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(54) Title: MEASUREMENT OF TOTAL MOLECULE IN A SAMPLE AND METHODS BASED THEREON

(57) Abstract

The measurement of the total amount of a target molecule is used in the detection, diagnosis, determining the severity of and staging of a variety of diseases or disorders and the monitoring of therapy. The measurements of the total amount of a target molecule is accomplished by treating a sample containing cells, such as a body fluid, with a non-ionic detergent in a manner that makes available for binding to one or more binding partners the total amount of a target molecule present in the cell membrane, the cytoplasm of the cell and/or in the soluble compartments of the body fluid. Once the total target molecule is available, it is measured using any of a number of assay techniques, preferably by sandwich enzyme immunoassay. The methods of the present application may be used to detect and measure molecules associated with microbial pathogens, in particular intracellular pathogens such as Chlamydia, as well as erythrocyte blood group antigens, tumor antigens, cytokines and cell adhesion molecules, for diagnosis, enumeration of cells expressing such molecules, and other methods.

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MEASUREMENT OF TOTAL MOLECULE IN A SAMPLE AND METHODS BASED THEREON

1. INTRODUCTION

The present invention is directed to methods for the direct measurement of the total quantity of a molecule in a sample. The molecule may be endogenous to the host cell, or a molecule associated with a pathogenic microorganism invading a cell or a tissue. The method of the present invention is applicable to diagnosis and to monitoring a subject's response to therapy in a variety of disease states.

2. BACKGROUND OF THE INVENTION

2.1. CELL SURFACE ANTIGENS

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 are molecules produced by intracellular parasites, such as bacteria or viruses. Cell-surface molecules are also present in the cytoplasm, and are often found in soluble or shed form in body fluids. Conventional immunoassays 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 molecule on the cell.

Well-known cell surface molecules with clinical significance include human leukocyte antigens such as HLA glycoproteins, B and T cell marker glycoproteins, and B and T cell receptor molecules (Bernard *et al.*, 1984, *Hum. Immunol. 11*:1-10; Knapp *et al.*, 1989, *Immunol. Today 10*:253:258; Gebel *et al.*, 1988, *ASHI Quarterly 12*:11; McMichael, A.J., ed., *Leukocyte Typing III: White Cell Differentiation Antigens*, 1987, Oxford University Press. Oxford, UK).

2.2. MICROBIAL PATHOGENS AND INTRACELLULAR GROWTH

For an overview of microbial pathogenesis, see Mims, C.A., *THE PATHOGENESIS OF INFECTIOUS DISEASE*, 2nd Ed., Academic Press, New York, 1982.

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. Table I lists microbial infections that are generally confined to epithelial surfaces.

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 associated with the microorganism in an epithelial cell, tissue or fluid sample.

Some of the more important microorganisms regularly establish systemic infections after traversing epithelial surfaces. These include intracellular and extracellular 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

TABLE I

Microbe	Respiratory/ conjunctiva	Urogenital	Skin	Gut
Viruses	Influenza Parainfluenza 1-4 Rhinoviruses Coronaviruses	Papillomas	Wart Molluscum contagiosum	Intestinal diarrheas
Chlamydias	Trachoma Inclusion conjunctivitis	TRIC agents (nonspecific urethritis)	--	--
Mycoplasma	*M. pneumoniae* (atypical pneumonia)	T strains	--	--
Bacteria	*Bordetella pertussis* *Corynebacterium diptheriae* *Corynebacterium multissimum*	Gonococcus *Gardnerella vaginalis*	Staphylococci	Most *Salmonella* Shigella
Fungi	*Candida albicans* (thrush)	*Candida albicans*	*Trichophyton spp* (athlete's foot, ringworm, etc.)	--
Protozoa	--	*Trichomonas vaginalis*	--	*Entamoeba, coli* *Giardia lamblia*

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 virus, smallpox virus, tubercle bacilli. Some bacteria such as *Mycobacterium tubersulosis*, *Salmonella typhi* or *Brucella abortus* do much of their multiplication in macrophages that have ingested them, though they are not obligate intracellular parasites.

Table II, below gives examples of infections in which microorganisms enter across epithelial surfaces and spread systemically.

TABLE II

Microbe	Respiratory/ conjunctiva	Urogenital	Skin	Gut
Viruses	Measles smallpox rubella varicella	Herpes simplex 2	Arboviruses	Enteroviruses Certain adenoviruses
Chlamydias	Psittacosis	Lymphogranuloma venereum	--	--
Bacteria	*Mycobacterium tuberculosis* *Pasteurella pestis*	*Treponema pallidum*	*B. anthracis*	*Salmonella* typhi
Rickettsias	Q fever	--	Typhus	Q fever ?
Fungi	Cryptococcosis Histoplasmosis	--	Maduramycosis	Blastomycosis
Protozoa	Toxoplasmosis	--	Malaria Trypanosomiasis	*Entamoeba histolytica*

Table III, below lists microorganisms that regularly multiply in macrophages.

TABLE III

Organism	Examples
Viruses	Herpes-type viruses, hepatitis virus of mice, measles, distemper, poxviruses, lymphocyte choriomeningitis virus, lactic dehydrogenase virus of mice
Rickettsia	*Rickettsia rickettsii, R. prowazeki*
Bacteria	*Mycobacterium tuberculosis, Mycobacterium leprae, Listeria monocytogenes, Legionella pneumophila*
Fungi	*Cryptococcus neoformans*
Protozoa	Leishmanias, Trypanosomes, Toxoplasmas

Such organisms multiply inside the cell while avoiding the intracellular defense mechanisms, such as lysosomal degradation. In some cases, the organisms multiply in phagosomes, while in others, they escape from the vacuole and multiply in the cytoplasm. The cells which harbor these organisms appear to give them biochemical and nutritional support.

An enzyme immunoassay for *Chlamydia trachomatis* is commercially available (CHLAMYDIAZYME® Diagnostic Kit from Abbott Laboratories, North Chicago, IL). This method utilizes a solid phase enzyme immunoassay which detects chlamydial antigen from urogenital, endocervical, conjunctival or nasopharyngeal swabs. The method is based on adsorption of antigen from the sample onto beads coated with an anti-chlamydial antibody which are then reacted with a rabbit antibody specific for a chlamydial antigen followed by an enzyme-conjugated anti-rabbit immunoglobulin antibody. The method does not employ detergent treatment of a sample, and is therefore limited to that amount of chlamydial antigen in the sample which exists in a form readily available for binding to the solid phase antibody.

Methods and devices for collecting and isolating microorganisms are known which utilize sample treating fluids which may contain a detergent, for example, saponin (Dorn, U.S. Patent 3,932,222; 1/13/76). However, use of a detergent to solubilize cells in a simple direct immunoassay method which measures the total amount of a microbial antigen in the sample, has not been disclosed.

It would be of great benefit in research, diagnostics, therapeutic and other endeavors, 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 containing host cells harboring the

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

2.3. TUMOR ANTIGENS

Immunodiagnostic approaches to cancer involve detection of "tumor antigens" that serve as markers for the presence of a preneoplastic or a neoplastic cell. Tumor antigens are classified into two broad categories: tumor-specific antigens (TSA) and tumor-associated antigens (TAA). See, for example, Klein, J., 1990, *Immunology*, Blackwell Scientific Publications, Inc., Cambridge, MA, pp. 419-428.

Simple, rapid and sensitive assays for analysis of a sample of cells, tissue or body fluids for the total amount of a tumor antigen are highly desirable for the detection and diagnosis of cancer, as well as for monitoring cancer therapy and research efforts relating to this area. The ability to detect low levels of a TSA or TAA early in the process of tumor development, when only few cells may express the antigen, is especially desirable.

2.4. RED BLOOD CELL ANTIGENS

Molecules on the surface of red blood cells (RBC), termed blood group antigens, encompass a wide variety of antigenic structures in many different species. What they have in common is their presence on red blood cells. Some blood group antigens, particularly those present on human RBC are among the serologically best-characterized substances known. A well-known representative of this group is the human ABO blood group which comprises antigens referred to as the ABHLe blood groups substances. The antigenicity of the ABH and Le (Lewis) blood group substances resides in oligosaccharide chains of varying length. Some chains are only a few sugar

residues long, while others contain up to 60 residues. The chains can exist in three forms: free, attached to proteins, or attached to lipids.

Free ABHLe oligosaccharides have been isolated from milk colostrum and urine. Protein bound ABHLe antigens are glycoproteins secreted by goblet cells, mucus glands of the gastrointestinal tract, genitourinary tract, and respiratory tract. In addition such glycoproteins are present in amniotic fluid, milk, sweat and tears. The oligosaccharide side chains are attached through N-acetyl-D-galactosamine (GalNAc) to a serine or threonine residue of the protein backbone. Lipid-bound ABHLe antigens are glycolipids incorporated into the plasma membrane of RBC and cells of other tissues including liver, spleen, kidney, pancreas and stomach. Here, the oligosaccharide is attached by a glucose (Glc) to a sphingolipid (consisting of N-acetyl-sphingosine or ceramide and fatty acids). Thus, in contrast to the ABHLe-active glycoproteins, which always contain GalNAc, glycolipids may or may not contain this sugar.

In addition to the ABHLe system, about 200 other blood group antigens belonging to more than 20 different systems are known in humans. Many other antigenic systems have been described in other mammals. The chemical structure of most of these antigens has not been elucidated. One human blood group system of clinical importance is the Rh system, which is quite complex, consisting of many antigens. Examples of other known human blood group systems include Duffy, Kidd, Lutheran, Kell, MN, Gerbich, P, I, Sda, and Pr.

Alterations in the expression of blood group antigens on cells other than RBC are associated with pre-malignant and tumorous conditions, in particular in cells of the gastrointestinal mucosa (Hakomori, S. *et al.*, *J. Natl. Canc. Inst.* *71*:231 (1983); Keshvara,

L. *et al.*, *Glycoconjugate J.* 9:16 (1992)). The enzyme known as A-transferase (α(1-3)N-acetylgalactosaminyl transferase) converts the H-glycolipid precursor to the mature form of A-glycolipid. In tumors, as compared to normal mucosal tissue, this conversion is markedly inhibited (Hakomori *et al.*, *supra*; Hakomori, S., *Adv. Cancer Res.* 52:257- (1989)). In cases of pre-malignant dysplasia of the oral epithelia, deletion of the A or B antigenic determinants has been noted (Hakomori *et al.*, 1983, *supra*). Thus, changes in ABH blood group glycolipids in epithelial cells bear an important relationship to carcinogenic events. On the basis of the above and other related observations, rapid and specific assays for analysis of samples of cells, tissue or body fluids for altered levels of blood group antigens or for altered activity in the blood group glycosyltransferases, evaluated by measuring the blood group antigen levels, are highly desirable for the diagnosis and monitoring of a variety of disease states, such as the development of gastrointestinal cancer.

Diminished expressions of Complement Receptor Type 1 (CD35 or CR1) on erythrocytes of patients with systemic lupus erythematosus (SLE) has been reported by investigators from several geographic regions, including Japan (Miyakawa *et al.*, 1981, *Lancet* 2:493-497; Minota *et al.*, 1984, *Arthr. Rheum.* 27:1329-1335), the United States (Iida *et al.*, 1982, *J. Exp. Med.* 307:981-986) and Europe (Walport *et al.*, 1985, *Clin. Exp. Immunol.* 59:547; Jouvin *et al.*, 1986, *Complement* 3:88-96; Holme *et al.*, 1986, *Clin. Exp. Immunol.* 63;41-48). Taken as a group, patients have an average number of receptors per cell that is 50-60% that of normal populations. An early report noted that CR1 number on erythrocytes varied inversely with disease activity, with lowest numbers occurring during periods of most severe manifestations of SLE, and higher

numbers being observed during periods of remission in the same patient (Iida *et al.*, 1982, *J. Exp. Med.* 155:1427-1438). CR1 number has also been found to correlate inversely with serum levels of immune complexes, with serum levels of C3d, and with the amounts of erythrocyte-bound C3dg, perhaps reflecting uptake of complement-activating immune complexes and deposition on the erythrocyte as an "innocent bystander" (Ross *et al.*, 1985, *J. Immunol.* 135:2005-2014; Holme *et al.*, 1986, *Clin. Exp. Immunol.* 63:41-48; Walport *et al.*, 1985, *Clin. Exp. Immunol.* 59:547). A patient with SLE lacking CR1 on erythrocytes was found to have an auto-antibody to CR1 (Wilson *et al.*, 1985, *J. Clin. Invest.* 76:182-190). Decreased titers of the anti-CR1 antibody coincided with improvement of the patient's clinical condition and with partial reversal of the receptor abnormality. Anti-CR antibody has been detected in two other SLE patients (Cook *et al.*, 1986, *Clin Immunol. Immunolpathol.* 38:135-138). Recently, acquired loss of erythrocyte CR1 in the setting of active SLE and hemolytic anemia was demonstrated by observing the rapid loss of the receptor from transfused erythrocytes (Walport *et al.*, 1987, *Clin. Exp. Immunol.* 69:501-507).

The relative loss of CR1 from erythrocytes has also been observed in patients with Human Immunodeficiency Virus (HIV) infections (Tausk, F.A., *et al.*, 1986, *J. Clin. Invest.* 78:977-982) and with lepromatous leprosy (Tausk, F.A., *et al.*, 1985, *J. Invest. Dermat.* 85:58s-61s).

Abnormalities of complement receptor expression in SLE are not limited to erythrocyte CR1. Relative deficiencies of total cellular CR1 of neutrophils and plasma membrane CR1 of B lymphocytes of the SLE patients have been shown to occur (Wilson *et al.*, 1986, *Arthr. Rheum.* 29:739-747).

In patients with Type IV SLE nephritis, all detectable CR1 antigen is lost from podocytes, whereas in less severe forms of SLE nephritis and in non-SLE types of proliferative nephritis, including membranoproliferative glomerulonephritis Types I and II, CR1 expression on glomerular podocytes does not differ from normal (Kazatchkine *et al.*, 1982, *J. Clin. Invest.* 69:900-912; Emancipator *et al.*, 1983, *Clin. Immunol. Immunopathol.* 27:170-175). However, patients having Type IV SLE nephritis do not have fewer numbers of erythrocyte CR1 than do SLE patients having other types of renal lupus or no nephritis (Jouvin *et al.*, 1986, *Complement* 3:88-96).

Simple, rapid and sensitive assays for analysis of biological samples, especially whole blood, for the total amount of complement receptor would be highly useful in the diagnosing and monitoring of the various disease states mentioned above. Alternatively, such detection is useful in the furtherance of research involving the complement receptor.

2.5. CYTOKINES

Cytokines are a large, diverse group of bioactive proteins and peptides generally having relatively low molecular weights which regulate a large number of cellular activities. For a recent reviews, see: Arai, K-I. *et al.*, 1992, *Allergy & Clin. Immunol. News* 4:113-120; Arai, K. *et al.*, 1990, *Ann. Rev. Biochem.* 59:783; Watanabe *et al.*, 1991, *Curr. Opin. Biotech.* 2:227. For example, cytokines regulate immunoglobulin production by B lymphocytes and the biosynthetic activities of various cell types.

Most cytokine research is based on measurement of the biological activity of cytokines in various bioassays. More recently, cytokine levels in fluids such as blood have been measured in immunoassays, such as ELISA, for example, utilizing a pair of appropriate

antibodies directed against the cytokine. This approach suffers from several limitations when used to detect cell-derived cytokines in serum or plasma because of their vanishingly low concentrations and extremely short half lives *in vivo*. The detectability of cytokines in serum or plasma 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 sampled compartment, *e.g.*, blood, (2) the cytokine's short half-life make the sampling time critical, and (3) cytokine proteins are usually unstable in plasma or serum during sample storage. These factors often render a cytokine undetectable even in a sample obtained from the particular site or tissue in which it is produced. In general, immunoassays of molecules present in serum are not useful for detecting proteins that are do not survive storage of the plasma or serum. Finally, most cytokines are known to be present in the extracellular and intracellular compartment. However, some cytokines such as TNF-α are also expressed on the membrane of producing cells (Kriegler *et al.*, 1988, *Cell 53(1)*:45-53). Presently, there are no available rapid and simple methods for simultaneously measuring the total amount of cytokine on the cell surface, the intracellular compartment and in soluble form in the fluid surrounding the cells.

Bioassays of cytokines *in vitro* typical measure the ability of a cytokine to stimulate or sustain growth or proliferation a cell line which is dependent on that cytokine for growth. Such assays suffer from the drawback of requiring at least many hours, and more typically, several days. The cell lines used in these bioassays are responsive not only to the cytokine being assayed but to other factors which may "contaminate" the sample. Thus, to attribute a

bioactivity to the suspected cytokine, it is often necessary to use a monoclonal antibody to neutralize the cytokine's activity. An additional important drawback of these bioassays is the influence that various inhibitory factors present in the sample, known or not yet discovered, may have on the measured bioactivity.

For all the foregoing reasons, there is a need in the art for improved assays to detect cytokines in samples of cells and body fluids.

In summary, the ability to easily detect in a sample of cells, tissue or a body fluid the total amount of a molecule, including a molecule associated with an intracellular pathogenic microorganism, would, in addition to serving as a tremendous research tool, also have great clinical potential for detection, diagnosis, staging and monitoring therapy in a number of disease states, such as infectious diseases, cancer and immunological disorders.

Conventional immunoassays of cell or tissue samples usually require several steps of sample processing, for example, to separate cells from blood, or to enrich a particular cell type. These steps typically avoid the use of detergents due to the potential deleterious effects of the detergent on subsequent binding of antibodies to an antigen in the sample.

3. SUMMARY OF THE INVENTION

The present invention provides methods for measurement of the total amount of a molecule (termed hereinafter "target molecule") in a sample in the presence of a non-ionic detergent, and the use of such measurements in the detection, diagnosis, staging, and determining the severity of diseases or disorders, monitoring of therapy, various research endeavors, or

enumeration of cells expressing the target molecule. Measurements of the total amount of a target molecule expressed by a cell, which is uniformly expressed, for example a platelet antigen, can be used to determine the approximate number of cells, *e.g.*, platelets, bearing the antigen in a sample such as a body fluid sample. Thus, thrombocytopenia and other disorders involving aberrant levels of platelets can be diagnosed. Because the level of certain target molecules change in the course of disease development and remission, measurement of the total amount of a target molecule is useful in monitoring the effectiveness of a treatment in a patient, in predicting therapeutic outcome or disease prognosis, and, in the case of lymphocyte target molecules, in evaluating and monitoring the immune status of a patient. When applied to a microbe-associated target molecule, the method of the present invention can serve in early diagnosis, prognosis, or in monitoring antimicrobial therapies. With blood group antigens such as the ABH antigens, such measurements may be used in blood typing as well as in early detection of pre-malignant changes in mucosal cells.

According to the present invention, the measurements of the total amount of a target molecule is accomplished by treating a sample containing cells, such as a body fluid, in a manner that make available for binding to a binding partner the total amount of the target molecule present in the cell membrane, the cytoplasm of the cell and/or in the soluble compartments of the sample. In a preferred aspect, the binding partner is an antibody or antibody fragment containing the binding domain thereof. The sample is preferably an original sample, *i.e.*, in the form substantially as it was obtained, and is treated with a non-ionic detergent prior to contact with its binding partner. Once the total target molecule is

made available, it is measured using any detection method known in the art, and preferably using any of a number of immunoassay techniques, most preferably by sandwich enzyme immunoassay (EIA).

Also provided is a method for typing blood by detecting an erythrocyte blood group antigen in a sample from a subject. Preferably, the blood group antigen is the A antigen. The above methods may also be used to measure the total amount of the blood group antigen.

In specific embodiments, the methods are used to determine in a sample the total amount of a target molecule, in which the target molecule is a cytokine, a tumor-specific or tumor-associated molecule, a cell adhesion molecule, a multidrug resistance marker, complement receptor, or cellular DNA (the latter preferably to detect polyploid cells).

The present invention is also directed to a method for detecting the presence of malignant or premalignant cells in gastrointestinal mucosal tissue, the tumor associated with a decrease in the blood group A antigen, wherein a decrease in the amount of specific binding or blood group A antigen is indicative of the presence of malignant or premalignant cells.

Also provided is another method for detecting the presence of malignant or premalignant cells in gastrointestinal mucosal tissue, the tumor associated with a decrease in the activity of the α(1-3)N-acetylgalactosaminyl transferase) enzyme in the cells of the tissue, wherein a decrease in said amount indicates the presence of malignant or premalignant cells.

Also provided is a process for monitoring the course of disease or the effect of a therapeutic treatment on a patient being treated for a disease associated with the presence of a target molecule,

wherein an improvement in patient condition (*e.g.*, remission), or positive response to therapy is accompanied by a decrease or increase in the total amount of the target molecule, or a disappearance thereof.

4. DETAILED DESCRIPTION OF THE INVENTION

4.1. TOTAL AMOUNT OF A TARGET MOLECULE

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

Any target molecule for which a binding partner is available can be detected and/or measured according to the present invention. The target molecule may be an "endogenous" target molecule, which as used herein, refers to a molecule that is encoded by the genome of the host cell or is produced by the host cell not due to the presence of an exogenous organism. Examples of endogenous target molecules are erythrocyte antigens, platelet antigens, cytokines, cell-adhesion molecules, multidrug resistance markers, cellular DNA and the like, which are discussed in more detail below.

The target molecule may also be an "exogenous" target molecule. As used herein, "exogenous target molecule" refers to a target molecule (a) encoded by a genome not of the host cell; or (b) 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 (see below).

The term "total amount of a target molecule" as used herein refers to the total amount of the target molecule in a sample, including target molecules which

may be present in or bound to the cell membrane, in any of a number of intracellular compartments including the cytoplasm and nucleus, and in the extracellular soluble compartments of the tissue, fluid or biological sample, which are made available for detection by use of the assay methods provided by the invention.

The soluble compartment may include an endogenous, spontaneously released, soluble target molecule or an exogenous, soluble target molecule. The exogenous target molecule may be one which was purposely administered to the subject, for example, a therapeutic cytokine.

In a sample comprising cultured cells, the total amount of target molecule includes the amount of target molecule present in the membrane, intracytoplasmic and cell culture media compartments of the sample.

In one embodiment, the present invention provides an assay method of a sample treated with non-ionic detergent, that measures the total amount of a target molecule present in all three of these compartments simultaneously.

In another embodiment, the method of the present invention measures the total amount of a target molecule in the intracytoplasmic and membrane compartments, where the cells have been rendered free of the released/soluble compartment, for example by washing the cells free of the serum or cell culture supernatant prior to solubilization with detergent.

In another embodiment, the method is used to measure the total amount of a target molecule in the membrane compartment, wherein the intracytoplasmic and soluble compartments have first been removed leaving just the cell membranes to be analyzed.

In yet another embodiment, the method is used to measure the total amount of a target molecule in the

released/soluble compartment, for example, the serum, body fluid or culture medium, from which the cells have first been removed prior to solubilization with detergent.

The total amount of target molecule may include the amount of the target molecule present in any one compartment or in any combination of compartments depending upon the nature of the sample. For example, if a sample of cells has been washed free of culture medium or serum/plasma (or other body fluid in which the cells were obtained), then the total amount of target molecule would include the membrane-associated and cytoplasmic compartments only.

Non-limiting examples of target molecules for which the present invention is intended to apply are proteins, peptides, glycoproteins, glycopeptides, glycolipids, polysaccharides, oligosaccharides, nucleic acids, and the like, or fragments thereof. Preferred target molecules according to the present invention are blood group antigens, microbe-associated molecules, cytokines, adhesion molecules, hormones (*e.g.*, insulin), and the like. In a specific embodiment, the invention provides a method for detecting and/or measuring the total amount of a target molecule which is intracellular (*i.e.*, a molecule which is not accessible on the cell surface, and thus, not, for example, a surface antigen). In another embodiment, the target molecule is a cell surface molecule. In a particular aspect, the target molecule is not a leukocyte cell surface marker (a "leukocyte cell surface marker" being an antigen or polypeptide found on the cell surface of a leukocyte).

Depending on the sample and nature of target molecule, and where the target molecule is distributed uniformly between different cells, the measurement of a total amount of target molecule as described herein can substitute for direct enumeration of cells

expressing the target molecule, and can be valuable in cell typing, monitoring the effect of a therapeutic treatment on a subject, detecting, diagnosing or staging a disease in a subject, in predicting therapeutic outcome or disease prognosis and in evaluating and monitoring immune status of patients. Furthermore, two or more distinct target molecules can be measured separately, preferably in a concurrent fashion. In a specific embodiment, the measured amount of the target molecule in a sample from a patient is compared to a baseline level of target molecule that is the level established to be present in a comparable sample not affected by the patient's disorder or from a patient without the disorder or from the patient at an earlier time.

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 surface receptors for the target molecules. Additional and specific examples of non-antibody binding partners are described in the subsections below. In addition, other non-antibody binding partners include those used as binding partners in the following assays: for assay of CD4 (or enumeration of $CD4^+$ cells), binding to HIV gp120 or gp160; for assay of CD8 (or enumeration of $CD8^+$ cells), binding to *Trypanosoma brucei*-derived lymphocyte-triggering factor (Olsson *et al.*, 1992, *Parasitology Today 8(7)*:237-239); for assay of V_β T cell antigen receptor or enumeration of cells expressing the same, binding to superantigen; for assay of T cell antigen receptor or cells expressing the same, binding to Concanavalin A, for assay of immunoglobulin or cells expressing the same, binding to SacI, for the assay of complement receptor etc.

In a preferred embodiment, the target molecule is an antigen, and detection and/or measurement of the target molecule is carried out by a method comprising immunospecific binding of the target molecule to at least one antibody. 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 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 and kits of this invention are applicable to human clinical and veterinary uses.

A sample which is subjected to testing according to the present invention is a sample derived from a biological cell or organism, and is preferably an "original sample" obtained from the subject and, in particular, which is not subjected to processing such as any fractionation steps (*e.g.*, fractionation to separate cells from extracellular fluid or one type of cell from another type, or to remove certain subcellular components (*e.g.*, membrane fraction, cytoplasmic fraction, nuclear fraction)). In another embodiment, the sample may be subjected to one or more processing steps, such as washing of and enrichment of cells, or isolation of the fluid portion of the sample.

The sample includes, but is not limited to, any biological fluid, preferably a body fluid. Examples of body fluids include, but not limited to, whole blood, serum, plasma, urine, synovial fluid, cranial or spinal fluid, saliva, tissue infiltrate, cervical or vaginal exudate, tissue infiltrate, pleural effusions, bronchoalveolar lavage fluid, gastric lavage fluid, small or large bowel contents, fecal preparations, and the like. Furthermore, a sample may

be a swab specimen from mucosal surface or a body orifice, for example, oral, anal, urogenital, endocervical, conjunctival or nasopharyngeal. In another embodiment, the biological fluid may be a cell culture medium or supernatant of cultured cells. The sample can be a biopsy specimen or other tissue sample. The sample can comprise any type of cell including but not limited to a blood cell (erythrocyte, neutrophil, eosinophil, monocyte, macrophage, T lymphocyte, B lymphocyte, etc.), epithelial cell, endothelial cell, neuron, glial cell, etc.

The method provided by the present invention, as described in more detail below, overcomes many of the limitations of prior art methods, in particular as regards the measurement or detection of cell-surface target molecules, which heretofore required cell isolation followed by direct or indirect immunofluorescence analysis by microscopy or flow cytometry. Limitations of the prior art procedures include the requirement for: (1) fresh samples, (2) fairly large sample sizes or a large number of cells, (3) enriched cell populations rather than whole tissue or blood, (4) extensive preparation time, and (5) expensive equipment, such as a flow cytometer. The methods provided herein overcome these limitations.

4.2. ENDOGENOUS TARGET MOLECULES

The present method is well-suited for the measurement of the total amount of an endogenous target molecule in a sample. The prior solubilization of the cells in a sample in order to make available the total amount of a cellular target molecule, such as but not limited to a cell-surface antigen, in a sample is a major improvement over existing approaches. The fact that the methods disclosed herein do not require fresh samples is also highly

advantageous for measuring a cell-surface target molecule. Each patient sample can be treated with the detergent preparation, as described herein, and stored frozen. This is especially useful for analysis of a series of samples obtained from the same patient over a period of time, as in a longitudinal study. All samples can then be thawed and analyzed concurrently. This is a definite improvement over flow cytometric analysis where fresh intact cells are generally required. It also eliminates or reduces variance in results that arises from interassay variability.

In a specific embodiment, the target molecule is not a leukocyte cell surface marker such as CD4, CD8, T cell antigen receptor, etc.

4.2.1. ERYTHROCYTE BLOOD GROUP AND TUMOR ANTIGENS

In a preferred embodiment, the present invention provides a method to detect or measure the total amount of an erythrocyte blood group antigen in a body fluid or cell or tissue sample. Thus, the invention provides a method of typing blood by detecting blood group markers on red blood cells by use of an assay of the invention. Such blood group markers include but are not limited to A, B, H, Lewis antigens, Rh system antigens, and others known in the art, for example, Duffy, Kidd, Lutheran, Kell, MN, Gerbich, P, I, Sda, and Pr (see Section 2.4 *supra*). Thus, the total amount of a blood group antigen from the ABO group, such as antigen A may be detected or measured by detergent treatment of a whole blood sample, for example, followed by assay using one or more binding partners to the blood group antigen, *e.g.*, anti-A antibodies. In another embodiment, the activity of A-transferase enzyme associated with premalignant changes in gastric mucosa may be measured by providing to the detergent treated blood or cell or tissue

sample the A precursor and assaying for the A antigen product formed by the action of A transferase in the sample. Assay of sequential samples for the A antigen or the A transferase, preferably samples of mucosal tissue from the gastrointestinal tract, is used to detect changes leading to carcinoma formation, or to monitor the therapy of a premalignant or malignant lesion associated with a fall in A antigen.

The methods of the present invention are useful for detecting changes in cells in their progression from a normal to a neoplastic or malignant state. Thus, the measurement in a sample of the total amount of a tumor-specific or tumor-associated antigen allows early detection of precancerous or cancerous stages. Because the method of the present invention can measure the total amount of an antigen in the cell-membrane, intracellular and extracellular fluid compartments simultaneously, in contrast to convention methods, less antigen need be present to pass the detection threshold. This would allow earlier diagnosis than is currently available. Furthermore, new classes of tumor-specific or tumor-associated molecules, for example, those that are only expressed intracellularly, can be identified and detected in a cell, tissue or body fluid sample using the present methods.

The present methods can therefore detect cells in the process of progression to neoplasia or cancer, including stages of non-neoplastic cell growth including hyperplasia, metaplasia, or most particularly, dysplasia, where expression of a new or normally unexpressed target molecule is associated with these steps. For review of such abnormal growth conditions, see, for example, Robbins *et al.*, *Basic Pathology*, 2d Ed., W.B. Saunders Co., Philadelphia, 1976, pp. 68-79.

Hyperplasia is a form of controlled cell proliferation involving an increase in cell number in a tissue or organ, without significant alteration in structure or function. For example, endometrial hyperplasia often precedes endometrial cancer. Metaplasia is a form of controlled cell growth in which one type of adult, or fully differentiated, cell substitutes for another type of adult cell. Metaplasia occurs in epithelial or connective tissue cells. Atypical metaplasia involves a somewhat disorderly metaplastic epithelium. The most disordered form of non-neoplastic growth is dysplasia, a frequent precursor of cancer, which occurs mainly in the epithelium, is the most disordered form of non-neoplastic cell growth. A dysplastic change involves the loss of individual cell uniformity and in the architectural orientation of cells. Dysplastic cells often have abnormally large, deeply staining nuclei, and exhibit pleomorphism. Dysplasia characteristically occurs at sites of chronic irritation or inflammation, typically in the cervix, respiratory passages, oral cavity, and gall bladder.

Alternatively or in addition to the presence of abnormal cell growth characterized as hyperplasia, metaplasia, or dysplasia, the presence of one or more characteristics of a transformed phenotype, or of a malignant phenotype, displayed *in vivo* or *in vitro* by a cell sample from a patient, can indicate the desirability of prophylaxis or therapy.

Characteristics of a transformed phenotype in a cell include morphological changes, looser attachment to the substratum, loss of contact inhibition, loss of anchorage-dependence, protease release, increased sugar transport, decreased serum requirement for *in vitro* growth, expression of fetal antigens, disappearance of particular cell surface proteins, etc. For a more detailed description of

characteristics associated with a transformed or malignant phenotype, see, for example, Robbins and Angell (*supra*), pp. 84-90. Such characteristics can be detected using the methods of the present invention when the change involves the appearance of a new tumor-specific antigen, the inappropriate or increased expression of a tumor-associated antigen, or the loss or diminution of a normal cellular antigen.

Non-limiting examples of pre-neoplastic lesions include leukoplakia, a benign-appearing hyperplastic or dysplastic lesion of the epithelium, Bowen's disease, a carcinoma *in situ*, fibrocystic disease (cystic hyperplasia, mammary dysplasia, particularly adenosis (benign epithelial hyperplasia)).

Tumor antigens which can thus be detected or measured according to the invention include but are not limited to carcinoma-associated antigen, breast cancer-associated antigen, melanoma-associated antigen, melanoma-associated pigmentation antigen, melanoma-associated proteoglycan; an erb, ras, myc, neu, or rel oncoprotein; oncoprotein p53, etc. (see, e.g., Klein, J., *supra*, pp. 419-428; see also Section 2.3 *supra*). Monoclonal antibodies to the above-listed antigens, and others, are commercially available (see *Linscott's Director of Immunological and Biological Reagents*, 1992, 7th Ed., Santa Rosa, CA) and are envisioned for use in immunoassays of the invention.

4.2.2. PLATELET TARGET MOLECULES

The present invention is useful in a method for determining the propensity of blood to clot in response to injury by measuring the total amount of a platelet-associated target molecule, or assessing the number of platelets, in a sample using an immunoassay. Current methods rely on counting platelets in blood, either microscopically, which is highly tedious, or in an automated counter. As provided herein, a blood

sample, or other platelet-containing sample, is treated with non-ionic detergent to solubilize and uniformly disperse platelet-associated target molecules. In a preferred aspect, the target molecule is a platelet antigen, and antibodies to platelet antigens, which are well-known in the art (see, for example, *Linscott's Director of Immunological and Biological Reagents, supra*, at p. 18) are used to measure the total platelet-associated antigen in an immunoassay. Binding partners other than antibodies can also be used. For example, the platelet glycoprotein GPIIb-IIIa binds to the sequence Arg-Gly-Asp-Ser (see Bacon-Baguley *et al.*, 1987, *J. Biol. Chem. 262:*1927-30). Thus, in one specific embodiment, it is envisioned that the Arg-Gly-Asp-Ser sequence or proteins comprising the same (*e.g.*, fibronectin) can be used as binding partners to detect and/or measure platelet GPIIb-IIIa. Methods of the invention for measuring platelet-associated target molecules are useful, for example, in the diagnosis of thrombocytopenia (decreased number of platelets) and in diagnosing, staging or monitoring therapy of any disease or disorder associated with altered platelet numbers relative to a baseline level. For example, such disorders include but are not limited to side effects of medical treatments. The baseline level is the level in a healthy individual or a standard level determined to be associated with disease absence or remission.

4.2.3. CELL ADHESION MOLECULES

The total amount of a cell adhesion molecule in a sample is detected or measured according to the methods described herein. Any known cell adhesion molecule for which a specific binding partner is available can be detected or measured, as the case may be. Examples of such molecules include molecules of

the β2 integrin family, including the leukocyte cell adhesion molecules, the complement receptor family such as CR3 and CR4, ICAM-1 and ICAM-2, and members of the β1 integrin family including the homing receptors such as CD44 and MEL-14, the VLA proteins, LFA-1, MAC-1, P150,95, and the like. Also included are members of the β3 integrin family, such as the vitronectin receptor family including GpIIB,IIIA and VNR. For a review, see: Hemler, M.E., *Ann. Rev. Immunol.* 8:365-400 (1990), which is hereby incorporated by reference in its entirety. Also detectable or measurable by the present method are the selectins, which represent another cell adhesion molecule family present on various cell types. Examples of selectins on endothelial cells include CD62 (also termed GMP140 or P-selectin), LECCAMs, ELAM-1 (also termed E-selectin), lymph node addressin (also termed MECCA-79), etc.

Target molecule-binding partner pairs other than antibody-antigen pairs, which can be used to detect or measure an adhesion molecule according to the invention include but are not limited to those set forth in Table IV (see Butcher, 1991, *Cell* 67:1033-36):

TABLE IV
BINDING PAIRS*

Leukocyte	Endothelial Cell
L-selectin	Lymph node addressin
CLA	ELAM-1
sialyl Lewis X	ELAM-1
sialyl Lewis X	CD62
Integrins:	
LFA-1	ICAM-1, ICAM-2
Mac-1	ICAM-1
VLA-4	VCAM-1

* The cell types which expresses each respective member of the binding pair is indicated by the column heading.

The cell types listed in Table IV above or subpopulations thereof can be used as sources of the respective member of the binding pair for purification of said member, *e.g.*, by immunoaffinity chromatography of blood cell extracts. (See also Larsen *et al.*, 1989, *Cell 59*:305-312 for a CD62 purification method; Lobb *et al.*, 1991, *J. Immunol. 147*:124, for ELAM-1; and Berg *et al.*, 1991, *J. Cell Biol. 114*:343, for lymph node addressin). Chemical synthesis, recombinant methods, or commercial sources may also be used to obtain the desired adhesion molecule binding partner. For example, ICAM-1, ICAM-2, and VCAM-1 have been cloned and sequenced (see Staunton *et al.*, 1988, *Cell 52*:925-933), European Patent Application Publication 387,688, published September 19, 1990; Osborn *et al.*, 1989, *Cell 59*:1203; Polte *et al.*, 1990, *Nucl. Acids Res. 18*:5901).

In a specific embodiment, measurement of adhesion molecules according to the invention can be used to diagnose, stage, determine the severity of, or monitor various diseases or disorders. In particular, such diseases or disorders in which an increased amount of an adhesion molecule is detected or measured relative to a baseline level include but are not limited to disorders involving an undesirable inflammatory response such as: inflammatory arthritis (rheumatoid arthritis, seronegative spondyloarthritides, juvenile rheumatoid arthritis, vasculitis, psoriatic arthritis, polydermatomyositis), systemic lupus erythematosus (SLE), asthma, inflammatory dermatoses (psoriasis, dermatitis herpetiformis, eczema, necrotizing and cutaneous vasculitis, bullous diseases), reperfusion injury, septic shock (sepsis), adult respiratory distress syndrome, tissue damage relating to tissue transplantation, thermal injury (burn), other autoimmune disorders [in addition to SLE and rheumatoid arthritis: glomerulonephritis, juvenile onset diabetes, multiple sclerosis, allergic conditions, autoimmune thyroiditis, allograft rejection (*e.g.*, rejection of transplanted kidney, heart or liver), Crohn's disease, graft versus host disease, post-pump syndrome or post-perfusion syndrome following cardiopulmonary bypass or hemodialysis], certain hematologic malignancies, pneumonia, post-ischemic myocardial inflammation and necrosis, barotrauma (decompression sickness), ulcerative colitis, inflammatory bowel disease, atherosclerosis, cytokine-induced toxicity, necrotizing enterocolitis, granulocyte-transfusion-associated syndromes, Reynaud's syndrome, multiple organ injury syndromes secondary to septicemia or trauma, and acute purulent meningitis or other central nervous system inflammatory disorders. In another embodiment, such diseases or disorders in which a decreased amount of

an adhesion molecule is detected or measured relative to a baseline level include but are not limited to leukocyte adhesion deficiency (Anderson and Springer, 1987, *Ann. Rev. Med. 38*:175-194), which involves an inherited deficiency in the integrins LFA-1, Mac-1, and p150,95; and diabetes mellitus, granulocytasthenia, and recurrent pyogenic infections, which have been reported to involve cell adherence defects (Gallin *et al.*, 1980, *Ann. Int. Med. 92*:520-538). The baseline level is that level established to be present in a comparable sample not affected by the disorder or from a patient without the disorder or from the patient at an earlier, healthy time.

In another specific embodiment, detection and/or measurement of a cell adhesion molecule in a sample according to the invention can be carried out in order to assess the metastatic potential of a tumor. Thus, the invention provides methods of determining metastatic or invasive potential of a malignant tumor in a subject by detecting and/or measuring the total amount of a cell adhesion molecule in a sample from a tumor-bearing subject. The detection or measurement of abnormal levels of a cell adhesion molecule relative to a baseline level in normal cells from the subject or to a standard level established for non-metastatic tumor cells or normal cells indicates that the tumor can metastasize. By way of example but not limitation, measurements of the following adhesion molecules are envisioned to be useful for determining metastatic potential of the indicated tumor types: LFA-1, the β1 integrin subunit CD29, CD31 (PECAM-1), and CD44, which are implicated in metastatic spread of lymphomas (Roos, E., 1991, *Cancer-Metastasis Rev. 10(1)*:33-48; carcinoembryonic antigen, which is associated with colorectal tumors which have a high risk for metastasis development (Johnson, J.P., 1991,

Cancer-Metastasis Rev. 10(1):11-22); ICAM-1, de novo expression of which is associated with development of metastatic potential in cutaneous melanoma (*id.*); VCAM-1, a decrease in which may be important in the development of metastatic potential of melanomas (Denton, K.J. et al., 1992, J. Pathol. 167(2):187-91); CD44, expression of a variant of which confers metastatic potential on pancreatic carcinoma cells (Gunthert et al., 1991, *Cell 65*:13-24), etc. (see also, *e.g.*, McCormick & Zetter, 1992, *Pharmacol. Ther. 53(2)*:239-60; Evans, C.W., 1992, *Cell Biol. Int. Rep. 16(1)*:1-10; Johnson, J.P., 1992, *Int. J. Clin. Lab. Res. 22(2)*:69-72).

4.2.4. CELLULAR DNA

The amount of double stranded and single stranded DNA (deoxyribonucleic acid) in a cell is a useful marker for the presence of malignant or pre-malignant cells. Normal euploid cells have only a single "copy" of cellular DNA and, in the human, 46 chromosomes, whereas malignant cells are often polyploid (diploid, tetraploid, etc.) having, for example, doubled or quadrupled the normal chromosome complement. Conventionally, polyploidy is assessed by flow cytometric analysis of total cellular DNA using DNA binding dyes. The present invention provides an assay for polyploidy by measuring the total amount of DNA present in a sample, preferably from a tissue or a suspension of single cells, in an assay following pretreatment of the cells with non-ionic detergent as described herein, for example with TRAx™ reagent, to make the DNA available for binding by a DNA binding partner. DNA binding partners include but are not limited to DNA binding proteins and anti-DNA antibodies, which are well-known in the art (see, for example, *Linscott's Directory*, *supra*, at p. 50). This method allows a laboratory lacking a flow cytometer to

detect polyploidy or measure the amount of DNA in a cell or cells by direct assay, thus providing a way of detecting a tumor in a cellular sample.

4.2.5. CYTOKINES

With respect to measurement of "total cytokine," the present invention provides a method for rapid detection of a cytokine present at the cell surface and/or intracellularly and/or in the extracellular fluid compartment of a biological sample. Preferably, the method is used to detect a cytokine in a sample of whole blood, including a stored sample of blood. The present method allows much greater reliability in measuring cytokine and flexibility in scheduling assay procedures to allow concurrent assay of multiple samples from the same or different subject, thereby decreasing interassay variability.

The method of the present invention overcomes the shortcomings of existing immunoassays or bioassays of cytokines, discussed above, by measuring the total amount of cytokine in a sample, which contains cells (including the membrane-bound and intracytoplasmic content) as well as the fluid, such as the blood, in which the cell is collected. The method of the present invention to measure a cytokine can provide information of cytokine level associated with the producing cells, allowing the approximation of the number of cytokine-producing cells in a sample.

Thus, the present invention also provides a method to estimate the number of cells producing a cytokine; a panel of control cells can be used as standards to determine a conversion factor for converting cytokine levels measured according to the invention into cell equivalent units. This estimate of cell number is useful for monitoring of disease (see *infra*).

Advantages of the present approach over those in the prior art include: (1) inclusion in the measurement of intracellular cytokine, which remains protected from serum proteases and is thus more stable over time, (2) the ability to measure total cytokines produced by cells which normally circulate in the body, such as leukocytes or lymphocytes, by performing an assay on a blood sample even when the affected area is strictly localized, and (3) the economy of measuring the total cytokine in a whole blood sample since it avoids the need for separations step for isolating plasma or serum, or for fractionating cells.

The methods of the present invention are useful for measuring the total amount of any cytokine, including, but not limited to interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 3 (IL-3), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 7 (IL-7), interleukin 8 (IL-8), interleukin 9 (IL-9), interleukin 10 (IL-10), interleukin 11 (IL-11), interleukin 12 (IL-12), monocyte chemoattractant protein-I (MCP-I), granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), tumor necrosis factor α (TNFα), tumor necrosis factor β (TNFβ), leukemia inhibitory factor (LIF), transforming growth factor α (TGF-α), transforming growth factor β1 (TGF-β1), transforming growth factor β2 (TGF-β2), transforming growth factor β3 (TGF-β3), interferon α (IFN-α), interferon β (IFN-β), interferon γ (IFN-γ), epidermal growth factor (EGF), acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor A (PDGF-A), platelet-derived growth factor B (PDGF-B), nerve growth factor β (NGF-β), HGF, insulin-like growth factor 1 (IGF-1), inhibin, and the like.

In a preferred embodiment, the present method of the present invention is used to measure total IL-4 in a blood sample to monitor the level of IL-4 producing lymphocytes in an allergy patient undergoing allergen immunotherapy; for example, in which the presence of or increased levels of IL-4 indicates an allergic response, or increased severity thereof and vice versa. In another embodiment, IL-2 can also be measured to detect T cell anergy (non-responsiveness), wherein a decrease in IL-2 is indicative of T cell anergy. In another embodiment, the method is used to monitor the level of lymphocytes producing one or more interferons in an infected patient. In another embodiment, the present method is used to monitor a transplant patient for the level of lymphocytes producing one or more interleukins, in particular during rejection episodes (correlating with increased expression of interleukins) and following immunosuppressive therapy. In yet another embodiment, total cytokine is measured as a means of monitoring cytokine-producing blood cells in patients with a hematological diseases in relation to therapy and/or disease relapse, or to monitor immune activation.

Any binding partner specific to the cytokine target molecule can be employed in the assay methods of the invention. In a specific embodiment, a cell surface receptor for the cytokine can be used. For example, the IL-2 receptor can be used to assay for IL-2 (see Rubin *et al.*, 1986, *J. Immunol.* *137*:3841-3844). In a preferred embodiment, an antibody binding partner is employed.

4.2.6. MULTIDRUG RESISTANCE MARKERS

In a specific embodiment, a multidrug resistance marker such as P-glycoprotein, the product of the MDR1 gene (Twentyman *et al.*, 1992, *J. Natl. Cancer Inst.* 84:58-60; Holzmayer *et al.*, 1992, *J. Natl. Cancer*

Inst. 84:1486-1491; Clarke *et al.*, 1992, *J. Natl. Cancer Inst.* 84:1506-1512) can be detected or measured by the assay of the invention. The invention provides a method for detecting multidrug resistance (*i.e.*, lack of sensitivity to drugs commonly used to treat cancer such as anthracyclines, vinca alkaloids, and epipodophyllotoxins) or a poor prognosis for a patient with a malignancy, by detecting or measuring the amount of P-glycoprotein in a sample from the patient containing or suspected of containing cancer cells. Such cancer cells include but are not limited to cells of leukemias, lymphomas, myelomas, breast cancers, esophageal carcinomas, childhood sarcomas, neuroblastomas, lung cancers, in particular small cell lung cancers, ovarian carcinomas, and other tumors, and, in particular, metastatic cancers. An increase in the measured amount of P-glycoprotein in the sample relative to the level of P-glycoprotein present in comparable cells which are not multidrug resistant or which are not cancer cells, indicates that the cells in the sample from the patient are multidrug resistant. Comparable cells are preferably cells of the same type as the cells present in the patient sample. Thus, the method of the invention has utility in monitoring the response of cancer patients to chemotherapy, or in determining the course of therapy.

Antibodies to P-glycoprotein are known in the art, *e.g.*, C219, JSB-1, and MRK16 (see *e.g.*, Wishart *et al.*, 1990, *Br. J. Cancer* 62:758-761; Thiebaut *et al.*, 1989, *J. Histochem. Cytochem.* 37:159-164; Schinkel *et al.*, 1991, *Cancer Res.* 51:2628-2635).

4.2.7. COMPLEMENT RECEPTOR MOLECULES

The present invention is particularly suitable for the detection or measurement of total amount of a complement receptor that may be found in a fluid, cell or tissue sample. Especially useful in this regard is

the detection of CR1 (CD35) or CR2 (CD21) on the surface of blood cells or other cells. Complement Receptor Type II (CR2) is present on the surface of cells such as B cells and T cells. An important source of CR1 is the red blood cell, which is notably quite fragile when compared to other blood cells. Heretofore, researchers have had to resort to use of conventional radioimmunoassay techniques, or would have to resort to instrumentation such as flow cytometry to explore the expression of CR1 or other red blood cell markers that might be of interest on a red blood cell. Such instrumentation methods have been fraught with difficulty, as such samples are simply not amenable to analysis using these particular art-accepted cell analysis techniques. The present invention provides an alternative assay which enables one to determine and measure the presence of CD35 in a safe and efficient manner. Measurement of CD35 is useful in many research and disease associated situations, such as those set forth in Section 2.4 above. In preferred embodiments, the CD35 detection is relevant to HIV infections, wherein alterations in amount of red blood cell CR1 could be measured as a function of the progression of the disease. Appropriate selection of a course of therapy and the success of such therapeutic intervention may also be tracked by measuring this red blood cell marker.

Binding partners suitable for use in the methods of the invention for detection of complement receptor are those capable of binding to all or a portion of the target complement receptor molecule. Especially preferred are antibodies to CD35, opsonins capable of binding to complement receptor, as for instance C3b, C4b, (binds CR1), iC3b (binds CR1 and CR2), C4d, C3d, C3dg (binds CR2), and the like. A good description of complement and various proteins that bind to complement receptor may be found in "Clinical Aspects

and Relevance to Disease", Paul Morgan, Academic Press, 1990.

4.3. EXOGENOUS TARGET MOLECULES

For detection of an exogenous target molecule, preferably a molecule associated with a pathogenic microorganism (*e.g.* an antigen of a microbial pathogen), any clinical specimen may be used, typically a sample of body fluid, a tissue specimen, or a swab from a mucosal surface. As used herein, a target molecule "associated with" a pathogenic microorganism shall mean a molecule whose presence is indicative of the presence of the microorganism, *e.g.*, a pathogen-specific surface or envelope antigen, core protein, nucleic acid binding protein, enzyme, etc. Swab specimens are obtained from sites suspected of containing the microorganism or containing cells within which the microorganism is growing. Non-limiting examples of such sites include oral, nasopharyngeal, conjunctival, urogenital, endocervical, anorectal, and the like. Such a swab may then be placed in a transport tube containing a buffer or, preferably, the lysing reagent (detergent). The swab is moistened with the lysis reagent and transported in this form to the laboratory for assay. The lysis reagent, containing the released antigens may be assayed directly or stored frozen, as described above.

The method of the present invention is especially advantageous for detecting the presence of, and thus diagnosing infection by, intracellular pathogens, since molecules associated with such pathogens may be sequestered within the cell and thus unavailable for detection by prior art assay methods. The assay methods of the invention can also be used to determine the severity of, stage, or monitor therapy of an infection by a microbial pathogen. For example,

measurement of the amount of a microorganism-associated target molecule in a sample can be used as an indication of the severity of infection; the number of infectious units/pathogens in the sample can be calculated from such amount by using a conversion factor previously determined using known amounts of infectious units, which reflects the relationship between the amount of target molecules and number of infectious units. Where the organism to be detected is an intracellular pathogen, it is essential that a sample contain a sufficient amount for detection of the pathogen-associated target molecule, or a sufficient number of infected cells. The number of cells required is a function of the nature of the microorganism, the quantity of the target molecule per cell, the type and affinity of the binding partner used in the assay, and the like, readily determinable by one skilled in the art. If the microbe-associated target molecule is not secreted or available on the infected cell surface, the cells must be treated to release the organism or its target molecule from the cell in a manner in which it is available for binding to a binding partner, and thus detectable, with preferably no further manipulation or with minimal need for further sample handling. Depending on the intracellular site of the target molecules, for example, free in the cytoplasm, or in vacuoles, vesicles or phagosomes, it is preferred to release the maximal amount of target molecule from such compartments. Treatment with detergent as described herein is the preferred method for making the target molecule available to a binding partner for detection.

One of ordinary skill in the art will appreciate the applicability of the present invention to measurement of any target molecule for which an appropriate binding partner is available. In a preferred embodiment, antibodies for a wide variety of

infectious microorganisms, or for antigens associated with these microorganisms, are commercially available and readily adaptable for use according to the methods disclosed herein. A non-limiting list of the organisms which can be detected (or whose toxic products can be detected) using the method of the present invention appears in Table V, below, and in Tables I, II, and III, *supra*.

Antibodies which allow detection of the above organisms can be found listed in *Linscott's Directory of Immunological and Biological Reagents*, Seventh Edition, 1992, Santa Rosa, CA, pp. 43-48. Also listed therein are the commercial sources for such antibodies. In particular, exemplary immunoassay configurations and antibodies for detection and/or measurement of a hepatitis B virus antigen are found in U.S. Patent No, 4,474,878 and U.S. Patent No. 4,642,285.

Binding partners which are not antibodies or derivatives or fragments thereof can also be used in the assays of the invention. For example, cell-surface receptors for microbial pathogen-associated target molecules can be used as binding partners for such target molecules to detect and/or measure the amount of the target molecules. As particular examples, ICAM-1 is a binding partner which can be used to detect and/or measure a rhinovirus-associated target molecule; CD4 is a binding partner for gp120 or gp160 associated with Human Immunodeficiency Virus (HIV), etc.

One major advantage of the present method is particularly relevant for infected samples. The minimum sample preparation required in the method of the present invention substantially lowers risk of creating aerosols that are hazardous to the laboratory worker. The solubilization procedure using concentrated detergent also inactivates enveloped

viruses, such as HIV-1, thereby making subsequent analysis safer.

TABLE V

BACTERIA	VIRUSES	OTHER
Bordetella pertussis	Adenoviruses	*Candida albicans*
Borrelia burgdorferi	Cytomegalovirus	*Chlamydia trachomatis*
Campylobacter jejunii	Hepatitis A, B, C	*Plasmodium falciparum*
Campylobacter pylori	Herpesviruses	*Pneumocystis carinii*
Mycobacteria spp.	HIV-1, HIV-2	*Toxoplasma gondii*
Mycoplasma spp.	Influenza	*Treponema pallidum*
Neisseria gonorrheae	Measles	*Trichomonas vaginalis*
Neisseria meningitidis	Mumps	
Yersinia pestis	Papilloma	
Gardnerella vaginalis	Respiratory syncytial	
Salmonella spp.	Varicella zoster	
Shigella spp.		

In a specific embodiment, the p24 antigen of HIV (the causative agent of AIDS) is detected or measured.

In a preferred embodiment of the invention, diagnosis of infection by *Chlamydia trachomatis* is carried out by detecting Chlamydial antigen (Schachter and Dawson, 1978, *Human Chlamydial Infections*, PSG Publishing Co., Inc., Littleton, MA) by the assay of the invention using samples which are urogenital, endocervical, conjunctival, or nasopharyngeal swab specimens.

4.4. SAMPLE PREPARATION AND ASSAY METHOD

The amount of total target molecule is determined in an assay wherein the target molecule is bound to a specific binding partner. In a preferred embodiment, the assay is an immunoassay wherein one or more epitopes of the target molecule (antigen) is recognized by a specific antibody or antibodies. In a preferred embodiment, two different epitopes of the

antigen are bound by two different antibodies in the course of the immunoassay.

The sample being analyzed, such as whole blood, is first treated to solubilize the cellular components (step 1). It is important that the cells be solubilized without damaging the integrity of the target molecule being measured, which would interfere with binding to its binding partner. A preferred solubilization method is to treat the cells with concentrated non-ionic detergent to efficiently lyse the cells, followed by dilution of the detergent-treated sample prior to assay. Other methods of solubilizing cells, such as repeated freeze-thaw cycles, sonication, hypotonic shock, or the addition of lower concentrations of detergents, are not as effective. Ionic detergents such as sodium dodecyl sulfate (SDS) are not effective, because they interfere with the subsequent target molecule-binding partner binding.

Non-ionic (includes zwitterionic) detergents for use in present invention include but are not limited to Triton X-100 (Octylphenol-polyethlene glycolther), Nonidet P-40 (NP-40, "Octylphenol-ethylene oxide condensate"), Tween-20 (Polyoxyethlene sorbiton monolaurate), CHAPS (3-[(C3-cholamido propyl-dimethyl-ammonio]1-propane sulfate, inner salt), CHAPSO (3-[(3-Cholamido propyl-dimethyl-ammonio]2 hydroxypropane sulfonate), BIGCHAP (N,N-Bis(3-D-gluconamido propyl)deoxycholamide), Deoxy-BIGCHAP (N,N-Bis(3-D-gluconamido propyl)deoxychlamide), (3-Sulfopropyl)dimethyl (3-methacrylamido-propyl ammonium inner salt), to mention a few. One skilled in the art may refer to publications such as various reagent catalogs for additional non-ionic detergents that may be appropriate for use in accordance with the present invention. One such reagent supplier is Sigma Chemical Company, located in St. Louis, Missouri, USA.

In a preferred embodiment, the sample is solubilized with Triton X-100, Nonidet P-40, Tween-20 and/or CHAPS. In a more preferred embodiment, the sample is solubilized with concentrated detergent to give a final detergent concentration of about 2% to about 4% in the detergent-treated sample.

According to the invention, the total volume of non-ionic detergent added to the sample should not dilute out the target molecule in the sample. Preferably, the volume of detergent does not exceed about 25% of the sample volume; more preferably, it does not exceed about 20%. The non-ionic detergent or detergents can be added to the original sample neat, or they can be prepared in a concentrated solution. In addition to the non-ionic detergent or detergents, the concentrated detergent solution can comprise distilled water or buffer. The concentration of non-ionic detergent or detergents in the solution will preferably be 5 times, and more preferably 6 times the final concentration of the non-ionic detergent or detergents after addition to the sample, i.e., in the detergent-treated sample.

In an even more preferred embodiment, more than one non-ionic detergent is used to treat the sample. For example, a relatively high concentration of Tween-20 and Triton X-100, or Triton X-100 and NP-40, or NP-40 and Tween-20 can be used. A prepared concentration of each detergent ranges from about 1% to about 2% after addition to the sample; where the total preferred concentration of detergent in the detergent-treated sample would then range from about 2% to about 4%. More preferably the total detergent concentration ranges from about 2% to about 3%; and even more preferably from about 2% to about 2.5%. In a specific preferred embodiment, the final detergent concentration in the sample is 1.5% Triton X-100 and 1% NP-40.

In certain preferred embodiments, the detergent concentration is a concentration that inactivates bacteria, viruses, or other pathogens. It is a particular advantage of the invention that the lytic concentrations of non-ionic detergent, such as the preferred ranges set forth above, are also typically pathogen-inactivating concentrations.

After solubilization for at least about one minute, preferably for about one to about five minutes, the sample is preferably diluted with buffer (step 2) prior to analysis for detection of target molecule. The dilution can be 2-fold; preferably it is 5-fold or greater. The buffer is chosen to be compatible with the assay for detecting the target molecule.

Dilution of the sample may be performed with any of a number of buffers well-known in the art. A preferred buffer is phosphate buffered saline, preferably supplemented with protein, with no stabilizers added.

In summary, an important component of the methods of the present invention is the simple direct technique used to solubilize cells in a sample using a non-ionic detergent, making the target molecule available to its binding partner while conserving recognition by its binding partner. For example, in the use of an immunoassay, the target molecule is made available to an antibody or a combination of a capture antibody and a detection antibody, while maintaining antigenic integrity of the antigen to be detected.

In one embodiment, a sample comprising 100 μl of whole blood is assayed for total amount of a target molecule. In a more preferred embodiment, wherein the target molecule is a blood cell antigen, a sample of 10-25 μl of whole blood is used. The amount of sample used is a function of the 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. In a specific embodiment, a blood sample is spotted on filter paper, and the filter paper is dried and then placed in a vial containing detergent for cell solubilization.

A solubilized sample may 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 interassay variability.

In a specific embodiment, a sample can be lyophilized by methods known in the art (see, *e.g.*, U.S. Patent No. 5,059,518) and then stored prior to detergent addition. At the desired time, the lyophilized sample can be retrieved, and detergent added directly to the sample for assaying according to the invention.

Any method of detecting and measuring target molecules may be used in the practice of this invention. Preferably, an immunoassay is carried out such as a sandwich or a competitive EIA (enzyme immunoassay) (see Examples, below). In a preferred embodiment, the immunoassay utilizes a CELLFREE® assay kit.

A modification of the above-described method can also be used to assay the total amount of a plurality of two or more target molecules. In yet a further embodiment, the relative amounts of two or more target molecules may be compared.

Measurement of the total amount of a target molecule is an improvement over measuring only the cell bound target molecule, the target molecule in a cell lysate or the soluble target molecule, for the following reasons. First, the measurement can provide information of the total amount of target molecule present in all three compartments, not just the amount

present in one or two compartments. Second, the measurement of total target molecule is simpler than other procedures that involve more cumbersome steps of sample preparation, complex equipment and time. Third, small quantities of sample, *e.g.*, 100 μl, and as little as 5-10 μl of whole blood, can be directly analyzed in a simple assay format without prior enrichment of particular fractions of the sample. The small volume of whole blood necessary has major benefits in use of the assays in the pediatric setting where the patients are infants and small children. The present methods represent a significant savings in cost per sample analyzed. Furthermore, elimination of a need for expensive equipment makes the method widely available to many laboratories or clinics, and improves its adaptability to non-laboratory conditions, such as field conditions.

4.5. ASSAY FORMATS AND KITS

This invention provides a method to prepare a sample to measure total target molecule. Many different assays and assay formats can be used to detect the target molecule of interest in the treated sample. Both competitive and non-competitive binding assays can be used. In preferred aspects, immunoassays are used, employing an antibody as a binding partner; these include but are not limited to the immunoassays used for measuring soluble antigens. A kit for carrying out the assay for total target molecule includes a kit with components that allow the measurement of multiple target molecules in one sample, for example the measurement of total antigen A and total Le^b, or total chlamydial antigen and total cytokine, etc. In another embodiment, a kit of this invention allows the measurement of total target molecule and measurement of soluble target molecule in the same sample, such as a blood sample.

Any procedure known in the art for the measurement of analytes can be used in the practice of the instant invention. Such procedures include but are not limited to competitive and non-competitive assay systems using techniques such as radioimmunoassays, enzyme immunoassays (EIA), preferably the enzyme linked immunosorbent assay (ELISA), "sandwich" immunoassays, precipitin reactions, gel diffusion reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, and immunoelectrophoresis assays, to name but a few. For examples of preferred immunoassay methods, see U.S. Patents No. 4,845,026 (July 4, 1989) and No. 5,006,459 (April 9, 1991).

In one embodiment, one or more binding partners used in an assay to bind a target molecule according to the invention is labeled; in another embodiment, such a first binding partner is unlabeled, and a labeled, second binding partner of the first binding partner is used to detect the bound first binding partner. As but one example of the latter embodiment, where a rat IgG monoclonal antibody is used to detect or measure total target molecule (antigen) in a sample by binding thereto, labeled goat anti-rat immunoglobulin can be used to detect the bound monoclonal antibody.

In a preferred embodiment, polyclonal and/or monoclonal antibodies can be used in sandwich immunoassays according to the invention. In a particular embodiment, a first binding partner which is not an antibody or antibody fragment or derivative is used, and a second binding partner which is an antibody or antibody fragment or derivative is used.

In an EIA, the enzyme which can be used to detectably label an antibody include, but are not

limited to, horseradish peroxidase, alkaline phosphatase, glucose-6-phosphate dehydrogenase, malate dehydrogenase, staphylococcal nuclease, Δ-V-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, asparaginase, glucose oxidase, β-galactosidase, ribonuclease, urease, catalase, glucoamylase and acetylcholinesterase.

It is also possible to label the detection antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labelling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The detecting antibody can also be detectably labeled using fluorescence emitting metals such as ^{152}Eu, or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester. Likewise, a bioluminescent compound may be used to label the antibody. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a biolumi-

nescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

Any other label known in the art may be used, *e.g.*, a radionuclide, etc.

In the assays of the present invention, an antigen or an antibody is preferably bound to a solid phase support or carrier. By "solid phase support or carrier" is intended any support capable of binding an antigen or antibodies. Well-known supports, or carriers, include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amyloses, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

In a preferred embodiment, an antibody-antigen-antibody sandwich immunoassay is done, *i.e.*, antigen is detected or measured by a method comprising binding of a first antibody to the antigen, and binding of a second antibody to the antigen, and detecting or measuring antigen immunospecifically bound by both the first and second antibody. In a specific embodiment, the first and second antibodies are monoclonal

antibodies. In this embodiment, if the antigen does not contain repetitive epitopes recognized by the monoclonal antibody, the second monoclonal antibody must bind to a site different from that of the first antibody (as reflected *e.g.*, by the lack of competitive inhibition between the two antibodies for binding to the antigen). In another specific embodiment, the first or second antibody is a polyclonal antibody. In yet another specific embodiment, both the first and second antibodies are polyclonal antibodies.

In a preferred embodiment, a "forward" sandwich enzyme immunoassay is used, as described schematically below. An antibody (capture antibody, Ab1) directed against the antigen is attached to a solid phase matrix, preferably a microplate. The sample is brought in contact with the Ab1-coated matrix and such that any antigen in the sample to which Ab1 is specific binds to the solid-phase Ab1. Unbound sample components are removed by washing. An enzyme-conjugated second antibody (detection antibody, Ab2) directed against a second epitope of the antigen binds to the antigen captured by Ab1 and completes the sandwich. After removal of unbound Ab2 by washing, a chromogenic substrate for the enzyme is added, and a colored product is formed in proportion to the amount of enzyme present in the sandwich, which reflects the amount of antigen in the sample. The reaction is terminated by addition of stop solution. The color is measured as absorbance at an appropriate wavelength using a spectrophotometer. A standard curve is prepared from known concentrations of the antigen, from which unknown sample values can be determined.

Other types of "sandwich" assays are the so-called "simultaneous" and "reverse" assays. A simultaneous assay involves a single incubation step as the antibody bound to the solid support and labeled

antibody are both added to the sample being tested at the same time. After the incubation is completed, the solid support is washed to remove the residue of fluid sample and uncomplexed labeled antibody. The presence of labeled antibody associated with the solid support is then determined as it would be in a conventional "forward" sandwich assay.

In the "reverse" assay, stepwise addition first of a solution of labeled antibody to the fluid sample followed by the addition of unlabeled antibody bound to a solid support after a suitable incubation period is utilized. After a second incubation, the solid phase is washed in conventional fashion to free it of the residue of the sample being tested and the solution of unreacted labeled antibody. The determination of labeled antibody associated with a solid support is then determined as in the "simultaneous" and "forward" assays.

In an embodiment wherein a binding partner is not an antibody (for example, where the binding partner is a receptor for the target molecule), assay configurations and labels which can be used include those described above for immunoassays as well as others known in the art.

Kits comprising one or more containers or vials containing components for carrying out the assays of the present invention are also within the scope of the invention. For instance, such a kit can comprises a detergent solution, preferably the Trax® lysing reagent (6% NP-40 and 9% Triton X-100 in 1X PBS). Also included in the kit are one or more binding partners, *e.g.*, an antibody or antibodies, preferably a pair of antibodies to the same antigen, for example a leukocyte, erythrocyte or bacterial antigen, which preferably do not compete for the same binding site on the antigen. In another embodiment, a kit can comprise more than one pair of such antibodies or

other binding partners, each pair directed against a different target molecule, thus allowing the detection or measurement of a plurality of such target molecules in a sample, for example, target molecules associated with several bacterial pathogens or with a bacterial and a viral pathogen. In a specific embodiment, one binding partner of the kit may be pre-adsorbed to the solid phase matrix, or alternatively, the binding partner and matrix are supplied separately and the attachment is performed as part of the assay procedure. The kit preferably contains the other necessary washing reagents well-known in the art. For EIA, the kit contains the chromogenic substrate as well as a reagent for stopping the enzymatic reaction when color development has occurred. The substrate included in the kit is one appropriate for the enzyme conjugated to one of the antibody preparations. These are well-known in the art, and some are exemplified below. The kit can optionally also comprise a target molecule standard; *i.e.*, an amount of purified target molecule that is the target molecule being detected or measured.

In a specific embodiment, a kit of the invention comprises in one or more containers: (1) a solid phase carrier, such as a microtiter plate coated with a first binding partner; (2) a detectably labeled second binding partner which binds to the same antigen as the first binding partner; (3) a standard sample of the target molecule recognized by the first and second binding partners; (4) concentrated detergent solution; and (5) optionally, diluent. In a specific embodiment, the target molecule is not a leukocyte cell surface marker.

4.6. ANTIBODIES

In one embodiment, one or more antibodies constitute the one or more binding partners used in

the assays of the invention, to detect and/or measure a target molecule which is an antigen. Antibodies useful in the detection of total antigen as described herein include polyclonal antibodies, monoclonal antibodies (mAbs), and chimeric antibodies (see below). Preferred antibodies are mAbs, which may be of any immunoglobulin class including IgG, IgM, IgE, IgA, and any subclass or isotype thereof. The term "antibody" is also meant to include both intact molecules as well as fragments thereof which bind the antigen, such as, for example, $F(ab')_2$, Fab', Fab and Fv. These fragments lack the Fc fragment of an intact antibody molecule, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody (Wahl *et al.*, 1983, *J. Nucl. Med.* *24*:316-325), properties which may be desirable for particular therapeutic or diagnostic utilities. It will be appreciated that these antigen-binding or epitope-binding fragments of the antibodies useful in the present invention may be used for the detection and quantitation of the total amount of an antigen, or cells expressing or harboring the antigen, as disclosed herein for intact antibody molecules. Such fragments are typically produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce $F(ab')_2$ fragments) or by reducing the disulfide bridges.

Various procedures well-known in the art may be used for the production of polyclonal antibodies to an epitope or antigen of interest. A host animal of any of a number of species, such as rabbits, goats, sheep, horse, cow, mice, rats, etc. is immunized by injection with an antigenic preparation which may be derived from cells or microorganisms, of may be recombinantly or synthetically produced products. Various adjuvants well-known in the art may be used to enhance the

production of antibodies by the immunized host, for example, Freund's adjuvant (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substance such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, liposomes, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and *Propionibacterium acnes*

A mAb specific for an epitope of an antigen of interest can be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975, Nature 256:495-497), and the more recent human B cell hybridoma technique (Kozbor *et al.*, 1983, Immunology Today 4:72) and EBV-hybridoma technique (Cole *et al.*, 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

The antibody used in the methods of the present invention may also be a chimeric antibody, preferably a mouse-human chimeric antibody, wherein the heavy and light chain variable regions are derived from a murine mAb and the constant regions are of human origin. Methods for producing chimeric antibody molecules are disclosed, for example, in Gorman *et al.*, PCT Pub.WO9206193 (4/16/92); Cabilly *et al.*, U.S. Patent 4,816,567 (3/28/89) and Eur. Patent Pub. EP125023 (11/14/84); Taniguchi *et al.*, EPO Pub. EP171496 (2/19/86); Morrison *et al.*, EPO Pub. EP173494 (3/5/86); Neuberger *et al.*, PCT Pub. WO8601533 (3/13/86); Kudo *et al.*, EPO Pub. EP184187 (6/11/86); Robinson *et al.*, PCT Pub. WO8702671 (5/7/87); Boulianne *et al.*, *Nature 312*:643-646 (1984); Morrison, *Science 229*:1202-1207 (1985); Neuberger *et al.*, *Nature 314*:268-270 (1985); Takeda *et al.*, *Nature 314*:452-454 (1985); Oi *et al.*, *BioTechniques 4*:214 (1986); and

Liu *et al.*, *Proc. Natl. Acad. Sci. USA* *84*:3439-3443 (1987).

MAbs or chimeric antibodies can be "humanized" by producing human constant region chimeras, where even parts of the variable regions, in particular the conserved or framework regions of the antigen-binding domain, are of human origin, and only the hypervariable regions are non-human. See for example, UK Patent Publication GB 2188638A ("Chimeric Antibodies"); Harris *et al.*, PCT Publication WO 9204381 (March 19, 1992); and Riechmann *et al.*, 1988, *Nature* 332:323-327.

In yet another embodiment, the antibody is a single chain antibody formed by linking the heavy and light chain fragment of the Fv region via an amino acid bridge, resulting in a single chain polypeptide (Bird, 1988, Science 242:423-426; Huston *et al.*, 1988, *Proc. Natl. Acad. Sci.* USA 85:5879-5883: and Ward *et al.*, 1989, *Nature* 34:544-546).

A recombinantly cloned antibody molecule or fragment may be prepared using methods well-known in the art. Recombinant DNA methodology (Sambrook, J. *et al.*, 1989, *MOLECULAR CLONING: A LABORATORY MANUAL*, 2d Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY) may be used to construct nucleic acid sequences which encode a mAb molecule, chain or epitope-binding fragment.

Antibody molecules may be purified by known techniques, *e.g.* immunoabsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof, etc.

Once specific antibodies are demonstrated to be suitable for use, other such suitable antibodies may be selected by virtue of their having different epitope specificity from the former antibodies. Epitope specificity can be ascertained, for example,

by observing the ability of a second antibody to inhibit binding of a first antibody to its antigen.

4.7. MONITORING THE EFFECT OF A THERAPEUTIC TREATMENT

In a subject undergoing therapeutic treatment that results in an increase or a decrease in the amount of an exogenous or endogenous target molecule, the amount of target molecule present in a sample may serve as a useful measure for the success or failure of the treatment. The sample may be derived from a body fluid, a cell sample or a tissue sample of the subject. Thus, the present invention provides a method for monitoring the effect of a therapeutic treatment in a subject which comprises measuring at suitable time intervals the amount of a target molecule. The total target molecule is compared to a "baseline" or "control" value which, depending on the target molecule, the disease, and the treatment, may be the amount of target molecule in a similar sample from a normal subject, from the patient prior to disease onset or during remission of disease, or from the patient prior to the initiation of therapy. One of ordinary skill in the art will readily discern the appropriate baseline value to use in a particular situation without undue experimentation.

In the case of an infectious disease, for example, a fall in the amount of a microbe-associated target molecule or its disappearance in a cell, tissue or body fluid sample is indicative of effective therapy. A cure of the disease is accompanied by a decrease to undetectable levels of the microbe-associated target molecule. Lack of change, or a rise, in the target molecule is indicative that the therapy is not successful. In a preferred embodiment, a subject being treated for *Chlamydia trachomatis* infection is tested sequentially after initiation of

therapy using the present method to detect the amount of a chlamydia-associated target molecule present in sequential samples taken from the subject.

The therapeutic treatments the response to which may be evaluated according to the present invention include but are not limited to radiotherapy, chemotherapy and drug therapy, vaccine administration, immunosuppressive, immuno-restorative or immunostimulatory regimens, and the like.

4.8. DETECTING, DIAGNOSING OR STAGING A DISEASE IN A SUBJECT

In another embodiment of the present invention, measurement of the total amount of a target molecule is used to detect, diagnose or stage a disease or disorder in a subject. A disease or disorder, associated with a particular target molecule is detected, diagnosed or staged by measuring the total amount of the target molecule in a sample, and comparing that to a baseline level, as described above. Thus a microbial infection, including but not limited to those listed in Tables I-III and Table V is diagnosed by the detection of the target molecule associated with a microbial pathogen. Thrombocytopenia is detected by the decrease in the amount of a platelet-associated target molecule in a blood sample compared to a baseline amount. Development of a pre-malignant lesion or cancer is detected by the appearance of a tumor-specific or tumor-associated target molecule (*e.g.*, antigen) in cells of a sample. In a preferred embodiment, a pre-malignant change in the gastrointestinal mucosa is detected by measuring the total amount of the blood group A antigen or the level of the A-transferase enzyme in a sample. Similarly, the progression of the disease, or the regression in response to therapy is monitored by measuring changes in the appropriate

target molecule, up or down, over time. Armed with the teachings presented herein, and a binding partner for a target molecule associated with a disease or disorder, one of ordinary skill in the art will be able to apply the method to the detection, diagnosis, staging or monitoring of any disease without undue experimentation.

4.9. DETECTING OR DIAGNOSING OF DISEASE OR MONITORING OF RESPONSE TO TREATMENT IN PATIENTS BY MEASURING THE TOTAL AMOUNT OF A PLURALITY OF TARGET MOLECULES

The present invention also provides for the detecting or staging of disease, or the monitoring of treatment by measuring the total amount in a sample of a plurality of at least two target molecules. For example, a target molecule associated with a microbial pathogen may be measured in conjunction with one or more T cell surface markers, for example, CD35, CD4 and CD8, either in soluble form or in total form, to diagnose, detect, stage or monitor treatment of a disease or disorder such as those discussed above. The measurement of the soluble and/or total levels of a T cell marker serves as a measure of the function of the immune system, which may be modified in parallel with the disease course or treatment efficacy.

In one embodiment, a disease caused by HIV-1 or HIV-2 infection may be monitored by measurements of one or more leukocyte surface markers, preferably total CD4, as well as detecting the total HIV antigen in a blood sample, a blood cell sample or in a select population of T cells and/or monocytes. The response to an AIDS therapeutic, for example, azidodeoxythymidine, dideoxyinosine, γ interferon, β interferon, soluble CD4, an HIV vaccine, etc., can be monitored using such a method.

In another specific embodiment, both (1) the p24 antigen of HIV, and (2) an erythrocyte blood group

antigen, are detected and/or measured according to the invention.

5. EXAMPLE: DETECTION OF *CHLAMYDIA TRACHOMATIS*

5.1. CLINICAL SAMPLE

A clinical specimen suspected of containing the organism is obtained on a swab from a site thought to be infected, including urogenital, endocervical, conjunctival and nasopharyngeal sites. In males, urine is also tested. A swab is placed in a transport tube containing the TRAx™ lysing reagent (6% NP-40 and 9% Triton X-100 in 1x PBS) diluted 1:6 in a volume of 200 µl. The swab is moistened with the lysis reagent and transported to the laboratory for assay. Once the sample arrives in the laboratory, a volume of sample diluent, about 800 µl is added to the tube, the contents mixed, and the swab is discarded. The contents of the tube are then subjected to the immunoassay.

The quality of the sample is important for detection of this organism by immunoassay. Since Chlamydia are obligate intracellular parasites, it is essential that a sample contain a sufficient number of infected cells, and that the organisms can be released from these cells for detection. The TRAx™ lysing reagent disrupts epithelial cells and their internal vesicles which contain chlamydial reticulate bodies, which increases the yields of antigen for the immunoassay. Standard TRAx™ immunoassay methods are then used.

5.2 IMMUNOASSAY

The immunoassay is performed as follows:

1. 100 µl of sample is added to duplicate wells of a 96-well microplate coated with antibody to a Chlamydial antigen, such as the antibodies in the CHLAMYDIAZYME® assay kit from Abbott

Laboratories; positive control wells receive 100 µl of a known concentration of chlamydial antigen; negative control wells receive 100 µl of the assay buffer.

2. 100 µl of an enzyme-conjugated antibody to chlamydial antigen, or, as in the CHLAMYDIAZYME® assay kit, an unlabeled anti-chlamydial antibody followed by an enzyme-conjugated anti-immunoglobulin reagent, is added to all wells. A horse radish-peroxidase enzyme is preferred.
3. The plate is incubated for 1-3 hours at room temperature.
4. The wells are washed at least 3 times with TRAx™ washing solution (PBS + 0.05% Tween)
5. 200 µl of substrate/chromagen (for horseradish peroxidase, the substrate is urea peroxide and the chromogen is o-phenylene diamine·2HCl (OPD)) is added to each well, including to 2 "substrate blank" wells.
6. The plate is incubated for 15-30 minutes to allow for the generation of the colored reaction product.
7. The color is read in a microplate reader which is blanked against the substrate blanks.

The cut-off value for designating a sample as being positive for the antigen is 0.1 O.D. unit greater than the mean of the negative control values. Any value less than or equal to this is considered negative. Samples which fall between the cut-off value and the positive range are considered equivocal and may be retested.

6. EXAMPLE: DETECTION OF BLOOD GROUP A ANTIGENS AND A-TRANSFERASE ACTIVITY

To detect low concentrations an antigen, especially a low molecular weight antigen such as

blood group A antigen, competitive immunoassay is preferred to a sandwich assay because of the lack of multiple antigenic binding sites on the target molecule. In cells preparations such as premalignant lesions or tumors, the level of antigen A is declining. Thus, to measure the levels of A antigen or to detect the activity of the A transferase enzyme, a competitive assay is selected. Unlike the sandwich EIA, where a capture antibody is bound to a solid support, in the present competitive assay, a synthetic blood group A determinant is conjugated to bovine serum albumin (BSA), using conventional methods (for example, O'Sullivan *et al.*, *Anal. Biochem. 100*:100 (1979)) and used to coat microplates. Synthetic BSA conjugates of oligosaccharide blood group antigens are commercially available from Chembiomed, Ltd., Edmonton, Alberta, CANADA, or from ELA Technologies, Inc.

6.1. SAMPLE TREATMENT

One volume of the TRAx™ detergent solution, containing 6% NP-40 and 9% Triton X-100 in 1x PBS) is mixed with 5 volumes of fresh EDTA-treated whole blood or another body fluid or cell sample, followed by gentle mixing. This method of cell lysis is superior to solubilization or disruption of cells by hypotonic treatment or sonication. After 5 min., the treated sample is diluted 1:5 in the standard TRAx™ sample diluent (4% BSA, 1% sucrose, 0.01% thimerosal in 1x PBS, pH 7.5) to reduce the possible effects of detergent on the assay performance. Samples are assayed fresh or stored at -70°C prior to analysis. The samples are assayed in a one step format.

6.2. PLATE COATING

Microplates are coated with 300 μl of synthetic blood group A determinant, Fucα1→2GalNAcα1→3Galβ→, conjugated to BSA in a solution of phosphate buffered saline (PBS), 5 mM $MgCl_2$, 0.01% thimerosal. The mixture is incubated for 16 hours at 4°C, following which the plates are blocked for 2 hours at 37°C with 5% BSA in PBS, 0.01% thimerosal. The plates are then washed 3 times with PBS, 0.05% Tween-20, 0.01% thimerosal, air dried for 1 hour at room temperature and stored at 4°C. Plates are used directly with no additional washing required prior to use.

6.3. ASSAY

To assess A-transferase activity by immunoassay, a substrate for the enzyme which is linked by the enzyme to A precursor material in the sample is provided. As A antigen is produced as a result of the enzymatic reaction, it competes with the immobilized A antigen for binding to labeled antibody.

Thus, the immunoassay is performed by adding to the coated wells a test sample and a "conjugate solution" containing a substrate for A-transferase, UDP-GalNAc (Sigma Chemical Co.) and a horse radish peroxidase (HRP)-conjugated mAb specific to the blood group A antigen or to the A-transferase enzyme, such as the anti-A-transferase mAb from Chembiomed, Inc., Edmonton, CANADA, in a solution of 100 mM sodium cacodylate (pH 6.8), 50 mM $MnCl_2$, 1% BSA, 0.01% thimerosal.

The sample solution (50 μl) and 50 μl of conjugate solution are added to the plate, mixed and incubated, with shaking for 3 hours at room temperature. The plate is then washed 3 times with PBS, 0.05% Tween-20, 0.01% thimerosal, and is blotted dry. The standard TRAx™ OPD substrate in citric acid, sodium phosphate, 0.05% thimerosal, is added, 100

μl/well and allowed to incubate for 30 min at room temperature. The reaction is stopped by the addition of 50 μl 2N H_2SO_4 to each well.

The absorbance is then read at 490 nm on an EIA plate reader. Standards used in the assay are dilutions of purified A-transferase from a mammalian source. In the present assay, human serum is the source for A transferase. The standard is prepared from freshly drawn blood which has been allowed to clot for about 2 hours at room temperature followed by refrigeration overnight at 4°C and centrifugation to remove the clot. The A-transferase is purified from the serum by affinity chromatography on an anti-A-transferase antibody affinity column, and is stored frozen in aliquots at -70°C.

6.4. ASSAY OF BLOOD GROUP A ANTIGEN IN A SAMPLE

A antigen, or any other blood group antigen, is measured in a similar assay, either competitive or sandwich type. In the competitive assay, as described above, a blood sample being tested for A (or any other blood group) antigen, after detergent treatment as above, is added to the coated plates described in Section 6.1.2 in the presence of the anti-A mAb conjugated to HRP. Inhibition of binding of the antibody to the immobilized A antigen is an indication that A antigen is present in the sample. The concentration can be obtained by running a parallel assay to generate a standard curve using known concentrations of A antigen.

In the sandwich assay, the plate is coated with an anti-A mAb (rather than with the A antigen-BSA complex), as above. After several washes, the test sample, a detergent treated blood sample, is added and any A antigen is allowed to bind to the immobilized antibody. After further washes to remove unbound antigens, each well receives HRP-conjugated anti-A

antibody. This "detection" antibody is either the same as the immobilized antibody or is a different mAb that binds to a different epitope of the antigen. After allowing this antibody to bind, and additional washes, substrate is added as above, and the colored reaction product is measured. Over a certain concentration range, the amount of color generated is directly proportional to the amount of antigen in the sample. The concentration of antigen in the sample is quantitated by interpolation on a standard curve generated by assaying known quantities of A antigen.

7. EXAMPLE: DETECTION OF CD35 IN WHOLE BLOOD SAMPLES

CD35 (complement receptor) was detected in whole blood samples from normal individuals and from HIV+ individuals. The samples were obtained from BioRan, Cambridge, Massachusetts (normals) and Maryland Medical Labs (HIV+). Plasma and lysate samples were tested in each case. A microtiter plate format was used.

7.1 Plate Coating: 100 μL of Anti-CD35 polyclonal antibody (unpurified), in a solution of phosphate buffered saline (PBS), was coated onto 96 well microtiter plates obtained from NUNC. The mixture was incubated for 24 hours at room temperature, following which the plates were blocked for 24 hours at room temperature with 1% BSA (bovine serum albumin) in PBS, 0.05% Tween-20, 0.01% Thimerosal, and stored at 4°C with the blocking solution in the wells.

7.2 Sample Treatment: One Volume of TRAx™ detergent solution containing 6% NP-40 and 9% Triton X-100 in 1X PBS, was mixed with 5 volumes of fresh EDTA-treated (ethylenediaminetetraacetic acid) whole blood. After about 5 minutes, the treated sample was diluted 1:5 in sample diluent (Tris Buffered Saline, 10%, Fetal Bovine Serum (FBS), 0.1% Tween-20, 100

μg/mL Murine IgG, 0.01% Gentamycin Sulfate, pH 7.4), which was 50 μL treated sample plus 200 μL sample diluent.

Plasma was obtained by centrifuging whole blood collected on EDTA and removing the plasma from the red blood cells prior to sample treatment.

7.3 Assay:

An immunoassay was prepared by adding to the control microtiter plates, 50 μL of the test sample (plasma or lysate in dilution) and 50 μL of a conjugate solution which contained a horseradish peroxidase (HRP-conjugated) antibody specific for CD35 in a solution of Tris Buffered Saline, 25% FBS, 0.15% NP-40, 0.01% Thimerosal, 0.01% Gentamycin Sulfate, pH 7.4).

The combination was mixed and incubated (with shaking at 150 rpms) for 2 hours at room temperature.

The plates were washed 3 times and blotted dry. Standard substrate (commercially available TMB Substrate Reagent) was mixed in equal parts with a peroxidase substrate reagent (this combination is available as paired reagents) and added at 100 μL/well and allowed to incubate for 30 minutes at room temperature. The reaction was stopped by the addition of 0.18M H_2SO_4 to each well.

Absorbance was read at 450 nM on a Molecular Devices EIA plate reader. Standard curves were generated by spiking diluent (IL-2R Standards Diluent) with ever-increasing concentrations of soluble complement receptor obtained from T Cell Sciences, Inc. Cambridge, Massachusetts. A summary of the measurements are set forth in the table below. The lysate sample values have been corrected for dilution by multiplying by six. The results indicate that total CD35 may be detected both in normal whole blood samples, as well as HIV+ whole blood samples.

TABLE VI

CD35 (CR1), EDTA				
Sample	Status	Plasma (ng/mL)	Lysate (ng/mL)	OD450
1	normal	31	258	1.266
2	normal	30	531	1.084
3	normal	30	418	1.122
4	normal	34	614	1.014
5	normal	20	425	1.100
8/20 A	HIV+	70	781	1.222
B	HIV+	56	468	1.160
C	HIV+	54	407	0.988
D	HIV+	81	416	0.956
E	HIV+	42	474	1.160
F	HIV+	51	570	0.877
G	HIV+	126	822	1.194

*Lysates diluted 1:10 in wash buffer, blanked vs. wash buffer.

8.0 EXAMPLE: ICAM DETECTION

Sample preparation and assay procedure was carried out pursuant to that detailed in Section 7.0 above, with the exception that the samples were diluted much more in this series of assays. Changes to the procedure are synopsized below:

Stds. - 1.0 mL DD H_2O

store at 1-8°C for 24 hours, then -70°C

Diluted Samples 1:100 (10 µL + 990 µL)

* did not dilute standards

25 µL sample

75 µL conjugate

| 2 hours, 150 rpm, room temp.

Washed 3X
added 100 μL chromogen solution
(1 tab per 5 mL)
| 30', RT
50 μL 2N H_2SO_4
Read at 490 nm

1:6 1mL whole blood + 0.2mL lysis buffer (1:1:2) = lysate
50μL lysate + 200 μL samples diluent 1:5
dilute 1:16 (total diluent 1:96)
1:16 .125 mL + 1.875 mL

The results were as follows:

TABLE VII

Comparison of Plasma and Lysate Values for ICAM-1

Sample		Hem'crit (%)	Plasma (ng/mL)	Lysate (ng/mL)
7-29	1	N/A	258	145
7-29	2	N/A	250	148
7-29	3	N/A	320	176
7-29	4	N/A	248	122
7-29	5	N/A	422	229
8-5	1	40	279	138
8-5	2	41	245	150
8-5	3	40	290	166
8-5	4	38	369	219
8-5	5	40	394	228
8-6	6	39	342	204
8-7	7	45	326	173
8-8	8	46	373	199
8-9	9	40	702	410
8-10	10	43	227	115

The references cited above are all incorporated by reference herein, whether specifically incorporated or not.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the inventions following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for diagnosing an infection by a microbial pathogen in a subject, comprising the steps of:

(a) solubilizing any cells in a sample from the subject by treating said sample with non-ionic detergent;

(b) contacting said solubilized sample with at least one binding partner specific for a molecule associated with a microbial pathogen under conditions which allow specific binding; and

(c) detecting any specific binding that occurs of a component in the sample with said at least one binding partner, wherein the detection of specific binding indicates the presence of an infection by the microbial pathogen in the subject.

2. The method according to claim 1 wherein said microbial pathogen is an intracellular microbial pathogen.

3. The method according to claim 1 wherein said microbial pathogen is selected from the group consisting of *Bordetella pertussis, Borrelia burgdorferi, Campylobacter jejunii, Campylobacter pylori, Neisseria gonorrheae, Neisseria meningitidis, Yersinia Pestis, Mycobacteria, Mycoplasma, Treponema pallidum, Pneumocystis carinii, Candida albicans, Toxoplasma gondii, Chlamydia trachomatis, Plasmodium falciparum, Trichomonas vaginalis*, Adenoviruses, Cytomegalovirus, Hepatitis virus A, Hepatitis virus B, Hepatitis virus C, Herpesviruses, HIV-1, HIV-2, Influenza virus, Measles virus, Mumps virus,

Papilloma virus, Respiratory syncytial virus, and Varicella zoster virus.

4. The method according to claim 3 wherein said microbial pathogen is *Chlamydia trachomatis*.

5. The method according to claim 1 wherein said sample is a body fluid selected from the group consisting of whole blood, serum, plasma, urine, synovial fluid, cranial fluid, spinal fluid, saliva, tissue infiltrate, cervical exudate, vaginal exudate, tissue infiltrate, pleural effusion, bronchoalveolar lavage fluid, gastric lavage fluid, and bowel contents.

6. The method according to claim 1 wherein said sample is a swab specimen from a mucosal surface or a body orifice.

7. The method according to claim 6 wherein said swab specimen is a specimen obtained from a site selected from the group consisting of oral, anorectal, urogenital, endocervical, conjunctival and nasopharyngeal.

8. The method according to claim 1 wherein said detergent is non-ionic detergent, and wherein, said solubilized sample is diluted after step (a) prior to said contacting of step (b).

9. The method according to claim 8 wherein said non-ionic detergent is a mixture of about 1% Triton X-100 and about 1% Nonidet P-40 after said diluting.

10. A method according to claim 8 which further comprises storing the solubilized sample at a

temperature of less than about -20°C prior to step (b).

11. The method according to claim 1 in which the sample has not been subjected to a fractionation step.

12. The method according to claim 8 in which the sample has not been subjected to a fractionation step.

13. A method for determining the severity of an infection by a microbial pathogen in a subject, comprising the steps of:

(a) solubilizing any cells in a sample from the subject by treating said sample with non-ionic detergent;

(b) contacting said solubilized sample with at least one binding partner specific for a molecule associated with a microbial pathogen under conditions which allow specific binding; and

(c) measuring the amount of any specific binding that occurs of a component in the sample with at least one binding partner, wherein the amount of specific binding indicates the amount of said molecule in the sample, and wherein the amount of said molecule in the sample indicates the approximate number of infectious units of the pathogen in the sample; and

(d) calculating the approximate number of infectious units of the pathogen in the sample, wherein the approximate number of infectious units indicates the severity of infection.

14. A method for detecting or measuring the amount of target molecule on erythrocytes in a sample from a subject, comprising the steps of:

(a) solubilizing any cells in a sample from a subject by treating the sample with non-ionic detergent;

(b) contacting said solubilized sample with at least one binding partner specific for the target molecule suspected of being on the erythrocytes present in the sample, under conditions which allow specific binding; and

(c) 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 blood group antigen in the sample.

15. The method according to claim 14 wherein said target molecule is selected from the group consisting essentially of an ABO blood group antigen and a complement receptor.

16. The method according to claim 14 wherein said detergent is non-ionic detergent, and wherein said solubilized sample is diluted after step (b) prior to said contacting of step (c).

17. The method according to claim 16 wherein said non-ionic detergent is a mixture of about 1% Triton X-100 and about 1% Nonidet P-40 after said diluting.

18. The method according to claim 16 which further comprises storing the solubilized sample at a

temperature of less than about -20°C prior to step (b).

19. The method according to claim 14 wherein said sample comprises whole blood.

20. The method according to claim 14 wherein said sample comprises a biological fluid selected from the group consisting of whole blood, plasma, serum, blood cells, saliva, urine, synovial fluid, pleural effusions, tissue infiltrate, vaginal exudate, tumor infiltrate, amniotic fluid, spinal fluid, cranial fluid, bronchoalveolar lavage fluid, gastric lavage fluid, bowel contents and a fecal preparation.

21. The method according to claim 1 in which said at least one binding partner is an antibody.

22. The method according to claim 14 in which said at least one binding partner is an antibody.

23. The method according to claim 21 or 22 wherein said at least one antibody is a monoclonal antibody.

24. The method according to claim 21 or 22 wherein said at least one antibody is a polyclonal antibody.

25. The method according to claim 21 wherein said at least one antibody comprises a first antibody and a second antibody.

26. The method according to claim 22 wherein said at least one antibody comprises a first antibody and a second antibody.

27. A method according to claim 25 or 26 wherein said second antibody is detectably labeled.

28. A method according to claim 27 wherein said detectable label is an enzyme.

29. The method according to claim 25 or 26 wherein said first antibody is immobilized on a solid support.

30. The method according to claim 25 or 26 wherein each of said first and said second antibody is a polyclonal antibody.

31. The method according to claim 25 wherein each of said first and said second antibody is a monoclonal antibody, and wherein said first antibody and said second antibody bind to different sites on said molecule.

32. The method according to claim 26 wherein each of said first and said second antibody is a monoclonal antibody, and wherein said first antibody and said second antibody bind to different sites on said blood group antigen.

33. A method for diagnosing an infection by *Chlamydia trachomatis* in a subject, comprising the steps of:

(a) solubilizing any cells in a sample from the subject by treating said sample with non-ionic detergent, wherein said sample is a swab specimen obtained from a site selected from oral, anorectal, urogenital, endocervical, conjunctival and nasopharyngeal;

(b) contacting said solubilized sample with at least one antibody specific for an antigen of *Chlamydia trachomatis* under conditions which allow immunospecific binding; and

(c) detecting any immunospecific binding that occurs of a component in the sample with said at least one antibody, wherein the detection of immunospecific binding indicates the presence of an infection by *Chlamydia trachomatis* in the subject.

34. A method for detecting or measuring the amount of a cytokine in a sample from a subject, comprising the steps of:

(a) solubilizing any cells in a sample from a subject by treating the sample with non-ionic detergent;

(b) contacting said solubilized sample with at least one binding partner specific for a cytokine under conditions which allow specific binding; and

(c) 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 cytokine in the sample.

35. A method for determining the approximate number of cells expressing a cytokine in a sample from a subject comprising the steps of:

(a) determining the amount of a cytokine in the sample according to the method of claim 34, in which the amount of the cytokine indicates the approximate number of cells expressing the cytokine; and

(b) calculating the approximate number of cells expressing the cytokine in the sample from the amount determined in step (a).

36. A method for determining the amount of a platelet-associated molecule in a sample from a subject, comprising the steps of:

(a) solubilizing any cells in a sample from a subject by treating the sample with non-ionic detergent;

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

(c) measuring the amount of any specific binding that occurs of a component in the sample with said at least one binding partner, wherein the amount of specific binding indicates the amount of the platelet-associated molecule in the sample.

37. A method for determining the approximate number of platelets in a sample from a subject, comprising the steps of:

(a) determining the amount of a platelet-associated molecule in the sample according to the method of claim 36, in which the amount of the platelet-associated molecule indicates the approximate number of platelets expressing the molecule; and

(b) calculating the approximate number of platelets in the sample from the amount determined in step (a).

38. A method for diagnosing thrombocytopenia in a subject, comprising determining the number of platelets in a sample from the subject according to

the method of claim 37, in which an decreased number of platelets relative to a baseline level indicates the presence of thrombocytopenia.

39. A method for detecting the presence of a tumor in a subject, comprising the steps of:

(a) solubilizing any cells in a sample from the subject by treating said sample with non-ionic detergent;

(b) contacting said solubilized sample with at least one binding partner specific for a tumor-specific or tumor-associated molecule under conditions which allow specific binding; and

(c) detecting any specific binding that occurs of a component in the sample with said at least one binding partner, wherein the detection of specific binding indicates the presence of a tumor in the subject.

40. A method for detecting or measuring the amount of a cell adhesion molecule in a sample from a subject, comprising the steps of:

(a) solubilizing any cells in a sample from a subject by treating the sample with non-ionic detergent;

(b) contacting said solubilized sample with at least one binding partner specific for a cell adhesion molecule under conditions which allow specific binding; and

(c) 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 adhesion molecule in the sample.

41. A method for detecting polyploidy in cells of a subject, comprising the steps of:

(a) solubilizing any cells in a sample from the subject by treating said sample with non-ionic detergent;

(b) contacting said solubilized sample with at least one binding partner specific for DNA under conditions which allow immunospecific binding;

(c) measuring the amount of any specific binding that occurs of a component in the sample with said at least one binding partner, wherein the amount of specific binding indicates the amount of DNA in the sample;

(d) comparing the amount of DNA detected in step (c) with the amount of DNA in a similar sample of euploid cells,

wherein a sufficient increase in the amount of DNA in said sample is an indication of polyploidy.

42. The method according to claim 34, 36, or 39 in which the sample has not been subjected to a fractionation step.

43. The method according to claim 40 or 41 in which the sample has not been subjected to a fractionation step.

44. A method for detecting the presence of malignant or premalignant cells in gastrointestinal mucosal tissue, said malignant or premalignant cells associated with a decrease in the blood group A antigen, the method comprising the steps of:

(a) solubilizing any cells in a gastrointestinal mucosal sample by treating with non-ionic detergent;

(b) contacting said solubilized sample with at least one binding partner specific for the blood group A antigen under conditions which allow specific binding;

(c) measuring the amount of specific binding that occurs of a component in the sample with said at least one binding partner, wherein the amount of specific binding indicates the amount of the blood group A antigen in the sample; and

(d) comparing the amount of specific binding measured in step (c) or amount of blood group A antigen calculated from said measured amount of specific binding, with a baseline amount of specific binding or of blood group A antigen, respectively,

wherein a decrease in the amount of specific binding or blood group A antigen indicates the presence of malignant or premalignant cells.

45. A method for detecting the presence of malignant or premalignant cells in gastrointestinal mucosal tissue, said malignant or premalignant cells associated with a decrease in the activity of the α(1→3)N-acetylgalactosaminyl transferase enzyme in the cells of said tissue, comprising the steps of:

(a) solubilizing any cells in a gastrointestinal mucosal tissue sample by treating the sample with non-ionic detergent;

(b) contacting said solubilized sample with a blood group A antigen precursor under conditions wherein said enzyme catalyzes the formation of blood group A antigen from the precursor;

(c) contacting the reaction mixture of step (b) with at least one binding partner specific

for the blood group A antigen under conditions which allow specific binding;

(d) measuring the amount of specific binding that occurs of a component in the sample with said at least one binding partner, wherein the amount of specific binding indicates the amount of the blood group A antigen in the reaction mixture, and the amount of blood group A antigen indicates the amount of activity of α(1→3)N-acetylgalactosaminyl transferase enzyme present in said sample; and

(e) comparing the amount of (i) specific binding measured in step (d), (ii) blood group A antigen calculated from said measured amount of specific binding, or (iii) said activity calculated from said measured amount of specific binding, with a baseline amount of specific binding, blood group A antigen, or activity of α(1→3)N-acetylgalactosaminyl transferase enzyme, respectively,

wherein a decrease in said amount indicates the presence of malignant or premalignant cells.

46. A method for detecting or measuring the amount of P-glycoprotein in a sample from a subject, comprising the steps of:

(a) solubilizing any cells in a sample from a subject by treating the sample with non-ionic detergent;

(b) contacting said solubilized sample with at least one binding partner specific for P-glycoprotein under conditions which allow specific binding; and

(c) 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 P-glycoprotein in the sample.

47. A method for detecting multidrug resistance of cancer cells in a patient comprising detecting or measuring the amount of P-glycoprotein in a sample containing cancer cells from the patient according to the method of claim 46, in which the presence or an increased level of P-glycoprotein in the sample relative to the level of P-glycoprotein present in a sample containing comparable cells which are not multidrug resistant or which are not cancerous, indicates that the cancer cells are multidrug resistant.

48. A process for monitoring the effect of a therapeutic treatment on a patient being treated for a disease associated with the presence of a molecule, wherein a positive response to therapy is accompanied by a decrease in the amount of said molecule or a disappearance thereof, which method comprises:

(a) measuring the amount of said molecule in at least two samples from said patient according to a method comprising the following steps:

(1) solubilizing any cells in a sample from the patient by treating said sample with non-ionic detergent;

(2) contacting said solubilized sample with at least one binding partner specific for the molecule under conditions which allow specific binding; and

(3) measuring the amount of any specific binding that occurs of a component in the sample with said at least one binding partner, wherein the amount of

specific binding indicates the amount of said molecule in the sample,

wherein a first sample is obtained prior to the initiation of said treatment and at least one second sample is obtained after initiation of said treatment; and

(b) comparing the amount of said specific binding or the amount of said molecule calculated from said specific binding in said first sample with said at least one second sample,

wherein a decrease in the amount of said specific binding or of said molecule after treatment indicates a good response to therapy; and with the proviso that said molecule is not a leukocyte surface marker.

49. A process for monitoring the effect of a therapeutic treatment on a patient being treated for a disease associated with the presence of a molecule, wherein a positive response to therapy is accompanied by an increase in the amount of said molecule, which method comprises:

(a) measuring the total amount of said molecule in at least two samples from said patient according to a method comprising the following steps:

(1) solubilizing any cells in a sample from the patient by treating said sample with non-ionic detergent;

(2) contacting said solubilized sample with at least one binding partner specific for the molecule under conditions which allow specific binding; and

(3) measuring the amount of any specific binding that occurs of a component in the sample with said at least one binding partner, wherein the amount of

specific binding indicates the amount of said molecule in the sample,

wherein a first sample is obtained prior to the initiation of said treatment and at least one second sample is obtained after initiation of said treatment; and

(b) comparing the amount of said specific binding or the amount of said molecule calculated from said specific binding in said first sample with said at least one second sample,

wherein an increase in the amount of said specific binding or of said molecule after treatment indicates a good response to therapy; and with the proviso that said molecule is not a leukocyte surface marker.

50. A method for detecting or measuring the amount of a molecule in a sample from a subject, comprising the steps of:

(a) solubilizing any cells in a sample from a subject by treating the sample with non-ionic detergent, in which said sample has not been subjected to a fractionation step;

(b) contacting said solubilized sample with at least one binding partner specific for a molecule under conditions which allow specific binding; and

(c) 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 molecule in the sample;

with the proviso that said molecule is not a leukocyte cell surface marker.

51. A kit useful for the measurement of the total amount of a molecule associated with a microbial pathogen in a sample, comprising in one or more containers:

(a) non-ionic detergent; and

(b) a binding partner of a molecule associated with a microbial pathogen.

52. A kit useful for measuring the total amount of a chlamydial antigen in a sample, comprising in one or more containers:

(a) non-ionic detergent; and

(b) a first antibody to an antigen of *Chlamydia trachomatis*; and

(c) a second antibody to an antigen of *Chlamydia trachomatis*, said second antibody being detectably labeled.

53. A kit useful for measuring the total amount of a blood group antigen in a sample, comprising in one or more containers:

(a) non-ionic detergent; and

(b) an antibody to a blood group antigen.

54. A kit useful for the measurement of the total amount of a molecule in a sample, comprising in one or more containers:

(a) non-ionic detergent;

(b) a first binding partner of a molecule, bound to a solid support, with the proviso that said molecule is not a leukocyte cell surface marker;

(c) an enzyme-labeled second binding partner of said molecule;

(d) a chromogenic substrate for the enzyme; and

(e) buffers for diluting and washing components (a), (b) or (c).

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/10550

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :435/7.1, 7.2, 7.21, 7.22, 7.32, 7.9, 30; 252/89.1
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. : 435/7.1, 7.2, 7.21, 7.22, 7.32, 7.9, 30; 252/89.1

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, MEDLINE, BIOSIS, CHEM AB, DERWENT WPI, EMBASE, search terms: non ionic detergent, detergent, triton, nonidet, chaps, antibody, intracellular, assay, immunoassay

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	D.M. Weir et al.,"Handbook of Experimental Immunology, Volume 4, Applications of Immunological Methods in Biomedical Sciences", published 1986, Blackwell Scientific Publications (Oxford), see pages 115.1-115.13, see entire document.	1 - 3 , 8 , 1 1 - 12,21,23-25,27
Y	Molecular Immunology, Volume 22, No. 8, issued August 1985, S.K. Dower et al.,"Quantitative measurement of human Interleukin 2 receptor levels with intact and detergent-solubilized human T-cells", pages 937-947, see entire document.	1-54

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

*	Special categories of cited documents:	"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
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"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
"E"	earlier document published on or after the international filing date	"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
"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)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

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