymphocyte subsets in peripheral 10 blood associated with various autoimmune and immunodeficiency diseases, leukemias and lymphomas, and transplantation rejection. In the case of AIDS, it has been established that the CD4 molecule is the cell surface receptor for the human immunodeficiency virus (HIV) and it 15 has been shown possible to monitor the depletion of CD4 T lymphocytes which are characteristic of progression to AIDS.

Early methods for assaying lymphocyte subsets such as by plaque and cell-mediated functional assays 20 were highly technique dependent and not cell lineage specific. With the availability of monoclonal antibodies against cell surface markers, the measurement of cell surface CD4 by flow cytometry has expanded the study of lymphocyte subset identification for immune monitoring. 25 However, this method requires the use of complex procedures, expensive instrumentation and is labor intensive. TRAx™ CD4, is an enzyme immunoassay by which total CD4 protein may be determined from a whole blood lysate. The results obtained using this micotiter based 30 enzyme-immunoassay (EIA) correlate at greater than 88% with CD4 T lymphocyte counts as determined by flow cytometry.

The dried blood spot (DBS) specimen collection medium has proven to be a valuable method for 35 epidemiological studies. Neonatal heel stick specimens

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are collected routinely and have been shown to be a useful for the detection of PKU and hypothyroidism, sickle-cell anemia and other hemoglobinopathies, and HIV. In developing countries, adult finger stick DBSS have  
5 provided a way to sample populations and transport stable specimens to the testing laboratories. However, this technique has not heretofore been applied to techniques for determining cell-associated molecules which are contained within the cell or on the cell surface. Until  
10 now, a dried blood collection method has not been used for the determination of CD4 lymphocytes in blood.

#### SUMMARY OF THE INVENTION

Methods of assaying for cell associated antigens are provided. In one embodiment, a sample of whole blood  
15 is assayed for the total amount of a selected cell-associated antigen, for example, CD4. In a specific embodiment, a blood sample is spotted onto filter paper, the filter paper is dried and placed in a vial containing a selected diluent and lysis reagent, and incubated for a  
20 selected period of time at a selected temperature. A sample of a selected size is then recovered from the vial and assayed in a standard assay, for instance an immunometric assay. Cell associated antigens such as CD4, CD8, CD3, CD19, CD25 (IL-2R), CD2, CD56, CD54 (ICAM-1),  
25 intracellular viruses, cytokines and nucleic acids can be measured according the methods of the instant invention. Cell associated antigens are found either within the cell or on the cell surface, as opposed to soluble antigens which are in the blood plasma fraction. A device for use  
30 in the assay of the invention is also provided.

A further embodiment of the present invention provides for determining the approximate number of cells positive for a given cell associated molecule in a sample.

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According to this aspect of the present invention the methods of measuring a cell associated molecule can be used to enumerate cells that express a particular cell associated molecule. The present inventors have  
5 discovered that an amount of a cell associated molecule can be recovered from a sample collected on a paper or other suitable collection device that correlates with cell number. According to the present methods, an amount of cell associated molecule from a sample can be obtained  
10 that exhibits a linear correlation with measurements obtained according to conventional cell number detection methods. The present methods of the present invention offer an advantage over methods of the prior art.

According to the present methods, a sample  
15 is assayed for the total amount of a selected cell-associated molecule, for example, CD4. A sample, preferably a blood sample, more preferably a whole blood sample, is collected onto a suitable collection material, preferably, a filter paper. The filter paper is dried and  
20 the cell associated molecule is recovered. In a preferred aspect the cell associated molecule can be recovered by placing the paper or other suitable collection material in a vial containing a selected recovery agent, for instance, a diluent and lysis reagent, and incubated for a selected  
25 period of time at a selected temperature. A sample of a selected size is then recovered from the vial and assayed in a standard assay, for instance an immunometric assay. Detection of the cell associated sample by immunoassay provides an indication of the amount of a cell associated  
30 molecule present in a sample. According to the present invention the amount of a cell associated sample can then be correlated with a cell number.

**DESCRIPTION OF THE DRAWINGS****SUBSTITUTE SHEET (RULE 26)**

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Figure 1 is a graph showing the correlation of dried blood specimens to lysate in a CD4 TRAx™ assay. CD4 dried blood specimens values are in units per ml (U/ml). A unit is defined as the amount of CD4 found in  $10^3$  Jurkat 5 cells lysed with 1% NP-40.

Figure 2 is a graph showing the correlation of dried blood specimens in a CD4 TRAx™ assay to flow cytometry. CD4 dried blood specimens values are in units per ml (U/ml). A unit is defined as the amount of CD4 10 found in  $10^3$  Jurkat cells lysed with 1% NP-40. The conversion of U/ml to equivalent CD4 count is based upon the linear relationship established between U/ml and CD4 cell count as determined by flow cytometry. Data from clinical studies of patients with a broad range of CD4 15 levels was used to derive the conversion equation. The values for the standards for the TRAx™ assay kit are calibrated to CD4 T lymphocytes and specimen results are read directly from the standard curve and reported as cell/ $\mu$ l.

## 20 DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to the detection or measurement of the total amount of a cell associated molecule (hereinafter "target molecule") in a sample and the use of such detection or measurement in the 25 detection, diagnosis, staging, determination of severity, and therapy of diseases and disorders, and in the enumeration of cells expressing the target molecule. The methods and device of the invention allows detection or measurement of target molecules heretofore not readily 30 accessible for detection by binding to a binding partner such as an antibody.

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Any target molecule for which a binding partner is available can be detected or measured according to the present invention. The target molecule may be endogenous (a molecule that is encoded by the genome of the host cell 5 or is produced by the host cell). Examples of endogenous target molecules include cytokines, cell adhesion molecules, phenotypic markers, activation markers, and nucleic acids.

The target molecule may also be an exogenous 10 molecule (a molecule encoded by a genome not of the host cell or otherwise produced by an exogenous agent). Typical exogenous target molecules which may be measured according to the present invention are molecules associated with microbial pathogens such as viral 15 antigens.

Non-limiting examples of target molecules for which the present invention is intended to apply are proteins, peptides, glycoproteins, glycopeptides glycolipids, polysaccharides, oligosaccharides, nucleic 20 acids, pharmacological agents and their metabolites, and the like, or fragments thereof. Preferred target molecules according to the present invention are microbe-associated molecules, cytokines, adhesion molecules, pharmacological agents or their metabolites, and the like. 25 In a specific embodiment, the invention provides a method for detecting or measuring the total amount of a target molecule which is intracellular. In another embodiment, the target molecule is a cell surface molecule. As used herein, by "cell-associated molecule" is meant that the 30 molecule is attached to the cell surface or is intracellular.

Depending on the sample and nature of the target molecule, the measurement of a total amount of target molecule can substitute for direct enumeration of cells 35 expressing the target molecule and can be valuable in

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monitoring the effect of a therapeutic on a subject, detecting, diagnosing or staging a disease in a subject, and predicting therapeutic outcome or disease prognosis and in evaluating and monitoring immune status of 5 patients.

Binding partners for use in the assays of the invention include, but are not limited to, receptors for and antibodies to the target molecules. In particular, binding partners which are not antibodies include cell 10 surface receptors for the target molecules.

In a preferred embodiment, the target molecule is an antigen, and detection or measurement of the target molecule is carried out by a method of immunospecific binding of the target molecule to at least one antibody. 15 All antigens which carry an antigenic determinant or epitope which can be detected or quantitated in an immunoassay are intended to be within the scope of the present invention.

A preferred subject from which one may obtain 20 samples for the methods of the present invention is a vertebrate, including but not limited to, a mammal, fish, amphibian, reptile, bird, marsupial and most preferably, a human. Thus, the methods of this invention are applicable to human clinical and veterinary uses.

25 As used herein, a "sample" refers to a collection of cells in the milieu in which they were obtained, i.e., a biological fluid, or to membrane and/or intracytoplasmic components of the cells. Total molecules may be measured in samples derived from a biological 30 fluid, e.g., whole blood, plasma, serum, blood cells, saliva, urine, synovial fluid, pleural effusions, tumor and tissue infiltrates, amniotic fluid, spinal fluid or cranial fluid. In another embodiment, the biological fluid may be cell culture fluid. The sample can comprise 35 tissue, including interstitial fluid. Preferably, when

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the sample is a tissue sample, the tissue is treated to disrupt the connective tissue matrix, e.g., by trypsin digestion or homogenization. In another embodiment, the sample comprises cells derived from the foregoing sources.

5 In a preferred embodiment, the sample comprises a blood sample, and more preferably a whole blood sample. By whole blood is meant unfractionated blood as would be collected from a needle puncture.

The amount of sample used is a function of the  
10 type of target molecule and its abundance in the preparation, and can be determined by one of ordinary skill in the art without undue experimentation.

The method provided by the present invention, as described in more detail below, overcomes many of the  
15 limitations of prior art methods, and in particular, with regard to the measurement or detection of cell associated molecules, which heretofore required cell isolation followed by direct or indirect immunofluorescence analysis by microscopy or flow cytometry. Limitations of the prior  
20 art procedures included the requirement for fresh sample, fairly large sample size, enriched cell populations rather than whole tissue or blood, extensive preparation time and expensive equipment such as a flow cytometer. The methods provided herein overcome these limitations. Surprisingly,  
25 the methods and device as taught herein are suitable in assaying various components of fresh samples, i.e. whole blood, and in relatively low volumes of samples, i.e. about 20-100  $\mu$ l. Thus, sample collection involves techniques as simple as a fingerstick. The samples are  
30 then stable enough to be transported by mail or otherwise, to a central site for analysis, thus eliminating the need for fresh blood samples and phlebotomy.

In the method steps of the presently claimed invention, a selected amount of a liquid biological sample

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is collected onto a suitable collection material. In one embodiment the sample is spotted onto a paper collection device. Collection material suitable within the context of the present invention includes, without limitation 5 filter paper. Filter paper suitable for purposes herein are those generally known to the art and commercially available. Various grades are generally available and relate to the porosity of the paper, as well as its absorbency, consistency and the material it is made out 10 of.

By "spotted" as used herein, is meant that the sample is contacted with the, for example, filter paper collection material, and transferring all or a portion of the sample to the device. In the case of filter paper 15 this is accomplished by wicking as the sample is absorbed into the component fibers of the filter paper.

One skilled in the art should optimize these parameters to achieve overall evenness of the sample dispersion across a defined area of the filter paper, so 20 that when a dot is punched out, the concentration of the sample will be relatively uniform on a sample to sample basis. In certain preferred embodiments, for example when leukocyte markers are the target molecules in a whole blood sample, filter paper comprising 100% cotton fibers 25 is preferred. In this scenario, mean serum uptake of about 1.4 to about 1.7  $\mu$ l per filter paper spot, with about 1.5 to about 1.6  $\mu$ l is particularly preferred. Serum uptake is a conventional measurement of absorbance, and thus absorbency may be determined and optimized using 30 this technique. It is generally preferred that absorption time be within about one minute, with 5-30 seconds more preferred. Consistency may be measured by wicking time and evenness of flow. Porosity relates to both absorbing and consistency, as the pore size and pore distribution of 35 the paper affects these aforementioned parameters. In

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certain embodiments, synthetic fabrics, both woven and nonwoven, such as polyester, or Gortex™ may offer advantages in overall assay performance.

Amounts of sample suitable for this purpose are relatively small, that is to say that amounts ranging from about 150 microliters to about 10 microliters are preferred, with about 80 microliters to about 40 microliters particularly preferred. The sample so transferred is preferentially allowed to dry under room temperature conditions that may generally range from about 18°C to about 29°C, with about 20°C to about 26°C being more typical, or at about 30°C to 37°C for a lesser period of time, if long-term stability of the sample is not required. This drying step is generally from about one to four hours. At this point, the sample may be stored until it is appropriate for further analysis, or may be immediately subjected to further handling as provided herein. The dried sample may be sent to a central testing facility for such further handling, or may be subjected to such steps on the site the sample was taken.

A suitable amount of the dried sample is removed from the spotted area and contacted with a selected diluent combined with a lysis reagent for a period of time sufficient to solubilize said sample. In general, a 1/4 inch diameter dot holds approximately 12  $\mu$ l of sample, which may be a suitable sample size for certain assay conditions, while more or less may be required for other assays. One skilled in the art may determine their needs in this regard through routine optimization. For example, smaller dots could be used for analytes of relatively low concentrations, i.e. less than about  $10^{-11}$ , while multiple dots (about 5.5 mm in diameter) could be used for higher concentrations, i.e. greater than about  $10^{-11}$ . By "solubilize" is meant that solid portions of the biological sample (i.e. intact cells) are lysed, allowing

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the target molecules to be released into the diluent and lysis reagent. In preferred embodiments, this period of time is at least about two hours, at room temperature. Suitable diluents include physiologically compatible buffers, such as phosphate-buffered saline, saline, Dulbecco's Media, and the like. A particularly preferred buffer comprises phosphate buffered saline, preferably supplemented with at least one protein, such as a bovine-derived serum protein. suitable lysis reagents comprise at least one non-ionic detergent. Of these may be mentioned non-ionic detergents in the Triton series such as Triton X-100, X-114, X-305, X-405, those of the Brij series such as Brij 35, 56, 58, those of the Tween series, such as Tween 20, Tween 80, Nonidet P-40 (NP40), and zwitterionic detergents such as CHAPS and CHAPSO, and the like. Preferred for use herein is a combination of Triton X-100 and NP40, in relatively high concentrations. Preferred concentrations for contact with the sample range from about 6-10% and 4-7%, respectively, with about 9% and 6%, respectively, particularly preferred when leukocyte markers are the target molecule (all measured on a concentration basis that is not a final concentration when considering the dilution factor of the liquid sample). The solubilized sample may be processed further in accordance with the method taught herein or stored until further handling is desired, and when stored, preferably frozen at least about -20°C, with about -20°C to at least about -70°C more preferred, and at least about -70°C particularly preferred.

30 A selected amount of said sample so solubilized is collected and contacted with at least one binding partner specific for the target molecule sought to be detected or enumerated. This step may be performed in accordance with well-accepted immunoassay detection kits and the like for this purpose. As an example, in the case

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of analysis for detection of the CD4 receptor, a TRAX™ CD4 Assay Kit, available from T Cell Diagnostics, Inc., Cambridge, MA, (see, International Patent Application No. WO92/08981, published May 29, 1992, by Rittershaus, C.) 5 may be utilized for this purpose by following the assay protocol provided with that kit. This may be accomplished with other such conventional assay techniques or even commercially available assay kits for the assay of other cell surface receptors and the like.

10 In a preferred embodiment, capillary or venous blood samples are collected on filter paper and dried. A suitable area is taken from the dried blood spot, for example, a 5.5 mm (0.25 inch) dot is punched from the dried blood spot and the analyte eluted from the paper 15 with a buffered detergent solution for at least 2 hours at room temperature.

Fifty microliters (50  $\mu$ l) of the eluate is then assayed in a standard TRAX™ CD4 microtiter plate ELISA assay which is intended for the determination of CD4 cell 20 counts in liquid blood samples. Assay CVs are less than 8% for both intra and inter assay. Assay sensitivity is about 86-130 cells/ $\mu$ l, and particularly 86 cells per  $\mu$ l, and correlation with flow cytometry is approximately 90%. Samples are stable for several months when stored with 25 desiccant at 4°C. The solubilized sample may also be stored frozen after detergent treatment, for example at -20°C, preferably at -70°C. This has the advantage of allowing the concurrent analysis of multiple samples taken at different times in a single assay, thus reducing 30 interassay variability. The methods of the invention show great promise in areas where access to flow cytometry is limited.

The filter paper collection device of the invention may be provided in a separate packaged format 35 suitable for "in field" retrieval of biological samples.

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Also provided will be a container to retain the sample once the paper is spotted, which helps to contain the possible infectivity of the sample. Additionally, either in conjunction with the filter paper collection device or  
5 separately, the remaining reagents useful for the practice of the present invention can be conveniently provided in a kit. The essential elements of this portion of a kit of the invention are a concentrated non-ionic detergent solution and a means for immunologically detecting the  
10 presence of an antigen.

The kit can comprise any immunological detection means known in the art, i.e. sandwich assay formats, competitive immunoassay formats, and the like. Preferably, the immunological detection means are a first  
15 capture antibody specific for the cell-associated molecule, and a second detectable antibody specific for the cell-associated molecule. The first antibody can be provided on a solid support, such as a glass or plastic bead, membrane, a plastic stick, a microwell, a glass or  
20 plastic test tube, or other solid supports known in the art for immunoassays. Alternatively, the first antibody can be provided for later immobilization. The first and second antibodies can be provided as a lyophilized preparation for reconstitution, or as a concentrated  
25 solution. The second antibody can be detectable by labeling means. In a preferred embodiment, the labeling means is an enzyme.

The detergent solution is provided in a container having sufficient volume to hold the amount of  
30 solution necessary for the number of assays contemplated by the kit. Preferably, the detergent solution comprises two or more non-ionic detergents. In a preferred embodiment, the detergent solution comprises 9% Triton X-100 and 6% N-40 in distilled water. That particular

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detergent concentration is optimum with respect to an assay for both CD4 and CD8.

Preferably, the kit comprises a cell-associated molecule standard, either in solution or lyophilized for 5 reconstitution. More preferably, the kit comprises a standard comprising a known number of cell positive for the molecule of interest. Thus, the activity in an assay for the cell-associated molecule in an unknown sample can be directly compared with a known sample, and the number 10 of cells in the unknown sample that are positive for the molecule enumerated thereby.

According to the present invention, the number of cells positive for a leukocyte marker in a sample can be determined by (1) determining the amount of a cell 15 associated molecule in a sample according to the methods of the present invention, and (2) calculating the total number of cells positive for the cell associated molecule from the amount of the cell associated molecule. In one embodiment, the calculation can be made by extrapolating 20 from a standard curve of total cell associated molecule versus total number of cells positive for the cell associated molecule. In another embodiment a formula can be derived from a standard curve, and the amount of a cell associated molecule plugged into the formula.

25 The kit can further comprise a reagent reactive with label, when the second antibody is detectable by attachment of a label to the antibody. For example, when the label is an enzyme, the kit can provide the enzyme substrate. The kit can also provide a dilution buffer, in 30 final concentration, high concentration for dilution or dry for reconstitution.

In yet a further embodiment, a kit provides immunological detection means for more than one molecule. In a preferred embodiment, the kit comprises immunological 35 detection means for both CD4 and CD8 antigens.

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The invention is further illustrated by the following, non-limiting examples.

**EXAMPLES****Example 1 Assay Protocol**

5 After a skin puncture or finger prick, whole blood is spotted onto Schleicher and Schuell, Inc. (Keene, NH) grade 903 filter paper. Circular marks are filled with about 60  $\mu$ l of blood. The paper is dried for about two hours at room temperature and either used immediately  
10 or stored with a desiccant, in a closed container at about 4°C.

A 0.25 inch dot is punched from a dried blood spot and placed into a 12x75 mm glass tube. 100  $\mu$ l of Eluting Buffer is added. The Eluting Buffer is a 6:1  
15 solution of TRAx™ CD4 Sample Diluent (T Cell Diagnostics, Inc; Cambridge, MA) and TRAx™ CD4 Lysis Reagent (T Cell Diagnostics, Inc; Cambridge, MA) e.g., 1.0 ml Sample Diluent (a buffered solution with bovine-derived serum proteins and thimerosal) plus 0.2 ml Lysis Reagent (6%  
20 Nonidet P-40 (NP-40) and 9% Triton X-100 in 1X PBS). The glass tube is then covered and placed on a shaker at 200 rpm for at least 2 hours at room temperature. At the end of this period, 50  $\mu$ l is removed from the glass tube after carefully squeezing the dot with the tip of the pipette  
25 and mixing the solution. The 50  $\mu$ l sample is added to a standard TRAx™ CD4 assay following the protocol in the TRAx™ CD4 Assay Kit insert (T Cell Diagnostics, Inc; Cambridge, MA).

**Example 2 TRAx™ CD4 Assay****SUBSTITUTE SHEET (RULE 26)**

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The TRAx™ CD4 Assay is a sandwich EIA for the quantitative measurement of total CD4 protein and for the enumeration of CD4 lymphocytes in human blood. Results obtained using this assay have been demonstrated to correlate to absolute CD4 T lymphocyte counts as determined by flow cytometry combined with hematological data. A simple pretreatment of whole blood specimens release the CD4 protein from the lymphocytes. Specimens can then be assayed by EIA and/or stored at -20°C to -70°C for later study. The assay is performed in microtiter wells which have been pre-coated with monoclonal antibody to human CD4 protein. A second anti-CD4 monoclonal antibody conjugated to horseradish peroxidase is pipetted into the wells, followed by standards, controls or specimens. The solubilized CD4 protein present in the standards, controls or specimens binds to the antibody coated on the plate while the conjugated antibody binds to a second epitope on the CD4 molecule completing the sandwich. After an incubation period, the wells are washed thoroughly to remove unreacted components and an enzyme-specific chromogen solution is added. After an additional short incubation, the reaction is terminated and the absorbance at 490 nm measured. The colored endpoint which results is directly proportional to the amount of CD4 protein present and is equivalent to the number of CD4 T lymphocytes in the original specimen. A standard curve is prepared from standards and control and specimen values are determined from the standard curve and reported as cells/ $\mu$ l.

**Example 3            CD4 TRAx™ Paper Dot Assay-Spike and Recovery**

Five fresh whole blood samples were collected in EDTA and one half of the volume of each sample was

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depleted of CD4. This was used to dilute the remaining half of the sample so as to create the addition of a known amount of CD4, when measured. The addition was at four different concentrations. These were spotted on S&S 903 5 filter paper according to the procedure described in Example 1, as well as the high specimen (neat) and depleted portions of the sample. The dilutions performed cover the range of the standard curve. The assay is performed according to the procedures described in the 10 foregoing examples. The recoveries were calculated based on the amount of recovered divided by the amount added (determined from the neat and depleted values). Results are shown in Table 1. The recovery for the addition of a known amount of CD4 into five separate fresh whole blood 15 spotted samples over the range of the standard curve averaged 98%.

TABLE 1

	SAMPLE	CD4 ADDED U/ml	RECOVERED CD4 U/ml	%CD4 RECOVERED	AVERAGE RECOVERY
	929	80			
5	1:1.5	54	52	96	101%
	1:2	43	46	107	
	1:3	31	31	100	
	1:4	24	24	100	
	0	6			
10	931	82			
	1:1.5	56	54	96	92%
	1:2	43	41	95	
	1:3	30	27	90	
	1:4	23	20	87	
15	0	4			
	932	101			
	1:1.5	69	65	94	94%
	1:2	53	49	92	
	1:3	38	35	92	
20	1:4	29	29	100	
	0	6			
	933	64			
	1:1.5	44	44	100	105%
	1:2	34	36	106	
25	1:3	24	25	104	
	1:4	19	21	110	
	0	4			
	934	92			



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SAMPLE	CD4 ADDED U/ML	RECOVERED CD4 U/ML	%CD4 RECOVERED	AVERAGE RECOVERY
1:1.5	63	64	102	97%
1:2	50	46	92	
1:3	36	32	89	
1:4	28	29	104	
5 0	7			

**Example 4      CD4 TRAx™ Paper Dot Assay-Linearity**

Eleven fresh whole blood samples were collected in EDTA and spotted onto S&S 903 filter paper according to the procedure described in Example 1. A portion of each sample was saved for flow determinations and for lysate preparation. The dilutions performed on the spots cover the range of the standard curve. The assay is performed according to procedures described in Examples 1 and 2. The recoveries are calculated based on the neat value. Results are shown in Table 2. The recovery for parallel dilution of eleven fresh whole blood spotted samples over the range of the standard curve averaged 112%.

TABLE 2

SAMPLE	DILUTION	U/ML MEASURED	U/ML CORRECTED	RECOVERY	AVERAGE RE-COVERY
761	NEAT	78.9	78.9	-	111%
	1:2	41.0	82.0	104%	
	1:3	30.9	92.7	118%	
	1:4	22.2	88.8	112%	
5 816	neat	88.2	88.2	-	109%
	1:2	45.1	90.2	102%	
	1:3	33.0	99.0	112%	
	1:4	24.6	98.4	112%	
817	neat	144.6	144.6	-	118%
	1:2	75.5	151.0	104%	
	1:3	55.1	165.3	114%	
	1:4	43.1	172.4	119%	
	1:6	32.7	196.2	136%	
830	neat	77.5	77.5	-	120%
	1:2	43.1	86.2	111%	
	1:3	29.9	89.7	116%	
	1:4	26.0	104.0	134%	
832	neat	81.5	81.5	-	112%
	1:2	51.6	103.2	127%	
	1:3	26.3	78.9	96.8%	
	1:4	22.6	90.4	111%	

SAMPLE	DILUTION	U/ML MEASURED	U/ML CORRECTED	RECOVERY	AVERAGE RE-COVERY
883	neat	75.4	75.4	-	113%
	1:2	40.2	80.4	107%	
	1:3	30.4	91.2	121%	
	1:4	21.1	84.4	112%	
884	neat	56.7	56.7	-	101%
	1:2	30.3	60.0	106%	
	1:3	18.8	56.4	99.5%	
	1:4	13.9	55.6	98.0%	
886	neat	65.5	65.5	-	113%
	1:2	35.6	71.2	109%	
	1:3	25.7	77.1	118%	
	1:4	18.3	73.2	112%	
888	neat	68.1	68.1	-	114%
	1:2	40.3	80.6	118%	
	1:3	25.8	77.4	114%	
	1:4	18.5	74.0	109%	
5 891	neat	97.6	97.6	-	103%
	1:2	54.3	108.6	111%	
	1:3	33.0	99.0	101%	
	1:4	23.7	94.8	97.1%	
896	neat	127.8	127.8	-	116%
	1:2	72.0	144.0	113%	
	1:3	48.8	146.4	115%	

SAMPLE	DILUTION	U/ML MEASURED	U/ML CORRECTED	RECOVERY	AVERAGE RE-COVERY
	1:4	35.5	142.0	111%	
	1:6	26.7	160.2	125%	
	1:8	18.2	145.6	114%	

**Example 5 CD4 TRAx™ Paper Dot Assay-Intraassay Precision**

Within one assay, 10 samples are assayed in triplicate (i.e., 3 spots for each sample), with the 5 results as shown in Table 3. The average correlation value (CV) for the triplicate (spots) of the above 10 samples is 4.1%.

TABLE 3

	SAMPLE	1	2	3	AVE. n=3	CV n=3
10	772	67.1	64.7	67.7	66.5	2.4%
	773	47.4	46.7	45.1	46.4	2.5%
	774	69.2	66.8	69.9	68.6	2.3%
	775	89.6	93.4	102.7	95.2	7.1%
15	776	47.9	45.8	44.4	46.0	3.8%
	777	54.7	52.1	54.7	53.8	2.7%
	778	37.4	39.2	41.9	39.5	5.8%
	779	66.8	68.5	67.5	67.6	1.3%
	780	48.6	53.9	53.6	52.0	5.8%
20	781	90.4	95.4	105.3	97.0	7.8%

**Example 6 CD4 TRAx™ Paper Dot Assay-Interassay Variation**

Four samples were assayed for interassay variation. The results are shown in Table 4.

TABLE 4

5	DATE	SAMPLE 997	SAMPLE 998	SAMPLE 1000	SAMPLE 1001
	3/16/93	105.6	87.0	101.5	76.9
	3/17/93	106.1	76.7	99.3	84.3
	3/18/93	108.4	74.4	102.8	76.8
	3/19/93	110.6	74.1	98.4	80.1
10	3/23/93	91.0	71.0	95.8	77.2
	3/23/93	97.5	75.2	93.4	71.9
	3/25/93	120.6	80.7	100.8	81.9
	3/26/93	109.5	80.9	99.5	83.2
	3/30/93	101.5	74.2	94.2	75.9
15	3/30/93	100.3	79.7	92.2	72.3
	3/31/93	109.6	75.4	93.9	76.1
	3/31/93	98.9	65.4	92.9	73.9
	4/02/93	97.2	67.6	94.3	73.5
	4/06/93	116.5	73.9	97.9	85.6
20	4/07/93	102.8	72.5	90.6	79.5
	4/08/93	113.6	81.7	95.8	77.0
	4/13/93	106.2	86.5	95.6	73.0
	4/16/93	114.3	76.6	88.9	77.1
	4/20/93	107.3	72.7	98.8	-
25	4/20/93	-	79.0	-	-

An average CV of 5.9 was determined as follows:

sample	n	mean	SD	CV
997	19	106.2	7.4	7.0
998	20	76.3	5.5	7.2
30 1000	19	96.1	3.8	4.0
1001	18	77.6	4.1	5.3

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**Example 7      CD4 TRAx™ Paper Dot Assay-Correlation of  
Dried Blood Specimens to Lysate**

The correlation of dried blood specimens to lysate was determined. The results are shown in Table 5 and Figure 1.

TABLE 5

	SAMPLE	DBS (U/ml)	LYSATE (U/ml)
	762	45.9	86.8
10	763	64.9	116.9
	764	69.4	124.5
	765	43.9	91.3
	766	60.4	105.2
	767	74.8	128.0
15	768	75.3	141.6
	769	63.0	118.4
	770	61.4	114.8
	771	49.3	97.3
	772	66.5	133.3
20	773	46.4	89.0
	774	68.6	141.7
	775	95.2	167.9
	776	46.0	89.4
	777	53.8	104.0
25	778	39.5	71.8
	779	67.6	120.5
	780	52.0	93.6
	781	97.0	168.2
	783	56.3	94.6
30	784	68.6	126.0
	785	77.4	141.8

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SAMPLE	DBS (U/Ml)	LYSATE (U/Ml)
786	33.4	53.5
787	47.9	85.8
798	72.4	127.5
815	72.6	108.1
5 816	103.4	155.2
817	162.0	263.7
818	71.1	108.4
830	97.7	152.9
831	85.7	126.0
10 832	113.2	157.5
833	33.5	52.2
758	77.3	146.6
759	58.3	103.8

15 **Example 8 CD4 TRAx™ Paper Dot Assay-Correlation of Dried Blood Spots to Flow Cytometry**

The correlation of dried blood specimen results to that obtained with flow cytometry was determined. The results are shown in Table 6 and Figure 2.

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TABLE 6

SAMPLE	DBS U/ml	FLOW ABS. CD4 cells/ml	WBL U/ml
762	45.9	681	86.3
5 763	64.9	941	116.9
764	69.4	1218	124.5
765	43.9	839	91.3
766	60.4	764	105.2
767	74.8	1097	128
10 768	75.3	1292	141.6
769	63.0	1165	118.4
770	61.4	921	114.8
771	49.3	771	97.3
772	66.0	1037	133.3
15 773	45.4	631	89
774	66.9	1126	141.7
775	90.8	1397	167.9
776	46.0	715	89.4
777	51.2	843	104
20 778	38.6	524	71.8
779	64.0	1135	120.5
780	49.6	713	93.6
781	93.8	1567	168.2
783	55.4	590	94.6
25 784	73.2	1200	126
785	76.4	1347	141.8
786	33.8	421	53.5
787	47.9	1025	85.8

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SAMPLE	DBS U/Ml	FLOW ABS. CD4 cells/ml	WBL U/ml
798	72.4	1058	127.5
815	72.6	920	108.1
816	103.4	1731	155.2
817	162.0	2408	263.7
5 818	71.1	11.02	108.4
821	64.6	657	105.3
823	76.7	1058	118.4
824	84.3	1126	136.6

A correlation of 0.93 was found as shown in Figure 2.

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What is Claimed is:

1. A method for detecting or measuring the amount of a cell-associated molecule in a sample from a subject comprising the steps of:
  - 5 (a) spotting a selected amount of a liquid biological sample from a subject onto a filter paper collection device;
  - (b) contacting the filter paper collection device with a selected diluent and lysis reagent for a  
10 selected period of time at a selected temperature to produce a solubilized sample;
  - (c) recovering a selected amount of said solubilized sample;
  - (d) contacting said solubilized sample with at  
15 least one binding partner specific for a cell-associated molecule under conditions which allow specific binding; and
  - (e) detecting or measuring the amount of any specific binding that occurs of a component in the sample  
20 with said at least one binding partner, wherein the detection or amount of specific binding indicates the presence or amount of the cell-associated molecule in the sample.
2. The method of claim 1 wherein said cell-  
25 associated molecule is selected from the group consisting of CD4, CD8, CD3, CD19, CD25, CD2, CD56 and CD54.
3. The method of claim 2 wherein said cell-associated molecule is CD4.

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4. The method of claim 1 wherein said cell-associated molecule comprises a cytokine.
5. The method of claim 1 wherein said cell-associated molecule is an intracellular viral antigen.
- 5 6. The method of claim 1 wherein said cell-associated molecule comprises nucleic acids.
7. The method of claim 1 wherein said cell-associated molecule comprises a pharmacological agent or metabolite thereof.
- 10 8. The method of claim 1 wherein said selected diluent comprises a buffer and lysis reagent.
9. The method of claim 1 wherein said selected buffered lysis reagent comprises a non-ionic detergent.
10. The method of claim 9 wherein said non-  
15 ionic detergent comprises Triton X-100 and Nonidet P-40.
11. The method of claim 1 wherein the filter paper collection device is contacted with the diluent and lysis reagent for at least 2 hours at about room temperature.
- 20 12. The method of claim 1 wherein said selected amount of solubilized sample comprises 50  $\mu$ l.
13. The method of claim 1 wherein spotting the blood onto the collection device comprises collecting the

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blood sample onto the filter paper and drying it to produce a dried blood spot.

14. The method of claim 13 wherein a 0.25 inch diameter portion of the dried blood spot is removed.

5           15. The method of claim 13 wherein said 0.25 inch diameter portion is then contacted with the diluent and lysis reagent in step (b).

16. The method of claim 1 wherein said filter paper collection device comprises grade 903 filter paper 10 having circular markings indicated thereupon.

17. The method of claim 16 wherein said circular markings are capable of containing about 60  $\mu$ l of blood.

18. The method of claim 1 wherein said liquid 15 biological sample is selected from the group consisting of saliva, urine, pleural effusions, tissue infiltrates, spinal fluid, synovial fluid, and blood.

19. A method for enumerating an approximate number of cells that are positive for a cell associated 20 molecule in a sample comprising:

(a) determining the amount of a cell associated molecule according to the method of claims 1-3 and 11-17; and

(b) calculating the approximate number of cells 25 positive for the cell associated molecule from the amount of cell associated molecule determined in step (a).

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20. A method of claim 17 comprising the steps of:

(a) spotting a selected amount of a liquid biological sample from a subject onto a filter paper collection device;

(b) contacting the filter paper collection device with a selected diluent and lysis reagent for a selected period of time at a selected temperature to produce a solubilized sample;

10 (c) recovering a selected amount of said solubilized sample;

(d) contacting said solubilized sample with at least one binding partner specific for a cell-associated molecule under conditions which allow specific binding;

15 (e) detecting or measuring the amount of any specific binding that occurs of a component in the sample with said at least one binding partner, wherein the detection or amount of specific binding indicates the presence or amount of the cell-associated molecule in the  
20 sample; and

(f) calculating the approximate number of cells from the amount of cell associated molecule in the sample.

21. The method of claim 22, wherein the biological sample comprises whole blood.

25 22. The method of claim 21, wherein the cell associated molecule is CD4.

23. A kit for enumerating an approximate number of cells positive for a cell associated molecule comprising:

30 (a) a paper collection device;

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- (b) a detergent; and
- (c) an immunological detection means.

FIG-1

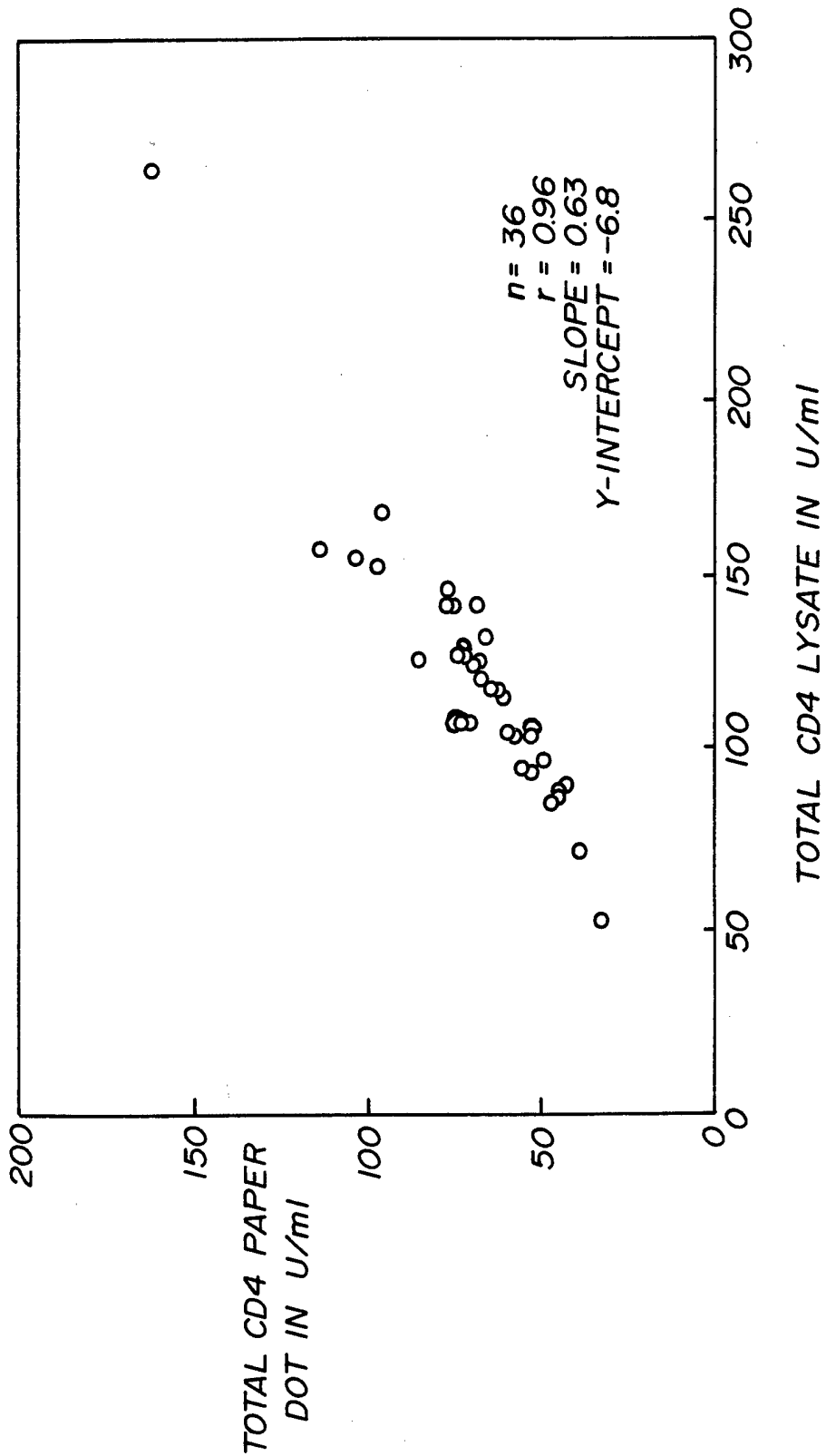
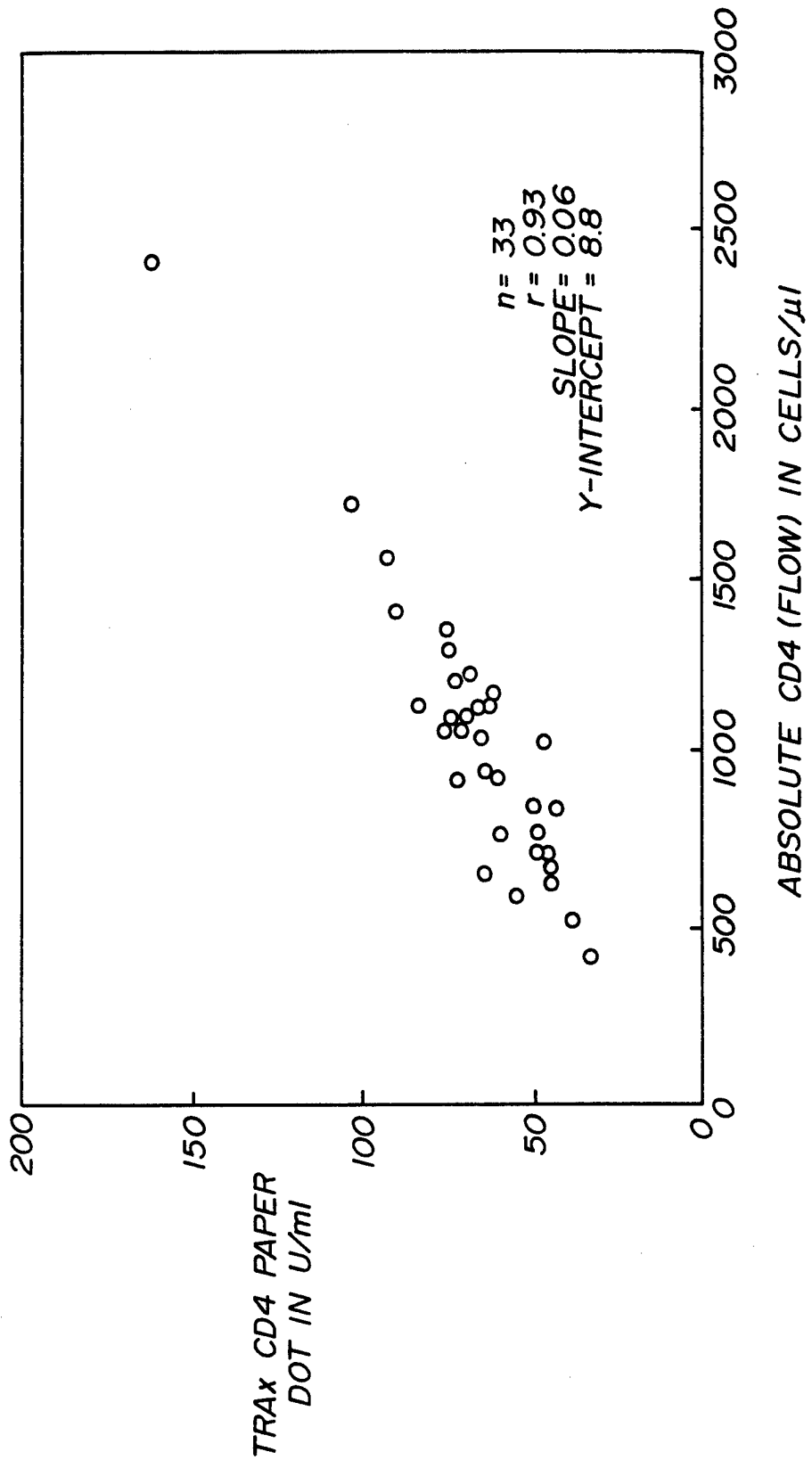


FIG-2





## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/05303**A. CLASSIFICATION OF SUBJECT MATTER**IPC(5) :C12Q 1/70  
US CL :435/5

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)

U.S. : 435/5, 7.1, 270, 810, 805; 436/522; 530/389.4, 389.6; 536/23.1, 23.5, 23.72; 930/221; 935/76, 77

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)

APS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF CLINICAL MICROBIOLOGY, Volume 30, Number 11, issued November 1992, Yourno et al., "A Novel Polymerase Chain Reaction Method for Detection of Human Immunodeficiency Virus in Dried Blood Spots on Filter Paper", pages 2887-2892, see pages 2887-2891.	1-23
Y	US, A, 4,381,295 (KUNG ET AL.) 26 April 1983, see entire document.	1-23
Y	US, A, 4,752,562 (SHELMAN ET AL.) 21 JUNE 1988, see entire document.	1-23
Y	US, A, 5,149,624 (GABRIEL) 22 September 1992, see entire document.	1-23

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

\* Special categories of cited documents:

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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

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Date of the actual completion of the international search

25 JULY 1994

Date of mailing of the international search report

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