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(54) **Title**: ASSAYS FOR IGFBP7 HAVING IMPROVED PERFORMANCE IN BIOLOGICAL SAMPLES

(57) **Abstract**: The invention provides IGFBP7 immunoassays with improved clinical performance, particularly when used in the evaluation of renal injuries. The immunoassays rely on the selection and use of antibodies and antibody pairs that exhibit improved assay performance when used in complex clinical specimens such as biological fluids, and particularly when used in rapid assay formats such as lateral flow test devices.

ASSAYS FOR IGFBP7 HAVING IMPROVED PERFORMANCE IN BIOLOGICAL SAMPLES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 61/900,942, filed November 6, 2013, and to U.S. Provisional Application No. 62/054,324, filed September 23, 2014, and to U.S. Provisional Application No. 62/064,380, filed October 15, 2014, each of which is hereby incorporated in its entirety including all tables, figures, and claims.

BACKGROUND

[0002] The following discussion of the background of the invention is merely provided to aid the reader in understanding the invention and is not admitted to describe or constitute prior art to the present invention.

[0003] IGFBP7 (human precursor Swiss Prot entry Q16270) is a secreted protein which is involved in regulation of of insulin-like growth factor expression in tissue and which modulates IGF binding to its receptors. It also reportedly stimulates prostacyclin production and cell adhesion. IGFBP7 suppresses growth and colony formation of prostate and breast cancer cell lines through an IGF independent mechanism, which causes a delay in the G1 phase of the cell cycle, and increased apoptosis. IGFBP7 is expressed in a wide range of normal human tissues and it usually shows reduced expression in cancer cell lines of prostate, breast, colon, and lung origin.

[0004] In addition, WO2011/097539 and WO2011/075744, each of which is hereby incorporated by reference in its entirety including all tables, figures and claims, describe the use of IGFBP7 for evaluating the renal status of a subject both individually and in multimarker panels. In particular, IGFBP7 levels measured by immunoassay are shown to correlate to risk stratification, diagnosis, staging, prognosis, classifying and monitoring of the renal status.

[0005] Signals obtained from specific binding assays such as immunoassays are a direct result of complexes formed between one or more binding species (e.g., antibodies) and the target biomolecule (i.e., the analyte) and polypeptides containing the necessary epitope(s) to which the antibodies bind. Immunoassays are often able to “detect" an analyte; but because an antibody epitope is on the order of 8 amino acids, an immunoassay configured to detect a marker of interest will also detect polypeptides

related to the marker sequence, so long as those polypeptides contain the epitope(s) necessary to bind to the antibody or antibodies used in the assay. While such assays may detect the full length biomarker and the assay result be expressed as a concentration of a biomarker of interest, the signal from the assay is actually a result of all such "immunoreactive" polypeptides present in the sample. Such binding assays may also detect immunoreactive polypeptides present in a biological sample that are complexed to additional species, such as binding proteins, receptors, heparin, lipids, sugars, etc., provided that those additional species do not interfere in binding between the binding species and the target biomolecule. Typically, however, specific binding assays are formulated using purified analyte, and complex formation and fragmentation patterns are not considered. This is particularly true where the identity of such additional binding species are unknown.

SUMMARY

[0006] It is an object of the invention to provide IGFBP7 immunoassays with improved clinical performance, particularly when used in the evaluation of renal injuries. Specifically, we describe the selection and use of antibodies and antibody pairs that exhibit improved assay performance when used in complex clinical specimens such as biological fluids, and particularly when used in rapid assay formats.

[0007] In a first aspect, the present invention relates to a monoclonal antibody which specifically binds human IGFBP7 and is suitable for use in a sandwich immunoassay. The antibody specifically binds to a polypeptide consisting of LIWNKVKRGHYGVQRTELL PGDRDNL (SEQ ID NO: 1) or SSSSSDTCGPCEPASCPPLP (SEQ ID NO: 2).

[0008] In a related aspect, the present invention relates to an antibody pair which specifically binds human IGFBP7 and is suitable for use in a sandwich immunoassay, the antibody pair comprising a first monoclonal antibody which specifically binds to a polypeptide consisting of LIWNKVKRGHYGVQRTELLPGDRDNL (SEQ ID NO: 1) and a second monoclonal antibody which specifically binds to a polypeptide consisting of SSSSSDTCGPCEPASCPPLP (SEQ ID NO: 2).

[0009] In another related aspect, the present invention relates to a monoclonal antibody which specifically binds human IGFBP7 and is suitable for use in a sandwich immunoassay. The antibody specifically binds to a conformational epitope of IGFBP7. Conformational epitopes are formed by residues that are sequentially discontinuous but

close together in three-dimensional space in the IGFBP7 protein. An example of such an antibody, referred to as 1D6, is described below.

[00010] In a related aspect, the present invention relates to an antibody pair which specifically binds human IGFBP7 and is suitable for use in a sandwich immunoassay, the antibody pair comprising a first monoclonal antibody which specifically binds to a conformational epitope of IGFBP7, and a second monoclonal antibody which specifically binds to a polypeptide consisting of LIWNKVKRGHYGVQRTELLPGDRDNL (SEQ ID NO: 1) or SSSSSDTCGPCEPASCPPLP (SEQ ID NO: 2).

[0110] In certain embodiments, an antibody of the present invention comprises one or both of (i) a light chain variable region having an amino acid sequence of SEQ ID NO: 9 or a sequence at least 90% identical to SEQ ID NO: 9, and (ii) a heavy chain variable region having an amino acid sequence of SEQ ID NO: 10 or a sequence at least 90% identical to SEQ ID NO: 10, wherein the antibody specifically binds human IGFBP7. In preferred embodiments, the antibody is that which is referred to herein as IC9E4.1.

[00011] In other embodiments, an antibody of the present invention comprises one or both of (i) a light chain variable region having an amino acid sequence of SEQ ID NO: 11 or a sequence at least 90% identical to SEQ ID NO: 11, and (ii) a heavy chain variable region having an amino acid sequence of SEQ ID NO: 12 or a sequence at least 90% identical to SEQ ID NO: 12, wherein the antibody specifically binds human IGFBP7. In preferred embodiments, the antibody is that which is referred to herein as 1D6.

[00012] In certain embodiments, an antibody pair of the present invention comprises (i) a first antibody which comprises one or both of (i) a light chain variable region having an amino acid sequence of SEQ ID NO: 11 or a sequence at least 90% identical to SEQ ID NO: 11, and (ii) a heavy chain variable region having an amino acid sequence of SEQ ID NO: 12 or a sequence at least 90% identical to SEQ ID NO: 12, wherein the antibody specifically binds human IGFBP7; and (ii) a second antibody which comprises one or both of (i) a light chain variable region having an amino acid sequence of SEQ ID NO: 9 or a sequence at least 90% identical to SEQ ID NO: 9, and (ii) a heavy chain variable region having an amino acid sequence of SEQ ID NO: 10 or a sequence at least 90% identical to SEQ ID NO: 10, wherein the antibody specifically binds human IGFBP7. In preferred embodiments, the antibody pair comprises a first antibody referred to herein as 1D6 and a second antibody referred to herein as IC9E4.1.

[00013] The phrase "specifically binds to a polypeptide consisting of" a particular sequence as used herein is not intended to mean that the antibody does not bind to a longer polypeptide comprising the sequence, or to a shorter polypeptide that is a subset of the sequence. Rather, this phrase is simply intended to mean that the antibody will bind to the particular recited polypeptide.

[00014] Antibodies for use in the claimed methods may be obtained from a variety of species. For example, the antibodies of the present invention may comprise immunoglobulin sequences which are rabbit, mouse, rat, guinea pig, chicken, goat, sheep, donkey, human, llama or camelid sequences, or combinations of such sequences (so-called chimeric antibodies). Antibodies for use in the present invention may be identified by their performance in immunoassays, and then further characterized by epitope mapping in order to understand the epitopes which are relevant to that performance.

[00015] Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and nonconformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents. Preferably, an epitope for each antibody is contained within SEQ ID NO: 1 or SEQ ID NO: 2, which is a sequence obtained from the human IGFBP7 sequence. In certain embodiments, the first monoclonal antibody comprises at least one, and preferably 2, 3, or 4 consecutive "critical residues" for binding to IGFBP7. A "critical residue" is defined as an amino acid of SEQ ID NO: 1 (or SEQ ID NO: 2) that, when changed to an alanine, reduces binding of an antibody by at least 50%, and more preferably at least 75%, relative to its binding to SEQ ID NO: 1 (or SEQ ID NO: 2) itself. In preferred embodiments, the at least one critical residue is at least one residue in the sequence TELLPGDRD (SEQ ID NO: 3) or at least one residue in the sequence EPASC (SEQ ID NO: 4).

[00016] Such monoclonal antibodies may be conjugated to a signal development element or immobilized on a solid support. In an example of a sandwich assay, a first antibody (detectably labeled) and a second antibody (immobilized at a predetermined zone of a test device) form sandwich complexes with IGFBP7 in the sample at a predetermined zone of a test device. In sandwich assays, the first and second antibodies can be the same (particularly when polyclonal antibodies are used) or different. Thus, the

antibodies of the invention may be used in sandwich pairs, or may be used individually with another binding entity which is not a monoclonal antibody such as a polyclonal antibody or an aptamer.

[00017] The antibodies of the present invention can be used as reagents in test kits for detecting IGFBP7 in biological samples. Such a test kit may, for example, comprise a disposable test device configured to generate a detectable signal related to the present or amount of human IGFBP7 in a biological sample. Alternatively, such a test kit may be formulated for performing an assay in a clinical analyzer which does not utilize a disposable test device. Preferably, the test kit is an in vitro diagnostic. The term "in vitro diagnostic" as used herein refers to a medical device which is a reagent, reagent product, calibrator, control material, kit, instrument, apparatus, equipment, or system, whether used alone or in combination, intended by the manufacturer to be used *in vitro* for the examination of specimens, including blood and tissue donations, derived from the human body, solely or principally for the purpose of providing information concerning a physiological or pathological state, or concerning a congenital abnormality, or to determine the safety and compatibility with potential recipients, or to monitor therapeutic measures.

[00018] In certain embodiments, the immunoassay is performed in a lateral flow format. Lateral flow tests are a form of immunoassay in which the test sample flows in a chromatographic fashion along a bibulous or non-bibulous porous solid substrate. Lateral flow tests can operate as either competitive or sandwich format assays. Preferred lateral flow devices are disposable, single use test devices. A sample is applied to the test device at an application zone and transits the substrate, where it encounters lines or zones which have been pretreated with an antibody or antigen. The term "test zone" as used herein refers to a discrete location on a lateral flow test strip which is interrogated in order to generate a signal related to the presence or amount of an analyte of interest. The detectable signal may be read visually or obtained by inserting the disposable test device into an analytical instrument such as a reflectometer, a fluorometer, or a transmission photometer. This list is not meant to be limiting. Sample may be applied without pretreatment to the application zone, or may be premixed with one or more assay reagents prior to application. In the latter case, the antibody may be provided in a separate container from the disposable test device.

[00019] An antibody of the present invention may be diffusively immobilized to a surface within a disposable test device, such that the antibody dissolves into a sample when the sample contacts the surface. In a sandwich assay format, this diffusively bound antibody may bind to its cognate antigen in the sample, and then be immobilized at a detection zone when the antigen is bound by a second antibody non-diffusively bound at the detection zone. In a competitive format, its cognate antigen in the sample may compete for binding to the diffusively bound antibody with a labeled antigen provided as an assay reagent.

[00020] A kit of the invention can further comprise a calibration to relate the detectable signal to a concentration of IGFBP7. By way of example, a calibration curve may be provided on an electronic memory device which is read by the analytical instrument which receives the disposable test device, such as a ROM chip, a flash drive, an RFID tag, etc. Alternatively, the calibration curve may be provided on an encoded label which is read optically, such as a 2-D bar code, or transmitted via a network connection. The analytical instrument can then use this calibration curve to relate a detectable signal from an assay into an IGFBP7 concentration.

[00021] In certain embodiment, an assay method performed using the antibody pair of the present invention provides a signal related to the present or amount of human IGFBP7 in a biological sample, wherein the minimum detectable concentration of IGFBP7 in the assay method is 20 ng/mL or less, more preferably 10 ng/mL or less, 5 ng/mL or less, 1 ng/mL or less, and most preferably 0.1 ng/mL or less.

[00022] In related aspects, the present invention provides methods for determining the presence or amount of human IGFBP7 in a biological sample, comprising:

performing an immunoassay on the biological sample with a first monoclonal antibody and a second monoclonal antibody which together form sandwich complexes with human IGFBP7, wherein the immunoassay provides a detectable signal that is related to the presence or amount of human IGFBP7 in the biological sample bound in the sandwich complexes; and

relating the detectable signal to the presence or amount of human IGFBP7 in the biological sample. Preferably, the minimum detectable concentration of IGFBP7 in the immunoassay is 20 ng/mL or less, more preferably 10 ng/mL or less, 5 ng/mL or less, 1 ng/mL or less, and most preferably 0.1 ng/mL or less.

[00023] In particularly preferred embodiments, the first monoclonal antibody binds to a polypeptide consisting of SEQ ID NO: 1, and the second monoclonal antibody binds to the polypeptide consisting of SEQ ID NO: 2, in each case with an affinity of at least 10^8 M^{-1}.

[00024] Preferred assay methods comprise performing an immunoassay that detects human IGFBP7. Such immunoassays may comprise contacting said body fluid sample with an antibody that detects the marker, and detecting binding to that antibody. Preferably, the body fluid sample is selected from the group consisting of urine, saliva, blood, serum, and plasma, and most preferably urine.

[00025] With regard to the antibodies of the present invention, the invention also relates to nucleic acids encoding such antibodies, and antibody-expressing cell lines expressing such antibodies in additional aspects.

[00026] The details of one or more embodiments of the disclosure are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the disclosure will be apparent from the description and drawings, and from the claims.

DETAILED DESCRIPTION

[00027] Definitions

[00028] As used herein, the terms "Insulin-like growth factor-binding protein 7" and "IGFBP7" refer to one or more polypeptides present in a biological sample that are derived from the Insulin-like growth factor-binding protein 7 precursor (Swiss-Prot Q16270 (SEQ ID NO: 5))

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        10         20         30         40         50         60
MERPSLRALL LGAAGLLLLL LPLSSSSSSD TCGPCEPASC PPLPPLGCLL GETRDACGCC

        70         80         90        100        110        120
PMCARGEGEP CGGGGAGRGY CAPGMECVKS RKRRKGKAGA AAGGPGVSGV CVCKSRYPVC

       130        140        150        160        170        180
GSDGTTYPSG CQLRAASQRA ESRGEKAITQ VSKGTCEQGP SIVTPPKDIW NVTGAQVYLS

       190        200        210        220        230        240
CEVIGIPTPV LIWNKVKRGH YGVQRTELLP GDRDNLAIQT RGGPEKHEVT GWVLVSPLSK

       250        260        270        280
EDAGEYECHA SNSQGQASAS AKITVVDALH EIPVKKGEGA EL
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[00029] The following domains have been identified in Insulin-like growth factor-binding protein 7:

Residues	Length	Domain ID
1-26	26	Signal peptide
27-282	256	Insulin-like growth factor-binding protein 7

[00030] Unless specifically noted otherwise herein, the definitions of the terms used are standard definitions used in the art of pharmaceutical sciences. As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a pharmaceutical carrier" includes mixtures of two or more such carriers, and the like.

[00031] The term “subject” as used herein refers to a human or non-human organism. Thus, the methods and compositions described herein are applicable to both human and veterinary disease. Further, while a subject is preferably a living organism, the invention described herein may be used in post-mortem analysis as well. Preferred subjects are humans, and most preferably “patients,” which as used herein refers to living humans that are receiving medical care for a disease or condition. This includes persons with no defined illness who are being investigated for signs of pathology.

[00032] Preferably, an analyte is measured in a sample. Such a sample may be obtained from a subject, or may be obtained from biological materials intended to be provided to the subject. For example, a sample may be obtained from a kidney being evaluated for possible transplantation into a subject, and an analyte measurement used to evaluate the kidney for preexisting damage. Preferred samples are body fluid samples.

[00033] The term “body fluid sample” as used herein refers to a sample of bodily fluid obtained for the purpose of diagnosis, prognosis, classification or evaluation of a subject of interest, such as a patient or transplant donor. In certain embodiments, such a sample may be obtained for the purpose of determining the outcome of an ongoing condition or the effect of a treatment regimen on a condition. Preferred body fluid samples include blood, serum, plasma, cerebrospinal fluid, urine, saliva, sputum, and pleural effusions. In addition, one of skill in the art would realize that certain body fluid samples would be more readily analyzed following a fractionation or purification procedure, for example, separation of whole blood into serum or plasma components.

[00034] The term "diagnosis" as used herein refers to methods by which the skilled artisan can estimate and/or determine the probability ("a likelihood") of whether or not a patient is suffering from a given disease or condition. In the case of the present invention, "diagnosis" includes using the results of an assay, most preferably an immunoassay, for a kidney injury marker of the present invention, optionally together with other clinical characteristics, to arrive at a diagnosis (that is, the occurrence or nonoccurrence) of an acute renal injury or ARF for the subject from which a sample was obtained and assayed. That such a diagnosis is "determined" is not meant to imply that the diagnosis is 100% accurate. Many biomarkers are indicative of multiple conditions. The skilled clinician does not use biomarker results in an informational vacuum, but rather test results are used together with other clinical indicia to arrive at a diagnosis. Thus, a measured biomarker level on one side of a predetermined diagnostic threshold indicates a greater likelihood of the occurrence of disease in the subject relative to a measured level on the other side of the predetermined diagnostic threshold.

[00035] Similarly, a prognostic risk signals a probability ("a likelihood") that a given course or outcome will occur. A level or a change in level of a prognostic indicator, which in turn is associated with an increased probability of morbidity (e.g., worsening renal function, future ARF, or death) is referred to as being "indicative of an increased likelihood" of an adverse outcome in a patient.

[00036] The term "lateral flow" as used herein refers to flow of reagents in a longitudinal direction through a substantially flat porous material. Such porous material is "substantially flat" if the thickness of the material is no more than 10% of the length and width dimensions.

[00037] The term "downstream region" as used herein relative to a first region of a device refers to which receives fluid flow after that fluid has already reached the first region.

[00038] The term "sample application region" as used herein refers to a portion of an assay device into which a fluid sample of interest is introduced for purposes of determining a component thereof.

[00039] Marker Assays

[00040] In general, immunoassays involve contacting a sample containing or suspected of containing a biomarker of interest with at least one antibody that specifically binds to

the biomarker. A signal is then generated indicative of the presence or amount of complexes formed by the binding of polypeptides in the sample to the antibody. The signal is then related to the presence or amount of the biomarker in the sample. Numerous methods and devices are well known to the skilled artisan for the detection and analysis of biomarkers. *See, e.g.*, U.S. Patents 6,143,576; 6,113,855; 6,019,944; 5,985,579; 5,947,124; 5,939,272; 5,922,615; 5,885,527; 5,851,776; 5,824,799; 5,679,526; 5,525,524; and 5,480,792, and *The Immunoassay Handbook*, David Wild, ed. Stockton Press, New York, 1994, each of which is hereby incorporated by reference in its entirety, including all tables, figures and claims.

[00041] The assay devices and methods known in the art can utilize labeled molecules in various sandwich, competitive, or non-competitive assay formats, to generate a signal that is related to the presence or amount of the biomarker of interest. Suitable assay formats also include chromatographic, mass spectrographic, and protein "blotting" methods. Additionally, certain methods and devices, such as biosensors and optical immunoassays, may be employed to determine the presence or amount of analytes without the need for a labeled molecule. *See, e.g.*, U.S. Patents 5,631,171; and 5,955,377, each of which is hereby incorporated by reference in its entirety, including all tables, figures and claims. One skilled in the art also recognizes that robotic instrumentation including but not limited to Beckman ACCESS®, Abbott AXSYM®, Roche ELECSYS®, Dade Behring STRATUS® systems are among the immunoassay analyzers that are capable of performing immunoassays. But any suitable immunoassay may be utilized, for example, enzyme-linked immunoassays (ELISA), radioimmunoassays (RIAs), competitive binding assays, and the like.

[00042] Antibodies or other polypeptides may be immobilized onto a variety of solid supports for use in assays. Solid phases that may be used to immobilize specific binding members include those developed and/or used as solid phases in solid phase binding assays. Examples of suitable solid phases include membrane filters, cellulose-based papers, beads (including polymeric, latex and paramagnetic particles), glass, silicon wafers, microparticles, nanoparticles, TentaGels, AgroGels, PEGA gels, SPOCC gels, and multiple-well plates. An assay strip could be prepared by coating the antibody or a plurality of antibodies in an array on solid support. This strip could then be dipped into the test sample and then processed quickly through washes and detection steps to generate a measurable signal, such as a colored spot. Antibodies or other polypeptides may be

bound to specific zones of assay devices either by conjugating directly to an assay device surface, or by indirect binding. In an example of the later case, antibodies or other polypeptides may be immobilized on particles or other solid supports, and that solid support immobilized to the device surface.

[00043] Biological assays require methods for detection, and one of the most common methods for quantitation of results is to conjugate a detectable label to a protein or nucleic acid that has affinity for one of the components in the biological system being studied. Detectable labels may include molecules that are themselves detectable (*e.g.*, fluorescent moieties, electrochemical labels, metal chelates, *etc.*) as well as molecules that may be indirectly detected by production of a detectable reaction product (*e.g.*, enzymes such as horseradish peroxidase, alkaline phosphatase, *etc.*) or by a specific binding molecule which itself may be detectable (e.g., biotin, digoxigenin, maltose, oligohistidine, 2,4-dintrobenzene, phenylarsenate, ssDNA, dsDNA, etc.).

[00044] Preparation of solid phases and detectable label conjugates often comprise the use of chemical cross-linkers. Cross-linking reagents contain at least two reactive groups, and are divided generally into homofunctional cross-linkers (containing identical reactive groups) and heterofunctional cross-linkers (containing non-identical reactive groups). Homobifunctional cross-linkers that couple through amines, sulfhydryls or react non-specifically are available from many commercial sources. Maleimides, alkyl and aryl halides, alpha-haloacyls and pyridyl disulfides are thiol reactive groups. Maleimides, alkyl and aryl halides, and alpha-haloacyls react with sulfhydryls to form thiol ether bonds, while pyridyl disulfides react with sulfhydryls to produce mixed disulfides. The pyridyl disulfide product is cleavable. Imidoesters are also very useful for protein-protein cross-links. A variety of heterobifunctional cross-linkers, each combining different attributes for successful conjugation, are commercially available.

[00045] In certain aspects, the present invention provides kits for the analysis of the described marker. The kit comprises reagents for the analysis of at least one test sample which comprise at least one antibody that specifically binds to the marker. The kit can also include devices and instructions for performing one or more of the diagnostic and/or prognostic correlations described herein. Preferred kits will comprise an antibody pair for performing a sandwich assay, or a labeled species for performing a competitive assay, for the analyte. Preferably, an antibody pair comprises a first antibody conjugated to a solid

phase and a second antibody conjugated to a detectable label, wherein each of the first and second antibodies that bind a kidney injury marker. Most preferably each of the antibodies are monoclonal antibodies. The instructions for use of the kit and performing the correlations can be in the form of labeling, which refers to any written or recorded material that is attached to, or otherwise accompanies a kit at any time during its manufacture, transport, sale or use. For example, the term labeling encompasses advertising leaflets and brochures, packaging materials, instructions, audio or video cassettes, computer discs, as well as writing imprinted directly on kits.

[00046] In certain embodiments, the marker assay is performed using a single-use disposable test device. Such test devices often take the form of lateral flow devices which are now familiar from the common use of over-the-counter pregnancy tests. Generally, these assay devices have an extended base layer on which a differentiation can be made between a sample addition region and an evaluation region. In typical use, the sample is applied to the sample addition region, flows along a liquid transport path which runs parallel to the base layer, and then flows into the evaluation region. A capture reagent is present in the evaluation region, and the captured analyte can be detected by a variety of protocols to detect visible moieties associated with the captured analyte. For example, the assay may produce a visual signal, such as color change, fluorescence, luminescence, and the like, when indicating the presence or absence of an analyte in a biological sample.

[00047] A sample addition region can be provided, for example, in the form of an open chamber in a housing; in the form of an absorbent pad; etc. The sample addition region can be a port of various configurations, that is, round, oblong, square and the like or the region can be a trough in the device.

[00048] A filter element can be placed in, on, or adjacent to the sample addition region to filter particulates from the sample, such as to remove or retard blood cells from blood so that plasma can further travel through the device. Filtrate can then move into a porous member fluidly connected to the filter. Suitable filters for removing or retarding cellular material present in blood are well known in the art. See, e.g., U.S. Patents 4,477,575; 5,166,051; 6,391,265; and 7,125,493, each of which is hereby incorporated by reference in its entirety. Many suitable materials are known to skilled artisans, and can include glass fibers, synthetic resin fibers, membranes of various types including asymmetric membrane filters in which the pore size varies from about 65 to about 15 μm, and

combinations of such materials. In addition, a filter element can comprise one or more chemical substances to facilitate separation of red blood cells from blood plasma. Examples of such chemical substances are thrombin, lectins, cationic polymers, antibodies against one or more red blood cell surface antigens and the like. Such chemical substance(s) which facilitate separation of red blood cells from plasma may be provided in the filter element by covalent means, nonspecific absorption, etc.

[00049] In certain embodiments, a label zone is located downstream of the sample receiving zone, and contains a diffusively located labeled reagent that binds to the analyte of interest or that competes with the analyte of interest for binding to a binding species. Alternatively, the label zone can be eliminated if the labeled reagent is premixed with the sample prior to application to the sample receiving zone. A detection zone is disposed downstream of from the label zone, and contains an immobilized capture reagent that binds to the analyte of interest.

[00050] The optimum pore diameter for the membrane for use in the invention is about 10 to about 50 μm. The membranes typically are from about 1 mil to about 15 mils in thickness, typically in the range of from 5 or 10 mils, but may be up to 200 mils and thicker. The membrane may be backed by a generally water impervious layer, such as a Mylar® polyester film (DuPont Teijin Films). When employed, the backing is generally fastened to the membrane by an adhesive, such as 3M 444 double-sided adhesive tape. Typically, a water impervious backing is used for membranes of low thickness. A wide variety of polymers may be used provided that they do not bind nonspecifically to the assay components and do not interfere with flow of the sample. Illustrative polymers include polyethylene, polypropylene, polystyrene and the like. Alternatively, the membrane may be self supporting. Other non-bibulous membranes, such as polyvinyl chloride, polyvinyl acetate, copolymers of vinyl acetate and vinyl chloride, polyamide, polycarbonate, polystyrene, and the like, can also be used. In various embodiments, the label zone material may be pretreated with a solution that includes blocking and stabilizing agents. Blocking agents include bovine serum albumin (BSA), methylated BSA, casein, nonfat dry milk. The device can also comprise additional components, including for example buffering agents, HAMA inhibitors, detergents, salts (e.g., chloride and/or sulfate salts of calcium, magnesium, potassium, etc.), and proteinaceous components (e.g., serum albumin, gelatin, milk proteins, etc.). This list is not meant to be limiting.

[00051] The device may further comprise various control locations which are read to determine that the test device has been run properly. By way of example, a procedural control zone may be provided separate from the assay detection zone to verify that the sample flow is as expected. The control zone is preferably a spatially distinct region at which a signal may be generated that is indicative of the proper flow of reagents. The procedural control zone may contain the analyte of interest, or a fragment thereof, to which excess labeled antibody used in the analyte assay can bind. In operation, a labeled reagent binds to the control zone, even when the analyte of interest is absent from the test sample. The use of a control line is helpful in that appearance of a signal in the control line indicates the time at which the test result can be read, even for a negative result. Thus, when the expected signal appears in the control line, the presence or absence of a signal in the capture zone can be noted. The device may further comprise a negative control area. The purpose of this control area is to alert the user that the test device is not working properly. When working properly, no signal or mark should be visible in the negative control area.

[00052] The outer casing or housing of such an assay device may take various forms. Typically, it will include an elongate casing and may have a plurality of interfitting parts. In a particularly preferred embodiment, the housing includes a top cover and a bottom support. The top cover contains an application aperture and an observation port. In a preferred embodiment, the housing is made of moisture impervious solid material, for example, a plastic material. It is contemplated that a variety of commercially available plastics, including, but not limited to, vinyl, nylon, polyvinyl chloride, polypropylene, polystyrene, polyethylene, polycarbonates, polysulfanes, polyesters, urethanes, and epoxies maybe used to construct a housing. The housing may be prepared by conventional methodologies, such as standard molding technologies that are well known and used in the art. The housing may be produced by molding technologies which include, but are not limited to, injection molding, compression molding, transfer molding, blow molding, extrusion molding, foam molding, and thermoform molding. The aforementioned molding technologies are well known in the art and so are not discussed in detail herein. See for example, Processes And Materials Of Manufacture, Third Edition, R. A. Lindsberg (1983) Allyn and Baron pp. 393-431.

[00053] If necessary, the colorimetric, luminescent, or fluorescent intensity of the detectable label being employed may be then evaluated with an instrument that is

appropriate to the label. By way of example, a fluorometer can be used to detect fluorescent labels; a reflectometer can be used to detect labels which absorb light, etc. The concentration of the analyte of interest in the samples may be determined by correlating the measured response to the amount of analyte in the sample fluid.

[00054] Assay Correlations

[00055] The terms "correlating" and "relating" as used herein in reference to the measurement of biomarkers in an assay refers to determining the presence, or more preferably the amount, of the biomarker in a sample based on the signal obtained from the assay. Often, this takes the form of comparing a signal generated from a detectable label on one species participating in the assay to a predetermined standard curve which can be used to convert the signal to a concentration or threshold amount of the biomarker.

[00056] The terms "correlating" and "relating" as used herein in reference to the use of biomarkers for diagnosis or prognosis refers to comparing the presence or amount of the biomarker(s) in a patient to its presence or amount in persons known to suffer from, or known to be at risk of, a given condition; or in persons known to be free of a given condition. Often, this takes the form of comparing an assay result in the form of a biomarker concentration to a predetermined threshold selected to be indicative of the occurrence or nonoccurrence of a disease or the likelihood of some future outcome.

[00057] Selecting a diagnostic threshold involves, among other things, consideration of the probability of disease, distribution of true and false diagnoses at different test thresholds, and estimates of the consequences of treatment (or a failure to treat) based on the diagnosis. For example, when considering administering a specific therapy which is highly efficacious and has a low level of risk, few tests are needed because clinicians can accept substantial diagnostic uncertainty. On the other hand, in situations where treatment options are less effective and more risky, clinicians often need a higher degree of diagnostic certainty. Thus, cost/benefit analysis is involved in selecting a diagnostic threshold.

[00058] Suitable thresholds may be determined in a variety of ways. For example, one recommended diagnostic threshold for the diagnosis of acute myocardial infarction using cardiac troponin is the 97.5th percentile of the concentration seen in a normal population. Another method may be to look at serial samples from the same patient, where a prior "baseline" result is used to monitor for temporal changes in a biomarker level.

[00059] Population studies may also be used to select a decision threshold. Receiver Operating Characteristic ("ROC") arose from the field of signal detection theory developed during World War II for the analysis of radar images, and ROC analysis is often used to select a threshold able to best distinguish a "diseased" subpopulation from a "nondiseased" subpopulation. A false positive in this case occurs when the person tests positive, but actually does not have the disease. A false negative, on the other hand, occurs when the person tests negative, suggesting they are healthy, when they actually do have the disease. To draw a ROC curve, the true positive rate (TPR) and false positive rate (FPR) are determined as the decision threshold is varied continuously. Since TPR is equivalent with sensitivity and FPR is equal to 1 - specificity, the ROC graph is sometimes called the sensitivity vs (1 - specificity) plot. A perfect test will have an area under the ROC curve of 1.0; a random test will have an area of 0.5. A threshold is selected to provide an acceptable level of specificity and sensitivity.

[00060] In this context, "diseased" is meant to refer to a population having one characteristic (the presence of a disease or condition or the occurrence of some outcome) and "nondiseased" is meant to refer to a population lacking the characteristic. While a single decision threshold is the simplest application of such a method, multiple decision thresholds may be used. For example, below a first threshold, the absence of disease may be assigned with relatively high confidence, and above a second threshold the presence of disease may also be assigned with relatively high confidence. Between the two thresholds may be considered indeterminate. This is meant to be exemplary in nature only.

[00061] In addition to threshold comparisons, other methods for correlating assay results to a patient classification (occurrence or nonoccurrence of disease, likelihood of an outcome, etc.) include decision trees, rule sets, Bayesian methods, and neural_network methods. These methods can produce probability values representing the degree to which a subject belongs to one classification out of a plurality of classifications.

[00062] Measures of test accuracy may be obtained as described in Fischer et al., Intensive Care Med. 29: 1043-51, 2003, and used to determine the effectiveness of a given biomarker. These measures include sensitivity and specificity, predictive values, likelihood ratios, diagnostic odds ratios, and ROC curve areas. The area under the curve ("AUC") of a ROC plot is equal to the probability that a classifier will rank a randomly chosen positive instance higher than a randomly chosen negative one. The area under the

ROC curve may be thought of as equivalent to the Mann-Whitney U test, which tests for the median difference between scores obtained in the two groups considered if the groups are of continuous data, or to the Wilcoxon test of ranks.

[00063] As discussed above, suitable tests may exhibit one or more of the following results on these various measures: a specificity of greater than 0.5, preferably at least 0.6, more preferably at least 0.7, still more preferably at least 0.8, even more preferably at least 0.9 and most preferably at least 0.95, with a corresponding sensitivity greater than 0.2, preferably greater than 0.3, more preferably greater than 0.4, still more preferably at least 0.5, even more preferably 0.6, yet more preferably greater than 0.7, still more preferably greater than 0.8, more preferably greater than 0.9, and most preferably greater than 0.95; a sensitivity of greater than 0.5, preferably at least 0.6, more preferably at least 0.7, still more preferably at least 0.8, even more preferably at least 0.9 and most preferably at least 0.95, with a corresponding specificity greater than 0.2, preferably greater than 0.3, more preferably greater than 0.4, still more preferably at least 0.5, even more preferably 0.6, yet more preferably greater than 0.7, still more preferably greater than 0.8, more preferably greater than 0.9, and most preferably greater than 0.95; at least 75% sensitivity, combined with at least 75% specificity; a ROC curve area of greater than 0.5, preferably at least 0.6, more preferably 0.7, still more preferably at least 0.8, even more preferably at least 0.9, and most preferably at least 0.95; an odds ratio different from 1, preferably at least about 2 or more or about 0.5 or less, more preferably at least about 3 or more or about 0.33 or less, still more preferably at least about 4 or more or about 0.25 or less, even more preferably at least about 5 or more or about 0.2 or less, and most preferably at least about 10 or more or about 0.1 or less; a positive likelihood ratio (calculated as sensitivity/(1-specificity)) of greater than 1, at least 2, more preferably at least 3, still more preferably at least 5, and most preferably at least 10; and or a negative likelihood ratio (calculated as (1-sensitivity)/specificity) of less than 1, less than or equal to 0.5, more preferably less than or equal to 0.3, and most preferably less than or equal to 0.1

[00064] Antibodies

[00065] The term "antibody" as used herein refers to a peptide or polypeptide derived from, modeled after or substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof, capable of specifically binding an antigen

or epitope. See, e.g. Fundamental Immunology, 3rd Edition, W.E. Paul, ed., Raven Press, N.Y. (1993); Wilson (1994; J. Immunol. Methods 175:267-273; Yarmush (1992) J. Biochem. Biophys. Methods 25:85-97. The term antibody includes antigen-binding portions, i.e., "antigen binding sites," (e.g., fragments, subsequences, complementarity determining regions (CDRs)) that retain capacity to bind antigen, including (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CHl domains; (ii) a F(ab')2 fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CHl domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., (1989) Nature 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Single chain antibodies are also included by reference in the term "antibody."

[00066] As used herein, “antibody variable domain” refers to the portions of the light and heavy chains of antibody molecules that include amino acid sequences of Complementarity Determining Regions (CDRs; ie., CDR1, CDR2, and CDR3), and Framework Regions (FRs). V_H refers to the variable domain of the heavy chain. V_L refers to the variable domain of the light chain. According to the methods used in this invention, the amino acid positions assigned to CDRs and FRs may be defined according to Kabat (Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, Md., 1987 and 1991)). Amino acid numbering of antibodies or antigen binding fragments is also according to that of Kabat.

[00067] An “isolated” antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the

antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

[00068] Ordinarily, an antibody may comprise heavy and/or light chain variable comprising an amino acid sequence having at least 75% amino acid sequence identity or similarity with the amino acid sequence of either the heavy or light chain variable domain of a parent antibody having known binding characteristics, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, and most preferably at least 95%. Identity or similarity with respect to this sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical (i.e same residue) or similar (i.e. amino acid residue from the same group based on common side-chain properties) with the species-dependent antibody residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the antibody sequence outside of the variable domain shall be construed as affecting sequence identity or similarity. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophilic: cys, ser, thr, asn, gln;

(3) acidic: asp, glu;

(4) basic: his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic: trp, tyr, phe.

[00069] While conservative substitutions are often preferred, non-conservative substitutions (which entail exchanging a member of one of these classes for a member of another class) are also contemplated.

[00070] Preferred therapeutic antibodies are IgG antibodies. The term “IgG” as used herein is meant a polypeptide belonging to the class of antibodies that are substantially encoded by a recognized immunoglobulin gamma gene. In humans this class comprises IgG1, IgG2, IgG3, and IgG4. In mice this class comprises IgG1, IgG2a, IgG2b, IgG3. The known Ig domains in the IgG class of antibodies are VH, Cγ1, Cγ2, Cγ3, VL, and CL.

IgG is the preferred class for therapeutic antibodies for several practical reasons. IgG antibodies are stable, easily purified, and able to be stored under conditions that are practical for pharmaceutical supply chains. *In vivo* they have a long biological half-life that is not just a function of their size but is also a result of their interaction with the so-called Fc receptor (or FcRn). This receptor seems to protect IgG from catabolism within cells and recycles it back to the plasma.

[00071] Antibodies are immunological proteins that bind a specific antigen. In most mammals, including humans and mice, antibodies are constructed from paired heavy and light polypeptide chains. The light and heavy chain variable regions show significant sequence diversity between antibodies, and are responsible for binding the target antigen. Each chain is made up of individual immunoglobulin (Ig) domains, and thus the generic term immunoglobulin is used for such proteins.

[00072] The term "specifically binds" is not intended to indicate that an antibody binds exclusively to its intended target since, as noted above, an antibody binds to any polypeptide displaying the epitope(s) to which the antibody binds. Rather, an antibody "specifically binds" if its affinity for its intended target is about 5-fold greater when compared to its affinity for a non-target molecule which does not display the appropriate epitope(s). Preferably the affinity of the antibody will be at least about 5 fold, preferably 10 fold, more preferably 25-fold, even more preferably 50-fold, and most preferably 100-fold or more, greater for a target molecule than its affinity for a non-target molecule. In preferred embodiments, Preferred antibodies bind with affinities of at least about 10^7 M^{-1}, and preferably between about 10^8 M^{-1} to about 10^9 M^{-1}, about 10^9 M^{-1} to about 10^{10} M^{-1}, or about 10^{10} M^{-1} to about 10^{12} M^{-1} .

[00073] Affinity is calculated as $K_d = k_{off}/k_{on}$ (k_{off} is the dissociation rate constant, K_{on} is the association rate constant and K_d is the equilibrium constant). Affinity can be determined at equilibrium by measuring the fraction bound (r) of labeled ligand at various concentrations (c). The data are graphed using the Scatchard equation: r/c = K(n-r): where r = moles of bound ligand/mole of receptor at equilibrium; c = free ligand concentration at equilibrium; K = equilibrium association constant; and n = number of ligand binding sites per receptor molecule. By graphical analysis, r/c is plotted on the Y-axis versus r on the X-axis, thus producing a Scatchard plot. Antibody affinity measurement by Scatchard

analysis is well known in the art. *See, e.g.*, van Erp *et al., J. Immunoassay* 12: 425-43, 1991; Nelson and Griswold, *Comput. Methods Programs Biomed.* 27: 65-8, 1988.

[00074] Antibodies of the invention may be further characterized by epitope mapping, so that antibodies and epitopes may be selected that have the greatest clinical utility in the immunoassays described herein. The term "epitope" refers to an antigenic determinant capable of specific binding to an antibody. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and nonconformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents. Preferably, an epitope is targeted which is present on the target molecule, but is partially or totally absent on non-target molecules.

[00075] In some embodiments, the antibody scaffold can be a mixture of sequences from different species. As such, if the antibody is an antibody, such antibody may be a chimeric antibody and/or a humanized antibody. In general, both "chimeric antibodies" and "humanized antibodies" refer to antibodies that combine regions from more than one species. For example, "chimeric antibodies" traditionally comprise variable region(s) from a mouse (or rat, in some cases) and the constant region(s) from a human. "Humanized antibodies" generally refer to non-human antibodies that have had the variable-domain framework regions swapped for sequences found in human antibodies. Generally, in a humanized antibody, the entire antibody, except the CDRs, is encoded by a polynucleotide of human origin or is identical to such an antibody except within its CDRs. The CDRs, some or all of which are encoded by nucleic acids originating in a non-human organism, are grafted into the beta-sheet framework of a human antibody variable region to create an antibody, the specificity of which is determined by the engrafted CDRs. The creation of such antibodies is described in, e.g., WO 92/11018, Jones, 1986, Nature 321:522-525, Verhoeyen et al., 1988, Science 239:1534-1536. "Backmutation" of selected acceptor framework residues to the corresponding donor residues is often required to regain affinity that is lost in the initial grafted construct (U.S. Pat. No. 5,530,101; U.S. Pat. No. 5,585,089; U.S. Pat. No. 5,693,761; U.S. Pat. No. 5,693,762; U.S. Pat. No. 6,180,370; U.S. Pat. No. 5,859,205; U.S. Pat. No. 5,821,337; U.S. Pat. No. 6,054,297; U.S. Pat. No. 6,407,213). The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region, typically that of a

human immunoglobulin, and thus will typically comprise a human Fc region. Humanized antibodies can also be generated using mice with a genetically engineered immune system. Roque et al., 2004, Biotechnol. Prog. 20:639-654. A variety of techniques and methods for humanizing and reshaping non-human antibodies are well known in the art (See Tsurushita & Vasquez, 2004, Humanization of Monoclonal Antibodies, Molecular Biology of B Cells, 533-545, Elsevier Science (USA), and references cited therein). Humanization methods include but are not limited to methods described in Jones et al., 1986, Nature 321:522-525; Riechmann et al., 1988; Nature 332:323-329; Verhoeyen et al., 1988, Science, 239:1534-1536; Queen et al., 1989, Proc Natl Acad Sci, USA 86:10029-33; He et al., 1998, J. Immunol. 160: 1029-1035; Carter et al., 1992, Proc Natl Acad Sci USA 89:4285-9, Presta et al., 1997, Cancer Res.57(20):4593-9; Gorman et al., 1991, Proc. Natl. Acad. Sci. USA 88:4181-4185; O'Connor et al., 1998, Protein Eng 11:321-8. Humanization or other methods of reducing the immunogenicity of nonhuman antibody variable regions may include resurfacing methods, as described for example in Roguska et al., 1994, Proc. Natl. Acad. Sci. USA 91:969-973. In one embodiment, the parent antibody has been affinity matured, as is known in the art. Structure-based methods may be employed for humanization and affinity maturation, for example as described in U.S. Ser. No. 11/004,590. Selection based methods may be employed to humanize and/or affinity mature antibody variable regions, including but not limited to methods described in Wu et al., 1999, J. Mol. Biol. 294:151-162; Baca et al., 1997, J. Biol. Chem. 272(16):10678-10684; Rosok et al., 1996, J. Biol. Chem. 271(37): 22611-22618; Rader et al., 1998, Proc. Natl. Acad. Sci. USA 95: 8910-8915; Krauss et al., 2003, Protein Engineering 16(10):753-759. Other humanization methods may involve the grafting of only parts of the CDRs, including but not limited to methods described in U.S. Ser. No. 09/810,502; Tan et al., 2002, J. Immunol. 169:1119-1125; De Pascalis et al., 2002, J. Immunol. 169:3076-3084.

[00076] In one embodiment, the antibody is a fully human antibody. "Fully human antibody" or "complete human antibody" refers to a human antibody having the gene sequence of an antibody derived from a human chromosome. Fully human antibodies may be obtained, for example, using transgenic mice (Bruggemann et al., 1997, Curr Opin Biotechnol 8:455-458) or human antibody libraries coupled with selection methods (Griffiths et al., 1998, Curr Opin Biotechnol 9:102-108).

[00077] Production of Antibodies

[00078] Monoclonal antibody preparations can be produced using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art and taught, for example, in Harlow et al., ANTIBODIES: A LABORATORY MANUAL, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling, et al., in: MONOCLONAL ANTIBODIES AND T-CELL HYBRIDOMAS, pp. 563-681 (Elsevier, N.Y., 1981) (both of which are incorporated by reference in their entireties). The term "monoclonal antibody" as used herein is not limited to antibodies produced through hybridoma technology. The term "monoclonal antibody" refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced.

[00079] Monoclonal antibodies derived from animals other than rats and mice offer unique advantages. Many protein targets relevant to signal transduction and disease are highly conserved between mice, rats and humans, and can therefore be recognized as self-antigens by a mouse or rat host, making them less immunogenic. This problem may be avoided when using rabbit as a host animal. See, e.g., Rossi et al., *Am. J. Clin. Pathol.,* 124, 295-302, 2005.

[00080] Methods for producing and screening for specific antibodies using hybridoma technology are routine and well known in the art. In a non-limiting example, mice can be immunized with an antigen of interest or a cell expressing such an antigen. Once an immune response is detected, e.g., antibodies specific for the antigen are detected in the mouse serum, the mouse spleen is harvested and splenocytes isolated. The splenocytes are then fused by well known techniques to any suitable myeloma cells. Hybridomas are selected and cloned by limiting dilution. The hybridoma clones are then assayed by methods known in the art for cells that secrete antibodies capable of binding the antigen. Ascites fluid, which generally contains high levels of antibodies, can be generated by inoculating mice intraperitoneally with positive hybridoma clones.

[00081] Adjuvants that can be used in the methods of antibody generation include, but are not limited to, protein adjuvants; bacterial adjuvants, e.g., whole bacteria (BCG, Corynebacterium parvum, Salmonella minnesota) and bacterial components including cell wall skeleton, trehalose dimycolate, monophosphoryl lipid A, methanol extractable

residue (MER) of tubercle bacillus, complete or incomplete Freund's adjuvant; viral adjuvants; chemical adjuvants, e.g., aluminum hydroxide, iodoacetate and cholesteryl hemisuccinateor; naked DNA adjuvants. Other adjuvants that can be used in the methods of the invention include, Cholera toxin, paropox proteins, MF-59 (Chiron Corporation; See also Bieg et al. (1999) "GAD65 And Insulin B Chain Peptide (9-23) Are Not Primary Autoantigens In The Type 1 Diabetes Syndrome Of The BB Rat," Autoimmunity, 31(1):15-24, which is incorporated herein by reference), MPL® (Corixa Corporation; See also Lodmell et al. (2000) "DNA Vaccination Of Mice Against Rabies Virus: Effects Of The Route Of Vaccination And The Adjuvant Monophosphoryl Lipid A (MPL)," Vaccine, 18: 1059-1066; Johnson et al. (1999) "3-O-Desacyl Monophosphoryl Lipid A Derivatives: Synthesis And Immunostimulant Activities," Journal of Medicinal Chemistry, 42: 4640-4649; Baldridge et al. (1999) "Monophosphoryl Lipid A (MPL) Formulations For The Next Generation Of Vaccines," Methods, 19: 103-107, all of which are incorporated herein by reference), RC-529 adjuvant (Corixa Corporation; the lead compound from Corixa's aminoalkyl glucosaminide 4-phosphate (AGP) chemical library, see also www.corixa.com), and DETOX™ adjuvant (Corixa Corporation; DETOX™ adjuvant includes MPL® adjuvant (monophosphoryl lipid A) and mycobacterial cell wall skeleton; See also Eton et al. (1998) "Active Immunotherapy With Ultraviolet B-Irradiated Autologous Whole Melanoma Cells Plus DETOX In Patients With Metastatic Melanoma," Clin. Cancer Res. 4(3):619-627; and Gupta et al. (1995) "Adjuvants For Human Vaccines—Current Status, Problems And Future Prospects," Vaccine, 13(14): 1263-1276, both of which are incorporated herein by reference).

[00082] Numerous publications discuss the use of phage display technology to produce and screen libraries of polypeptides for binding to a selected analyte. See, e.g, Cwirla et al., Proc. Natl. Acad. Sci. USA 87, 6378-82, 1990; Devlin et al., Science 249, 404-6, 1990, Scott and Smith, Science 249, 386-88, 1990; and Ladner et al., U.S. Pat. No. 5,571,698. A basic concept of phage display methods is the establishment of a physical association between DNA encoding a polypeptide to be screened and the polypeptide. This physical association is provided by the phage particle, which displays a polypeptide as part of a capsid enclosing the phage genome which encodes the polypeptide. The establishment of a physical association between polypeptides and their genetic material allows simultaneous mass screening of very large numbers of phage bearing different polypeptides. Phage displaying a polypeptide with affinity to a target bind to the target

and these phage are enriched by affinity screening to the target. The identity of polypeptides displayed from these phage can be determined from their respective genomes. Using these methods a polypeptide identified as having a binding affinity for a desired target can then be synthesized in bulk by conventional means. See, e.g., U.S. Patent No. 6,057,098, which is hereby incorporated in its entirety, including all tables, figures, and claims.

[00083] The antibodies that are generated by these methods may then be selected by first screening for affinity and specificity with the purified polypeptide of interest and, if required, comparing the results to the affinity and specificity of the antibodies with polypeptides that are desired to be excluded from binding. The screening procedure can involve immobilization of the purified polypeptides in separate wells of microtiter plates. The solution containing a potential antibody or groups of antibodies is then placed into the respective microtiter wells and incubated for about 30 min to 2 h. The microtiter wells are then washed and a labeled secondary antibody (for example, an anti-mouse antibody conjugated to alkaline phosphatase if the raised antibodies are mouse antibodies) is added to the wells and incubated for about 30 min and then washed. Substrate is added to the wells and a color reaction will appear where antibody to the immobilized polypeptide(s) are present.

[00084] The antibodies so identified may then be further analyzed for affinity and specificity in the assay design selected. In the development of immunoassays for a target protein, the purified target protein acts as a standard with which to judge the sensitivity and specificity of the immunoassay using the antibodies that have been selected. Because the binding affinity of various antibodies may differ; certain antibody pairs (e.g., in sandwich assays) may interfere with one another sterically, etc., assay performance of an antibody may be a more important measure than absolute affinity and specificity of an antibody.

[00085] Antibodies can also be produced using transgenic mice which are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For example, the human heavy and light chain immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. Alternatively, the human variable region, constant region, and diversity region may be introduced into mouse embryonic stem cells in addition to

the human heavy and light chain genes. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. In particular, homozygous deletion of the JH region prevents endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring which express human antibodies. The transgenic mice are immunized using conventional methodologies with a selected antigen, e.g., all or a portion of a polypeptide of the invention. Monoclonal antibodies directed against the antigen can be obtained from the immunized, transgenic mice using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg et al. (1995) "Human Antibodies From Transgenic Mice," Int. Rev. Immunol. 13:65-93, which is incorporated herein by reference in its entirety). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, e.g., International Publication Nos. WO 98/24893, WO 96/34096, and WO 96/33735; and U.S. Pat. Nos. 5,413,923, 5,625,126, 5,633,425, 5,569,825, 5,661,016, 5,545,806, 5,814,318, and 5,939,598, which are incorporated by reference herein in their entirety. In addition, companies such as Abgenix, Inc. (Freemont, Calif.) and Medarex (Princeton, N.J.) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

[00086] Recombinant Expression of Antibodies

[00087] Once a nucleic acid sequence encoding an antibody of the invention has been obtained, the vector for the production of the antibody may be produced by recombinant DNA technology using techniques well known in the art. Methods which are well known to those skilled in the art can be used to construct expression vectors containing the antibody coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. (See, for example, the techniques described in Sambrook et al, 1990, MOLECULAR CLONING, A

LABORATORY MANUAL, 2d Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. and Ausubel et al. eds., 1998, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, NY).

[00088] An expression vector comprising the nucleotide sequence of an antibody can be transferred to a host cell by conventional techniques (e.g., electroporation, liposomal transfection, and calcium phosphate precipitation) and the transfected cells are then cultured by conventional techniques to produce the antibody of the invention. In specific embodiments, the expression of the antibody is regulated by a constitutive, an inducible or a tissue, specific promoter.

[00089] The host cells used to express the recombinant antibodies of the invention may be either bacterial cells such as Escherichia coli, or, preferably, eukaryotic cells, especially for the expression of whole recombinant immunoglobulin molecule. In particular, mammalian cells such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus is an effective expression system for immunoglobulins (Foecking et al. (1986) "Powerful And Versatile Enhancer-Promoter Unit For Mammalian Expression Vectors." Gene 45:101-105; Cockett et al. (1990) "High Level Expression Of Tissue Inhibitor Of Metalloproteinases In Chinese Hamster Ovary Cells Using Glutamine Synthetase Gene Amplification," Biotechnology 8:662-667).

[00090] A variety of host-expression vector systems may be utilized to express the antibodies of the invention. Such host-expression systems represent vehicles by which the coding sequences of the antibodies may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express the antibodies of the invention in situ. These include, but are not limited to, microorganisms such as bacteria (e.g., E. coli and B. subtilis) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing immunoglobulin coding sequences; yeast (e.g., Saccharomyces pichia) transformed with recombinant yeast expression vectors containing immunoglobulin coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the immunoglobulin coding sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus (CaMV) and tobacco mosaic virus (TMV)) or transformed with recombinant

plasmid expression vectors (e.g., Ti plasmid) containing immunoglobulin coding sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 293T, 3T3 cells, lymphotic cells (see U.S. Pat. No. 5,807,715), Per C.6 cells (rat retinal cells developed by Crucell)) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

[00091] In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the antibody being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of an antibody, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the E. coli expression vector pUR278 (Ruther et al. (1983) "Easy Identification Of cDNA Clones," EMBO J. 2:1791-1794), in which the antibody coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye et al. (1985) "Up-Promoter Mutations In The Lpp Gene Of Escherichia coli," Nucleic Acids Res. 13:3101-3110; Van Heeke et al. (1989) "Expression Of Human Asparagine Synthetase In Escherichia coli," J. Biol. Chem. 24:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to a matrix glutathione-agarose beads followed by elution in the presence of free gluta-thione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

[00092] In an insect system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in Spodoptera frugiperda cells. The antibody coding sequence may be cloned individually into non-essential regions (e.g., the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (e.g., the polyhedrin promoter).

[00093] In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the antibody coding sequence of interest may be ligated to an adenovirus transcription/translation

control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the immunoglobulin molecule in infected hosts. (see e.g., see Logan et al. (1984) "Adenovirus Tripartite Leader Sequence Enhances Translation Of mRNAs Late After Infection," Proc. Natl. Acad. Sci. (U.S.A.) 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted antibody coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bitter et al. (1987) "Expression And Secretion Vectors For Yeast," Methods in Enzymol. 153:516-544).

[00094] In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERY, BHK, Hela, COS, MDCK, 293, 293T, 3T3, WI38, BT483, Hs578T, HTB2, BT20 and T47D, CRL7030 and Hs578Bst.

[00095] For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express an antibody of the invention may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the

introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the antibodies of the invention. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that interact directly or indirectly with the antibodies of the invention.

[00096] A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al. (1977) "Transfer Of Purified Herpes Virus Thymidine Kinase Gene To Cultured Mouse Cells," Cell 11:223-232), hypoxanthine-guanine phosphoribosyltransferase (Szybalska et al. (1962) "Genetics Of Human Cess Line. IV. DNA-Mediated Heritable Transformation Of A Biochemical Trait," Proc. Natl. Acad. Sci. (U.S.A.) 48:2026-2034), and adenine phosphoribosyltransferase (Lowy et al. (1980) "Isolation Of Transforming DNA: Cloning The Hamster Aprt Gene," Cell 22:817-823) genes can be employed in tk–, hgprt– or aprt– cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler et al. (1980) "Transformation Of Mammalian Cells With An Amplfiable Dominant-Acting Gene," Proc. Natl. Acad. Sci. (U.S.A.) 77:3567-3570; O'Hare et al. (1981) "Transformation Of Mouse Fibroblasts To Methotrexate Resistance By A Recombinant Plasmid Expressing A Prokaryotic Dihydrofolate Reductase," Proc. Natl. Acad. Sci. (U.S.A.) 78:1527-1531); gpt, which confers resistance to mycophenolic acid (Mulligan et al. (1981) "Selection For Animal Cells That Express The Escherichia coli Gene Coding For Xanthine-Guanine Phosphoribosyltransferase," Proc. Natl. Acad. Sci. (U.S.A.) 78:2072-2076); neo, which confers resistance to the aminoglycoside G-418 (Tachibana et al. (1991) "Altered Reactivity Of Immunoglobutin Produced By Human-Human Hybridoma Cells Transfected By pSV2-Neo Gene," Cytotechnology 6(3):219-226; Tolstoshev (1993) "Gene Therapy, Concepts, Current Trials And Future Directions," Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan (1993) "The Basic Science Of Gene Therapy," Science 260:926-932; and Morgan et al. (1993) "Human gene therapy," Ann. Rev. Biochem. 62:191-217). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), 1993, CURRENT PROTOCOLS

IN MOLECULAR BIOLOGY, John Wiley & Sons, NY; Kriegler, 1990, GENE TRANSFER AND EXPRESSION, A LABORATORY MANUAL, Stockton Press, NY; and in Chapters 12 and 13, Dracopoli et al. (eds), 1994, CURRENT PROTOCOLS IN HUMAN GENETICS, John Wiley & Sons, NY.; Colbere-Garapin et al. (1981) "A New Dominant Hybrid Selective Marker For Higher Eukaryotic Cells," J. Mol. Biol. 150:1-14; and hygro, which confers resistance to hygromycin (Santerre et al. (1984) "Expression Of Prokaryotic Genes For Hygromycin B And G418 Resistance As Dominant-Selection Markers In Mouse L Cells," Gene 30:147-156).

[00097] The expression levels of an antibody of the invention can be increased by vector amplification (for a review, see Bebbington and Hentschel, "The Use Of Vectors Based On Gene Amplification For The Expression Of Cloned Genes In Mammaian Cells," in DNA CLONING, Vol. 3. (Academic Press, New York, 1987)). When a marker in the vector system expressing an antibody is amplifiable, increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the nucleotide sequence of the antibody, production of the antibody will also increase (Crouse et al. (1983) "Expression And Amplification Of Engineered Mouse Dihydrofolate Reductase Minigenes," Mol. Cell. Biol. 3:257-266).

[00098] The host cell may be co-transfected with two expression vectors of the invention, the first vector encoding a heavy chain derived polypeptide and the second vector encoding a light chain derived polypeptide. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes both heavy and light chain polypeptides. In such situations, the light chain should be placed before the heavy chain to avoid an excess of toxic free heavy chain (Proudfoot (1986) "Expression And Amplification Of Engineered Mouse Dihydrofolate Reductase Minigenes," Nature 322:562-565; Kohler (1980) "Immunoglobulin Chain Loss In Hybridoma Lines," Proc. Natl. Acad. Sci. (U.S.A.) 77:2197-2199). The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

[00099] Once the antibody of the invention has been recombinantly expressed, it may be purified by any method known in the art for purification of an antibody, for example, by chromatography (e.g., ion exchange, affinity, particularly by affinity for the

specific antigen after Protein A, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

EXAMPLES

[000100] Example 1: Monoclonal Antibody development in rabbits

[000101] Female New Zealand Rabbits were immunized by subcutaneous injections (SQ) with antigen/adjuvant emulsions. Primary immunization was done with Complete Freund's Adjuvant and Incomplete Freund's Adjuvant was used for all subsequent boosts. Rabbits were injected SQ every three weeks at 250µg protein antigen per rabbit (alternating two sites, hips and scapulas). A test bleed was taken from the marginal ear vein seven days after the second boost. This test bleed (immune sera) was tested by indirect ELISA assay to determine if immune response of the rabbit was adequate for monoclonal antibody development. The best responding rabbit was given a final SQ boost and four days later was euthanized via exsanguination. The whole blood was collected via cardiac puncture. B cells producing antibody of interest were identified by indirect ELISA on target antigen and immunoglobulin genes were isolated. Heavy and light chains were cloned into separate mammalian expression vectors, transfected into HEK cells (transient transfection), and tissue culture supernatant containing rabbit monoclonal antibodies were harvested.

[000102] Example 2: Monoclonal Antibody development in mice

[000103] Female BALB/c mice (60 days old) were immunized by intraperitoneal injections (IP) with antigen/adjuvant emulsions as per standard operating procedure. Primary immunization was done with Complete Freund's Adjuvant and Incomplete Freund's Adjuvant was used for all subsequent boosts. Mice were injected IP every 3 weeks at 25µg antigen per mouse (total volume 125µL per mouse). Test bleeds were done by saphenous vein lancing 7 to 10 days after the second boost. This test bleed (immune sera) was tested by indirect ELISA assay to determine if the immune response of mice was adequate for fusion. The best 2 responding mice were given a final intravenous boost of 10µg antigen per mouse in sterile saline via lateral tail vein. 4 days after the IV boost the mice were euthanized and the spleens were harvested. Lymphocytes isolated from the spleen were used in the fusion process to produce hybridomas using the method of Kohler, G.; Milstein, C. (1975). "Continuous cultures of fused cells secreting antibody of

predefined specificity". Nature 256 (5517): 495–497. Hybridomas were generated using a PEG1500 fusion process.

[000104] Example 3: Screening of Antibodies with Patient Samples (Microtiter-based ELISA Method)

[000105] Materials:

96-well high bind ELISA plates-Costar 3590 (Corning)
ELISA coating buffer: PBS
ELISA wash buffer: PBS with 0.02% Tween-20
ELISA blocking Buffer (Thermo Pierce, catalogue number N502)
ELISA reagent diluent: 200 mM Tris, 1% BSA (BioFx), 0.05% Tween-20, pH 8.1
Neutravidin-HRP conjugate (Thermo Pierce, catalogue number 31001)
1-Step Ultra TMB substrate (R&D systems, catalogue number 34028)
Stop solution: 2N sulfuric acid
Capture antibodies
Biotin conjugated detection antibodies
Recombinant human IGFBP7 (Peprotech, catalogue number 410-02)
EXLx405 plate washer (Biotek)
Multiskan FC plate reader (Fisher Scientific)

[000106] Testing Procedure

[000107] Purified, recombinant IGFBP7 analyte was spiked into Reagent Diluent and serially diluted to generate a set of standard samples covering a range of concentrations. Frozen single-use aliquots of patient samples were thawed in a room temperature water bath for 10 minutes, and then diluted to desired level with Reagent Diluent.

[000108] 100 µL of 5 µg/mL Capture Antibody solution prepared in coating buffer was added to each well on a 96-well high bind ELISA plate and incubated over night at room temperature (22°C to 25°C). Each well was aspirated and washed three times with 300 µL of wash buffer using an autowasher. Then 250 µL of ELISA blocking buffer was added to each well. After an incubation of 2 hours at room temperature, the aspiration / wash step described above was repeated.

[000109] 100 µL of standard or patient samples was added to each well of the prepared plate and incubated at room temperature on a horizontal orbital shaker. After 2 hours of

incubation, the plate was washed as described above. Then 100 μL of 0.1 μg/mL detection antibody solution prepared in reagent diluent was added to each well. After incubation for 1 hour at room temperature, the plate was washed again. A 0.1 μg/mL solution of neutravidin-HRP conjugate was prepared in reagent diluent, and 100 μL of this solution was added to each well. The plate was incubated for 1 hour at room temperature and washed. 100 μL of 1-step ultra TMB substrate was added to each well, incubated at room temperature for 10 minutes protected from light, followed by 50 μL of stop solution. The optical density in each well was measured with a microplate reader set to a wavelength of 450nm.

[000110] Example 4: Screening of Antibodies with Patient Samples (Lateral Flow Strip Testing Method)

[000111] Materials:

Nitrocellulose membrane

Backing card

Sample pad

Wicking pad

Membrane blocking buffer: 10 mM Sodium phosphate, 0.1% sucrose, 0.1% BSA, 0.2% PVP-40, pH 8.0

Sample pad blocking buffer: 5 mM Borate, 0.1% Tween-20, 0.25% PVP-40, 0.5% BSA, pH 8.5

Running buffer J: 500 mM Tris, 0.2% 10G, 0.35% Tween-20, 0.25% PVP-40, pH 8.5

Fluorescently-conjugated antibodies

Test line antibodies

Goat-anti-mouse positive control antibodies

Recombinant human IGFBP7

[000112] Strip Assembly

[000113] Nitrocellulose membranes were striped with test line antibodies using an AD3050 aspirate dispense system, blocked with the membrane blocking buffer and dried at 37° C for 30 min. After curing over night in a desiccator, the striped and blocked nitrocellulose membranes were laminated onto backing cards with wicking pads and

sample pads pre-treated with the sample pad blocking buffer. The cards were cut into 5 mm wide test strips, which were then placed into cartridges.

[000114] Sample Preparation

[0100] Purified, recombinant IGFBP7 analyte was spiked into Running buffer J and serially diluted to generate a set of standard samples covering a range of concentrations. Frozen single-use aliquots of patient samples were thawed in a room temperature water bath for 10 minutes, and then diluted to desired level with Running buffer J.

[0101] Testing Procedure

[0102] 10 μL of fluorescently conjugated antibody (0.025 μg/μL) in PBS was added to 100 μL of sample. 100 μL of this solution was then loaded into the input port on the cartridge. Results was read at t=20 minutes using a fluorescence reader and associated software.

[0111] Example 5: Peptide Mapping

[0112] Materials**:** 96-well high bind microtiter plates, Neutravidin, biotinylated peptides, Unconjugated antibodies, mouse IgG, rabbit IgG, goat IgG, HRP conjugated to anti-mouse IgG HRP conjugate, anti-rabbit IgG HRP conjugate, anti-goat IgG HRP conjugate, TMB substrate, 2N sulfuric acid were used for epitope mapping experiments.

[0113] Neutravidin was immobilized in individual wells of 96-well high bind microtiter plate. The plates were washed to remove unreacted neutravidin followed by a blocking step. Biotinylated peptides were dissolved in an aqueous buffer to a concentration of 10 μg/mL. 50 μL of the peptide solutions were added to each well of neutravidin coated microtiter plates. These plates were incubated one hour at room temperature and then washed to remove unbound peptides. Unconjugated mouse and rabbit antibodies were diluted to 5 μg/mL and added to the plate at 100 μL/well. Anti-mouse IgG (in the mouse anti-IGFBP7) or anti-rabbit IgG (in the case of rabbit anti-IGFBP7) was added to neighboring wells as a negative control. Plates were incubated 1 hour at room temperature and washed. HRP conjugated to anti-mouse IgG (in the case of mouse anti-IGFBP7 and mouse IgG negative control), and HRP conjugated to anti-rabbit IgG (in the case of rabbit anti-IGFBP7 and rabbit IgG negative control) was diluted to 0.2 μg/mL and 100 μL was added to each well of the plate. These plates were incubated for 20 minutes at room temperature and washed. 100 μL/well of TMB substrate was added

and plates were incubated for 20 minutes while avoiding exposure to light. 50 µl/well of Stop solution (2N sulfuric acid) was added to each well and plates to stop the reaction. The absorbance was read on spectrophtometric 96-well microplate reader set to measure the optical density at 450 nm.

[0114] Example 6: Alanine scanning peptide mapping

[0115] Alanine scanning is a widely used mutagenesis approach in which residues in a target protein are systematically substituted for alanine at selected positions by site-directed mutagenesis, expressed, and assayed for function. Substitution with alanine residues eliminates side-chain interactions without altering main-chain conformation or introducing steric or electrostatic effects. Using automated mutagenesis protocols, every residue in a target polypeptide is changed to alanine, and critical residues that comprise each antibody binding domain can be determined.

[0116] Example 7: Results

[0117] Using the combined alanine scanning and peptide mapping results, unique IGFBP7 monoclonal antibodies were identified and selected based on analytical performance.

Antibody	Pepscan sequence	Astute Sequence (total region)
7G2.1	$_{210}$PGDRD$_{214}$ (SEQ ID NO: 6)	$_{201}$YGVQRTELLPGDRDNL$_{216}$ (SEQ ID NO: 6)
6D2.1	$_{206}$TELLPGDR$_{213}$ (SEQ ID NO: 3)	$_{191}$LIWNKVKRGHYGVQRT$_{206}$ (SEQ ID NO: 7)
1C9E4.1	$_{36}$EPASC$_{40}$ (SEQ ID NO: 4)	$_{25}$SSSSSDTCGPCEPASCPPLP$_{44}$ SEQ ID NO: 8)

[0118] Example 8: Sequencing data

[0119] Antibody IC9E4.1 was isotyped as a murine IgG1/kappa antibody. cDNA from the monocloncal cell line was obtained for sequencing by standard methods. The sequences of the heavy chain variable region and the light chain variable region were as follows:

[0120] V_{light} (SEQ ID NO: 9)

DVVMTQTPLT LSVTIGQPAS ISCKSSQSLL YSNGETYLHW LLQRPGQSPK 50
RLIYLVSKLD SGVPDRFTGS GSRTDFTLKI SRVEAEDLGV YYCAQGTHFP 100
HTFGGGTKLE

[0121] V_{heavy} (SEQ ID NO: 10)

QIQLVQSGPE LKKPGETVKI SCKASGYSFT DYSIHWVKQA PGKGLKWMGL 50
INTETGEPIY VDDFKGRFAF SLETSARTAY LQINNLKNED TATYFCARAY 100
YWAYWGQGTL V

[0122] Antibody 1D6

[0123] Antibody 1D6 was isotyped as a murine IgG1/kappa antibody. By epitope mapping, the 1D6 antibody was determined to bind to a conformational epitope of IGFBP7. cDNA from the monocloncal cell line was obtained for sequencing by standard methods. The sequences of the heavy chain variable region and the light chain variable region were as follows:

[0124] V_{light} (SEQ ID NO: 11)

QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMHWYQQKSG TSPKRWIYDT 50
SELASGVPAR FSGSGSGTSY SLTISSMEAE DAATYYCQQW SSSPFTFGSG 100
TKLEIKR

[0125] V_{heavy} (SEQ ID NO: 12)

QIQLVQSGPE LKKPGETVKI SCKASGYTFK KYGMNWVKQA PGKGLKWMGW 50
INTYTGEPIY ADDFKGRFAF SLETSASTAY LQISNLKNED TATYFCAREE
YGPFYAMDYW GQGTSVTVSS

[0126] While the invention has been described and exemplified in sufficient detail for those skilled in this art to make and use it, various alternatives, modifications, and improvements should be apparent without departing from the spirit and scope of the invention. The examples provided herein are representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Modifications therein and other uses will occur to those skilled in the art. These modifications are encompassed within the spirit of the invention and are defined by the scope of the claims.

[0127] The use of "or" herein means "and/or" unless stated otherwise. Similarly, "comprise," "comprises," "comprising" "include," "includes," and "including" are interchangeable and not intended to be limiting.

[0128] It is to be further understood that where descriptions of various embodiments use the term "comprising," those skilled in the art would understand that in some specific instances, an embodiment can be alternatively described using language "consisting essentially of" or "consisting of."

[0129] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs. Although any methods and reagents similar or equivalent to those described herein can be used in the practice of the disclosed methods and compositions, the exemplary methods and materials are now described.

[0130] All publications mentioned herein are incorporated herein by reference in full for the purpose of describing and disclosing the methodologies, which are described in the publications, which might be used in connection with the description herein. All patents and publications mentioned in the specification are indicative of the levels of those of ordinary skill in the art to which the invention pertains prior to the filing date of the disclosure. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior disclosure.

[0131] It will be readily apparent to a person skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

[0132] The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features,

modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

[0133] Other embodiments are set forth within the following claims.

WHAT IS CLAIMED IS:

1. An antibody which specifically binds human IGFBP7 selected from the group consisting of:

a monoclonal antibody which specifically binds to a polypeptide consisting of SEQ ID NO: 1, and a monoclonal antibody which specifically binds to the polypeptide consisting of SEQ ID NO: 2.

2. An antibody according to claim 1, wherein the monoclonal antibody is conjugated to a signal development element.

3. An antibody according to claim 1, wherein the second monoclonal antibody is immobilized on a solid support.

4. An antibody according to one of claims 1-3, wherein the antibody is provided as a reagent in a test kit for the detection of human IGFBP7, the test kit optionally comprising a disposable test device configured to generate a detectable signal related to the present or amount of human IGFBP7 in a biological sample.

5. An antibody according to claim 4, wherein the disposable test device is a lateral flow test device.

6. An antibody pair according to one of claims 4 or 5, wherein the monoclonal antibody is immobilized to a surface within the disposable test device.

7. An antibody according to one of claims 4 or 5, wherein the monoclonal antibody is provided in a separate container from the disposable test device.

8. An antibody according to one of claims 4-7, wherein the test kit comprises two different antibodies according to one of claims 1-3 selected to provide a sandwich antibody pair for the detection of human IGFBP7, the antibody pair consisting of a first monoclonal antibody which specifically binds to a polypeptide consisting of SEQ ID NO: 1, and a second monoclonal antibody which specifically binds to the polypeptide consisting of SEQ ID NO: 2.

9. An antibody according to claim 8, wherein the test kit further comprises a calibration to relate the detectable signal to a concentration of IGFBP7.

10. An antibody according to claim 9, wherein the calibration is a calibration curve provided on an electronic memory device.

11. An antibody according to one of claims 8-10, wherein each of the first and second monoclonal antibody is provided as a reagent in an *in vitro* diagnostic.

12. An antibody according to one of claims 4-11, wherein the kit is configured to perform an assay method which provides a signal related to the present or amount of human IGFBP7 in a biological sample, and wherein the minimum detectable concentration of IGFBP7 in the assay method is 20 ng/mL or less.

13. An antibody according to one of claims 1-12, wherein the antibody is a rabbit, mouse, chicken, goat, sheep, donkey, human, llama or camelid antibody.

14. An antibody according to one of claims 1-12, wherein the antibody is a rabbit antibody.

15. An antibody according to one of claims 1-14, wherein the antibody comprises at least one critical residue, defined as an amino acid of SEQ ID NO: 1 that, when changed to an alanine, reduces binding of the first and second monoclonal antibodies by at least 50% relative to binding to SEQ ID NO: 1, or as an amino acid of SEQ ID NO: 2 that, when changed to an alanine, reduces binding of the first and second monoclonal antibodies by at least 50% relative to binding to SEQ ID NO: 2.

16. An antibody pair according to claim 15, wherein the at least one critical residue is at least one residue in SEQ ID NO: 3 or 4.

17. A method for determining the presence or amount of human IGFBP7 in a biological sample, comprising:

performing an immunoassay on the biological sample with a first monoclonal antibody and a second monoclonal antibody which together form sandwich complexes with human IGFBP7, wherein the first monoclonal antibody specifically binds to a polypeptide consisting of SEQ ID NO: 1, wherein the second monoclonal antibody specifically binds to a polypeptide consisting of SEQ ID NO: 2, and wherein the immunoassay provides a detectable signal that is related to the presence or amount of human IGFBP7 in the biological sample bound in the sandwich complexes; and

relating the detectable signal to the presence or amount of human IGFBP7 in the biological sample.

18. A method according to claim 17, wherein the minimum detectable concentration of IGFBP7 in the immunoassay is 20 ng/mL or less.

19. A method according to one of claims 17 or 18, wherein the immunoassay is performed in a lateral flow format.

20. A method according to claim 17, wherein the immunoassay is an *in vitro* diagnostic.

21. A method according to one of claims 17-20, wherein the immunoassay is performed by applying the human patient sample to a disposable test device, and the detectable signal is obtained by inserting the disposable test device into an analytical instrument, wherein the sandwich complexes comprising the first and second monoclonal antibodies are immobilized for detection in a predetermined zone of the disposable test device, and wherein the analytical instrument detects the immobilized sandwich complexes to provide the detectable signal.

22. A method according to one of claims 17-21, wherein the first or the second monoclonal antibody is conjugated to a signal development element.

23. A method according to claim 22, wherein the first or the second monoclonal antibody that is conjugated to a signal development element forms a reaction mixture with the human patient sample, and the human patient sample is applied to the disposable test device by applying the reaction mixture to the disposable test device.

24. A method according to claim 22 or 23, wherein the first or the second monoclonal antibody that is not conjugated to a signal development element is immobilized at the predetermined zone of a solid support.

25. A method according to one of claims 17-24, wherein each of the first and second monoclonal antibody is independently selected from the group consisting of a rabbit, mouse, chicken, goat, sheep, donkey, human, llama and camelid antibody.

26. A method according to one of claims 17-24, wherein the first and second antibodies are independently a rabbit antibody or a mouse antibody.

27. A method according to one of claims 17-24, wherein the first monoclonal antibody comprises at least one critical residue, defined as an amino acid of SEQ ID NO: 1 that, when changed to an alanine, reduces binding of the first and second monoclonal antibodies by at least 50% relative to binding to SEQ ID NO: 1, and the second monoclonal antibody comprises at least one critical residue, defined as an amino acid of

SEQ ID NO: 2 that, when changed to an alanine, reduces binding of the first and second monoclonal antibodies by at least 50% relative to binding to SEQ ID NO: 2.

28. A method according to claim 25, wherein the at least one critical residue in the first monoclonal antibody is at least one residue in SEQ ID NO: 3 and the at least one critical residue in the second monoclonal antibody is at least one residue in SEQ ID NO: 4.

29. An isolated antibody comprising:

one or both of (i) a light chain variable region having an amino acid sequence of SEQ ID NO: 9 or a sequence at least 90% identical to SEQ ID NO: 9, and (ii) a heavy chain variable region having an amino acid sequence of SEQ ID NO: 10 or a sequence at least 90% identical to SEQ ID NO: 10, wherein the antibody specifically binds human IGFBP7.

30. An antibody according to claim 29, wherein the antibody has a light chain variable region having an amino acid sequence of SEQ ID NO: 9 and a heavy chain variable region having an amino acid sequence of SEQ ID NO: 10.

31. An antibody according to claim 29 or 30, wherein the antibody is a monoclonal antibody.

32. A nucleic acid, the sequence of which encodes:

an antibody comprising one or both of (i) a light chain variable region having an amino acid sequence of SEQ ID NO: 9 or a sequence at least 90% identical to SEQ ID NO: 9, and (ii) a heavy chain variable region having an amino acid sequence of SEQ ID NO: 10 or a sequence at least 90% identical to SEQ ID NO: 10, wherein the antibody specifically binds human IGFBP7.

33. A nucleic acid according to claim 32, wherein the antibody has a light chain variable region having an amino acid sequence of SEQ ID NO: 9 and a heavy chain variable region having an amino acid sequence of SEQ ID NO: 10.

34. An antibody-expressing cell line, the cell line expressing an antibody comprising one or both of (i) a light chain variable region having an amino acid sequence of SEQ ID NO: 9 or a sequence at least 90% identical to SEQ ID NO: 9, and (ii) a heavy chain variable region having an amino acid sequence of SEQ ID NO: 10 or a sequence at least

90% identical to SEQ ID NO: 10, wherein the antibody specifically binds human IGFBP7.

35. A cell line according to claim 34, wherein the antibody has a light chain variable region having an amino acid sequence of SEQ ID NO: 9 and a heavy chain variable region having an amino acid sequence of SEQ ID NO: 10.

36. An isolated antibody comprising:

one or both of (i) a light chain variable region having an amino acid sequence of SEQ ID NO: 11 or a sequence at least 90% identical to SEQ ID NO: 11, and (ii) a heavy chain variable region having an amino acid sequence of SEQ ID NO: 12 or a sequence at least 90% identical to SEQ ID NO: 12, wherein the antibody specifically binds human IGFBP7.

37. An antibody according to claim 29, wherein the antibody has a light chain variable region having an amino acid sequence of SEQ ID NO: 11 and a heavy chain variable region having an amino acid sequence of SEQ ID NO: 12.

38. An antibody according to claim 36 or 37, wherein the antibody is a monoclonal antibody.

39. A nucleic acid, the sequence of which encodes:

an antibody comprising one or both of (i) a light chain variable region having an amino acid sequence of SEQ ID NO: 11 or a sequence at least 90% identical to SEQ ID NO: 11, and (ii) a heavy chain variable region having an amino acid sequence of SEQ ID NO: 12 or a sequence at least 90% identical to SEQ ID NO: 12, wherein the antibody specifically binds human IGFBP7.

40. A nucleic acid according to claim 39, wherein the antibody has a light chain variable region having an amino acid sequence of SEQ ID NO: 11 and a heavy chain variable region having an amino acid sequence of SEQ ID NO: 12.

41. An antibody-expressing cell line, the cell line expressing an antibody comprising one or both of (i) a light chain variable region having an amino acid sequence of SEQ ID NO: 11 or a sequence at least 90% identical to SEQ ID NO: 11, and (ii) a heavy chain variable region having an amino acid sequence of SEQ ID NO: 12 or a sequence at least 90% identical to SEQ ID NO: 12, wherein the antibody specifically binds human IGFBP7.

42. A cell line according to claim 34, wherein the antibody has a light chain variable region having an amino acid sequence of SEQ ID NO: 11 and a heavy chain variable region having an amino acid sequence of SEQ ID NO: 12.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/64327

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68 (2015.01)
CPC - G01N 33/6893, 2800/347, 2333/4745
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)
IPC(8): C07K 16/00; C12P 21/08; A61K 39/00, 39/395; G01N 33/53; C12Q 1/68 (2015.01)
CPC: G01N 33/54353, 33/6893, 2800/347, 2333/4745; C07F 5/025; A61K 38/00, 2039/505; USPC: 530/391.1, 387.1, 386, 380, 350

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)
PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); Google; Google Scholar; PubMed; Dialog ProQuest; monoclonal, aantibody, binds, human, 'Insulin-like growth factor-binding protein 7,' 'IGFBP7'

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 2011/097539 A1 (ASTUTE MEDICAL, INC.) August 11, 2011; abstract; paragraph [0044], [0048], [0059], [0069], [0070], [0076], [0077], [0079], [0126]	1-4, 17, 18, 20 ----- 5/4/1-5/4/3, 19/17, 19/18
Y	US 2006/0263907 A1 (ZWEIG, SE) November 23, 2006; abstract; paragraph [0125]	5/4/1-5/4/3, 19/17, 19/18
X --- A	WO 2012/027723 A1 (STEMCENTRX, INC.) March 01, 2012; paragraph [00170]; Claims 3, 4, 8, 9	29, 31/29, 32, 34 ----- 30, 31/30, 33, 35
A	US 2002/0132983 A1 (JUNGHANS, RP) September 19, 2002; figure 4; page 13	30, 31/30, 33, 35
A	WO 2010/127294 A2 (ABBOTT LABORATORIES) November 04, 2010; abstract; page 15, lines 33-34; page 16, lines 5-11; Claims 1-2	30, 31/30, 33, 35
X — A	WO 2013/067517 A2 (TOLERA THERAPEUTICS, INC.) May 10, 2013; paragraphs [0075], [00104], [00128], [00135], [00136], [00140]; Claim 10	36, 38/36, 39, 41 ----- 37, 38/37, 40, 42
A	WO 2007/008547 A2 (BIOGEN IDEC MA INC.) January 18, 2007; paragraphs [0145], [0228]; Table 8	37, 38/37, 40, 42
A	WO 1992/11383 A1 (CELLTECH LIMITED) July 09, 1992; abstract; page 18, third paragraph; page 20, second paragraph; figure 4	37, 38/37, 40, 42

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"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)
"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
"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
"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
"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
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 March 2015 (26.03.2015)	08 APR 2015
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/64327

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 6-16, 21-28
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

-***-Please See Supplemental Page-***-

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/64327

-***-Continuation of Box No. III - Observations where unity of invention is lacking:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-5, 17-20 and 29-35 are directed toward antibodies which specifically bind human IGFBP7, and polypeptides consisting of SEQ ID NOs: 1 and 2; a method for determining the presence or amount of human IGFBP7 using said antibodies; an isolated antibody comprising a light chain variable region having an amino acid sequence of at least 90% identical to SEQ ID NO: 9, and (ii) a heavy chain variable region having an amino acid sequence at least 90% identical to SEQ ID NO: 10, wherein the antibody specifically binds human IGFBP7, a nucleic acid encoding the antibody, and an antibody-expressing cell line that expresses said antibody.

Group II: Claims 36-42 are directed toward an isolated antibody comprising: one or both of (i) a light chain variable region having an amino acid sequence at least 90% identical to SEQ ID NO: 11, and (ii) a heavy chain variable region having an amino acid at least 90% identical to SEQ ID NO: 12, wherein the antibody specifically binds human IGFBP7, a nucleic acid encoding the antibody, and an antibody-expressing cell line that expresses said antibody.

The inventions listed as Groups I-II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Group I include an isolated antibody comprising a light chain variable region having an amino acid sequence of at least 90% identical to SEQ ID NO: 9, and (ii) a heavy chain variable region having an amino acid sequence at least 90% identical to SEQ ID NO: 10, which are not present in Group II, the special technical features of Group II including an isolated antibody comprising: one or both of (i) a light chain variable region having an amino acid sequence at least 90% identical to SEQ ID NO: 11, and (ii) a heavy chain variable region having an amino acid at least 90% identical to SEQ ID NO: 12, not present in Group I.

Groups I-II share the technical features including antibodies having a light chain variable region and/or a heavy chain variable region, where the antibodies specifically bind human IGFBP7; a nucleic acid, the sequence of which encodes: an antibody comprising one or both of (i) a light chain variable region having an amino acid sequence, and (ii) a heavy chain variable region having an amino acid sequence, wherein the antibody specifically binds human IGFBP7; and an antibody-expressing cell line, the cell line expressing an antibody comprising one or both of (i) a light chain variable region having an amino acid sequence, and (ii) a heavy chain variable region having an amino acid sequence, wherein the antibody specifically binds human IGFBP7.

However, these shared technical features are previously disclosed by US 2012/0045391 A1 to Stanimirovic, et al. (hereinafter 'Stanimirovic'). Stanimirovic discloses antibodies (abstract) having a light chain variable region (paragraphs [0018], [0073], [0074]) and/or a heavy chain variable region (paragraphs [0018], [0073], [0074]), where the antibodies specifically bind human IGFBP7 (where the antibodies bind to IGFBP7 in human brain tumors; paragraph [0031]); a nucleic acid (paragraph [0088]), the sequence of which encodes (paragraph [0088]): an antibody (abstract) comprising one or both of (i) a light chain variable region (paragraphs [0018], [0073], [0074]) having an amino acid sequence (paragraph [0078]), and (ii) a heavy chain variable region (paragraphs [0018], [0073], [0074]) having an amino acid sequence (paragraph [0078]), wherein the antibody specifically binds human IGFBP7 (where the antibodies bind to IGFBP7 in human brain tumors; paragraph [0031]); and an antibody-expressing cell line (E. coli cells transformed with a vector encoding the antibody; paragraph [0113]), the cell line expressing an antibody (abstract) comprising one or both of (i) a light chain variable region (paragraphs [0018], [0073], [0074]) having an amino acid sequence (paragraph [0078]), and (ii) a heavy chain variable region (paragraphs [0018], [0073], [0074]) having an amino acid sequence (paragraph [0078]), wherein the antibody specifically binds human IGFBP7 (where the antibodies bind to IGFBP7 in human brain tumors; paragraph [0031]).

Since none of the special technical features of the Groups I-II inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by the Stanimirovic reference, unity of invention is lacking.

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权利要求书3页 说明书22页

(54)发明名称

以生物样品进行的针对IGFBP7的具有改进的性能的测定

(57)摘要

本发明提供特别是在用于评估肾损伤时具有改进的临床性能的IGFBP7免疫测定。所述免疫测定依赖于对在用于诸如生物流体的复杂临床样本中时,并且特别是在用于诸如侧向流动测试装置的快速测定形式中时展现改进的测定性能的抗体和抗体对的选择和使用。

CN 105793439 A

1.一种特异性结合人IGFBP7的抗体,其选自由以下组成的组:

特异性结合由SEQ ID NO:1组成的多肽的单克隆抗体,和特异性结合由SEQ ID NO:2组成的多肽的单克隆抗体。

2.根据权利要求1所述的抗体,其中所述单克隆抗体缀合于信号显现元件。

3.根据权利要求1所述的抗体,其中第二单克隆抗体固定在固体载体上。

4.根据权利要求1-3中的一项所述的抗体,其中所述抗体作为试剂提供在用于检测人IGFBP7的测试试剂盒中,所述测试试剂盒任选包括被配置来产生与生物样品中人IGFBP7的存在或数量相关的可检测信号的一次性测试装置。

5.根据权利要求4所述的抗体,其中所述一次性测试装置是侧向流动测试装置。

6.根据权利要求4或5中的一项所述的抗体对,其中所述单克隆抗体固定于所述一次性测试装置内的表面。

7.根据权利要求4或5中的一项所述的抗体,其中所述单克隆抗体提供在独立于所述一次性测试装置的容器中。

8.根据权利要求4-7中的一项所述的抗体,其中所述测试试剂盒包括根据权利要求1-3中的一项所述的被选择以提供用于检测人IGFBP7的夹心式抗体对的两种不同抗体,所述抗体对由特异性结合由SEQ ID NO:1组成的多肽的第一单克隆抗体和特异性结合由SEQ ID NO:2组成的多肽的第二单克隆抗体组成。

9.根据权利要求8所述的抗体,其中所述测试试剂盒还包括用以使所述可检测信号与IGFBP7的浓度相关联的校正。

10.根据权利要求9所述的抗体,其中所述校正是提供在电子存储装置上的校正曲线。

11.根据权利要求8-10中的一项所述的抗体,其中所述第一单克隆抗体和所述第二单克隆抗体各自作为试剂提供在体外诊断中。

12.根据权利要求4-11中的一项所述的抗体,其中所述试剂盒被配置来执行提供与生物样品中人IGFBP7的存在或数量相关的信号的测定方法,并且其中所述测定方法中IGFBP7的最小可检测浓度是20ng/mL或小于20ng/mL。

13.根据权利要求1-12中的一项所述的抗体,其中所述抗体是兔、小鼠、鸡、山羊、绵羊、驴、人、美洲驼或骆驼科动物抗体。

14.根据权利要求1-12中的一项所述的抗体,其中所述抗体是兔抗体。

15.根据权利要求1-14中的一项所述的抗体,其中所述抗体包含至少一个关键残基,其被定义为SEQ ID NO:1的在变为丙氨酸时,相对于与SEQ ID NO:1的结合使所述第一单克隆抗体和所述第二单克隆抗体的结合降低至少50%的氨基酸,或被定义为SEQ ID NO:2的在变为丙氨酸时,相对于与SEQ ID NO:2的结合使所述第一单克隆抗体和所述第二单克隆抗体的结合降低至少50%的氨基酸。

16.根据权利要求15所述的抗体对,其中所述至少一个关键残基是SEQ ID NO:3或4中的至少一个残基。

17.一种用于测定生物样品中人IGFBP7的存在或数量的方法,所述方法包括:

用与人IGFBP7一起形成夹心式复合物的第一单克隆抗体和第二单克隆抗体对所述生物样品进行免疫测定,其中所述第一单克隆抗体特异性结合由SEQ ID NO:1组成的多肽,其中所述第二单克隆抗体特异性结合由SEQ ID NO:2组成的多肽,并且其中所述免疫测定提

供与所述生物样品中结合在所述夹心式复合物中的人IGFBP7的存在或数量相关的可检测信号;以及

使所述可检测信号与所述生物样品中人IGFBP7的存在或数量相关联。

18.根据权利要求17所述的方法,其中所述免疫测定中IGFBP7的最小可检测浓度是20ng/mL或小于20ng/mL。

19.根据权利要求17或18中的一项所述的方法,其中所述免疫测定是以侧向流动形式进行。

20.根据权利要求17所述的方法,其中所述免疫测定是体外诊断性的。

21.根据权利要求17-20中的一项所述的方法,其中所述免疫测定是通过将人患者样品施加于一次性测试装置来进行,并且所述可检测信号是通过将所述一次性测试装置插入分析仪器中获得,其中包含所述第一单克隆抗体和所述第二单克隆抗体的所述夹心式复合物被固定以用于在所述一次性测试装置的预定区中检测,并且其中所述分析仪器检测所述固定的夹心式复合物以提供所述可检测信号。

22.根据权利要求17-21中的一项所述的方法,其中所述第一单克隆抗体或所述第二单克隆抗体缀合于信号显现元件。

23.根据权利要求22所述的方法,其中缀合于信号显现元件的所述第一单克隆抗体或所述第二单克隆抗体与所述人患者样品形成反应混合物,并且通过将所述反应混合物施加于所述一次性测试装置来将所述人患者样品施加于所述一次性测试装置。

24.根据权利要求22或23所述的方法,其中未缀合于信号显现元件的所述第一单克隆抗体或所述第二单克隆抗体固定在固体载体的预定区处。

25.根据权利要求17-24中的一项所述的方法,其中所述第一单克隆抗体和所述第二单克隆抗体各自独立地选自由兔、小鼠、鸡、山羊、绵羊、驴、人、美洲驼和骆驼科动物抗体组成的组。

26.根据权利要求17-24中的一项所述的方法,其中所述第一抗体和所述第二抗体独立地是兔抗体或小鼠抗体。

27.根据权利要求17-24中的一项所述的方法,其中所述第一单克隆抗体包含至少一个关键残基,其被定义为SEQ ID NO:1的在变为丙氨酸时,相对于与SEQ ID NO:1的结合使所述第一单克隆抗体和所述第二单克隆抗体的结合降低至少50%的氨基酸,并且所述第二单克隆抗体包含至少一个关键残基,其被定义为SEQ ID NO:2的在变为丙氨酸时,相对于与SEQ ID NO:2的结合使所述第一单克隆抗体和所述第二单克隆抗体的结合降低至少50%的氨基酸。

28.根据权利要求25所述的方法,其中所述第一单克隆抗体中的所述至少一个关键残基是SEQ ID NO:3中的至少一个残基,并且所述第二单克隆抗体中的所述至少一个关键残基是SEQ ID NO:4中的至少一个残基。

29.一种分离的抗体,其包含:

以下中的一个或两个:(i)具有氨基酸序列SEQ ID NO:9或与SEQ ID NO:9至少90%同一的序列的轻链可变区,和(ii)具有氨基酸序列SEQ ID NO:10或与SEQ ID NO:10至少90%同一的序列的重链可变区,其中所述抗体特异性结合人IGFBP7。

30.根据权利要求29所述的抗体,其中所述抗体具有轻链可变区,其具有氨基酸序列

SEQ ID NO:9;和重链可变区,其具有氨基酸序列SEQ ID NO:10。

31.根据权利要求29或30所述的抗体,其中所述抗体是单克隆抗体。

32.一种核酸,其序列编码:

包含以下中的一个或两个的抗体:(i)具有氨基酸序列SEQ ID NO:9或与SEQ ID NO:9至少90%同一的序列的轻链可变区,和(ii)具有氨基酸序列SEQ ID NO:10或与SEQ ID NO:10至少90%同一的序列的重链可变区,其中所述抗体特异性结合人IGFBP7。

33.根据权利要求32所述的核酸,其中所述抗体具有轻链可变区,其具有氨基酸序列SEQ ID NO:9;和重链可变区,其具有氨基酸序列SEQ ID NO:10。

34.一种抗体表达性细胞系,所述细胞系表达包含以下中的一个或两个的抗体:(i)具有氨基酸序列SEQ ID NO:9或与SEQ ID NO:9至少90%同一的序列的轻链可变区,和(ii)具有氨基酸序列SEQ ID NO:10或与SEQ ID NO:10至少90%同一的序列的重链可变区,其中所述抗体特异性结合人IGFBP7。

35.根据权利要求34所述的细胞系,其中所述抗体具有轻链可变区,其具有氨基酸序列SEQ ID NO:9;和重链可变区,其具有氨基酸序列SEQ ID NO:10。

36.一种分离的抗体,其包含:

以下中的一个或两个:(i)具有氨基酸序列SEQ ID NO:11或与SEQ ID NO:11至少90%同一的序列的轻链可变区,和(ii)具有氨基酸序列SEQ ID NO:12或与SEQ ID NO:12至少90%同一的序列的重链可变区,其中所述抗体特异性结合人IGFBP7。

37.根据权利要求29所述的抗体,其中所述抗体具有轻链可变区,其具有氨基酸序列SEQ ID NO:11;和重链可变区,其具有氨基酸序列SEQ ID NO:12。

38.根据权利要求36或37所述的抗体,其中所述抗体是单克隆抗体。

39.一种核酸,其序列编码:

包含以下中的一个或两个的抗体:(i)具有氨基酸序列SEQ ID NO:11或与SEQ ID NO:11至少90%同一的序列的轻链可变区,和(ii)具有氨基酸序列SEQ ID NO:12或与SEQ ID NO:12至少90%同一的序列的重链可变区,其中所述抗体特异性结合人IGFBP7。

40.根据权利要求39所述的核酸,其中所述抗体具有轻链可变区,其具有氨基酸序列SEQ ID NO:11;和重链可变区,其具有氨基酸序列SEQ ID NO:12。

41.一种抗体表达性细胞系,所述细胞系表达包含以下中的一个或两个的抗体:(i)具有氨基酸序列SEQ ID NO:11或与SEQ ID NO:11至少90%同一的序列的轻链可变区,和(ii)具有氨基酸序列SEQ ID NO:12或与SEQ ID NO:12至少90%同一的序列的重链可变区,其中所述抗体特异性结合人IGFBP7。

42.根据权利要求34所述的细胞系,其中所述抗体具有轻链可变区,其具有氨基酸序列SEQ ID NO:11;和重链可变区,其具有氨基酸序列SEQ ID NO:12。

以生物样品进行的针对IGFBP7的具有改进的性能的测定

[0001] 相关申请的交叉引用

[0002] 本申请要求2013年11月6日提交的美国临时申请号61/900,942以及2014年9月23日提交的美国临时申请号62/054,324和2014年10月15日提交的美国临时申请号62/064,380的权益,所述美国临时申请各自据此以包括所有表格、附图和权利要求的方式整体并入本文。

[0003] 背景

[0004] 以下对发明背景的讨论仅被提供以帮助读者了解本发明而非被承认描述或构成本发明的先前技术。

[0005] IGFBP7(人前体Swiss Prot条目Q16270)是一种涉及调控胰岛素样生长因子在组织中的表达,并且调节IGF结合它的受体的分泌蛋白质。据报道它也刺激前列环素产生和细胞粘附。IGFBP7通过IGF非依赖性机理抑制前列腺癌和乳腺癌细胞系的生长和集落形成,此导致细胞周期的G1期延迟和凋亡增加。IGFBP7在广泛范围的正常人组织中表达,并且它通常显示在前列腺、乳腺、结肠和肺来源的癌细胞系中的表达降低。

[0006] 此外,各自据此以引用的方式以包括所有表格、附图和权利要求的方式整体并入本文的WO2011/097539和WO2011/075744描述IGFBP7个别地以及于各组多标志物中用于评估受试者的肾状态的用途。具体地,显示通过免疫测定测量的IGFBP7水平与对肾状态的风险分层、诊断、分级、预测、分类和监测相关联。

[0007] 从诸如免疫测定的特异性结合测定获得的信号是在一种或多种结合物质(例如,抗体)与含有抗体结合所必需的表位的靶标生物分子(即,分析物)和多肽之间形成的复合物的直接结果。免疫测定常常能够“检测”分析物;但因为抗体表位约为8个氨基酸,所以被配置来检测目标标志物的免疫测定也将检测与标志物序列相关的多肽,只要那些多肽含有为结合用于测定中的一种或多种抗体所必需的表位即可。尽管此类测定可检测全长生物标志物,并且测定结果可表示为目标生物标志物的浓度,但由测定获得的信号实际上是样品中存在的所有此类“免疫反应性”多肽的结果。此类结合测定也可检测生物样品中存在的与诸如结合蛋白、受体、肝素、脂质、糖等的其它物质复合的免疫反应性多肽,前提是那些其它物质不干扰结合物质与靶标生物分子之间的结合。通常,然而,特异性结合测定是使用纯化的分析物制定,并且未考虑复合物形成和片段化样式。这在此类其它结合物质的身份未知时尤其确切。

[0008] 发明概述

[0009] 本发明的一个目标在于提供特别是在用于评估肾损伤时具有改进的临床性能的IGFBP7免疫测定。具体来说,我们描述对在用于诸如生物流体的复杂临床样本中时,并且特别是在用于快速测定形式中时展现改进的测定性能的抗体和抗体对的选择和使用。

[0010] 在第一方面,本发明涉及一种特异性结合人IGFBP7,并且适用于夹心式免疫测定中的单克隆抗体。所述抗体特异性结合由LIWNKVKRGHYGVQRTELLPGDRDNL(SEQ ID NO:1)或SSSSSDTCGPCEPASCPPLP(SEQ ID NO:2)组成的多肽。

[0011] 在一相关方面,本发明涉及一种特异性结合人IGFBP7,并且适用于夹心式免疫测

定中的抗体对，所述抗体对包含特异性结合由LIWNKVKRGHYGVQRTELLPGDRDNL(SEQ ID NO:1)组成的多肽的第一单克隆抗体和特异性结合由SSSSSDTCGPCEPASCPPLP(SEQ ID NO:2)组成的多肽的第二单克隆抗体。

[0012] 在另一相关方面，本发明涉及一种特异性结合人IGFBP7，并且适用于夹心式免疫测定中的单克隆抗体。所述抗体特异性结合IGFBP7的构象表位。构象表位由IGFBP7蛋白中在序列上不连续，但在三维空间中靠近在一起的残基形成。以下描述此类抗体的实例，被称为1D6。

[0013] 在一相关方面，本发明涉及一种特异性结合人IGFBP7，并且适用于夹心式免疫测定中的抗体对，所述抗体对包含特异性结合IGFBP7的构象表位的第一单克隆抗体，和特异性结合由LIWNKVKRGHYGVQRTELLPGDRDNL(SEQ ID NO:1)或SSSSSDTCGPCEPASCPPLP(SEQ ID NO:2)组成的多肽的第二单克隆抗体。

[0014] 在某些实施方案中，本发明的抗体包含以下中的一个或两个：(i)具有氨基酸序列SEQ ID NO:9或与SEQ ID NO:9至少90％同一的序列的轻链可变区，和(ii)具有氨基酸序列SEQ ID NO:10或与SEQ ID NO:10至少90％同一的序列的重链可变区，其中所述抗体特异性结合人IGFBP7。在优选实施方案中，抗体是在本文中被称为IC9E4.1的抗体。

[0015] 在其它实施方案中，本发明的抗体包含以下中的一个或两个：(i)具有氨基酸序列SEQ ID NO:11或与SEQ ID NO:11至少90％同一的序列的轻链可变区，和(ii)具有氨基酸序列SEQ ID NO:12或与SEQ ID NO:12至少90％同一的序列的重链可变区，其中所述抗体特异性结合人IGFBP7。在优选实施方案中，抗体是在本文中被称为1D6的抗体。

[0016] 在某些实施方案中，本发明的抗体对包含(i)第一抗体，其包含以下中的一个或两个：(i)具有氨基酸序列SEQ ID NO:11或与SEQ ID NO:11至少90％同一的序列的轻链可变区，和(ii)具有氨基酸序列SEQ ID NO:12或与SEQ ID NO:12至少90％同一的序列的重链可变区，其中所述抗体特异性结合人IGFBP7；和(ii)第二抗体，其包含以下中的一个或两个：(i)具有氨基酸序列SEQ ID NO:9或与SEQ ID NO:9至少90％同一的序列的轻链可变区，和(ii)具有氨基酸序列SEQ ID NO:10或与SEQ ID NO:10至少90％同一的序列的重链可变区，其中所述抗体特异性结合人IGFBP7。在优选实施方案中，所述抗体对包含在本文中称为1D6的第一抗体和在本文中称为IC9E4.1的第二抗体。

[0017] 如本文所用的短语“特异性结合由特定序列组成的多肽”不旨在意指抗体不结合包含所述序列的更长多肽或是所述序列的子组的更短多肽。相反，这个短语仅旨在意指抗体将结合特定所述多肽。

[0018] 用于要求保护的方法中的抗体可从多种物种获得。举例来说，本发明的抗体可包含是兔、小鼠、大鼠、豚鼠、鸡、山羊、绵羊、驴、人、美洲驼或骆驼科动物序列、或此类序列的组合(所谓嵌合抗体)的免疫球蛋白序列。用于本发明中的抗体可通过它们在免疫测定中的性能来鉴定，接着通过表位作图来进一步表征以了解与该性能相关的表位。

[0019] 表位通常由各组化学活性表面分子，诸如氨基酸或糖侧链组成，并且通常具有特定三维结构特征以及特定电荷特征。构象表位和非构象表位的区别在于在变性溶剂存在下，与前者而非与后者的结合丧失。优选地，供各抗体用的表位含在作为从人IGFBP7序列获得的序列的SEQ ID NO:1或SEQ ID NO:2内。在某些实施方案中，第一单克隆抗体包含至少1个，并且优选2、3或4个用于结合IGFBP7的连续“关键残基”。“关键残基”定义为SEQ ID NO:1

(或SEQ ID NO:2)的在变为丙氨酸时,相对于抗体与SEQ ID NO:1(或SEQ ID NO:2)自身的结合使抗体的结合降低至少50%,并且更优选至少75%的氨基酸。在优选实施方案中,至少一个关键残基是序列TELLPGDRD(SEQ ID NO:3)中的至少一个残基,或序列EPASC(SEQ ID NO:4)中的至少一个残基。

[0020] 可使此类单克隆抗体缀合于信号显现元件或固定在固体载体上。在夹心式测定的实例中,第一抗体(被可检测标记)和第二抗体(固定在测试装置的预定区处)与样品中的IGFBP7在测试装置的预定区处形成夹心式复合物。在夹心式测定中,第一和第二抗体可相同(特别是当使用多克隆抗体时)或不同。因此,本发明的抗体可以夹心对形式使用,或可个别地与不是单克隆抗体的另一结合实体(诸如多克隆抗体或适体)一起使用。

[0021] 本发明的抗体可用作用于检测生物样品中的IGFBP7的测试试剂盒中的试剂。此类测试试剂盒可例如包括被配置来产生与生物样品中人IGFBP7的存在或数量相关的可检测信号的一次性测试装置。或者,此类测试试剂盒可被制定以在不利用一次性测试装置的临床分析器中进行测定。优选地,测试试剂盒是体外诊断性的。如本文所用的术语"体外诊断性的"是指是试剂、试剂产品、校准物、对照物质、试剂盒、仪器、器具、设备或系统的医学装置,无论单独或组合使用都由制造商意图在体外用于检查源于人体的样本,包括血液和组织捐献物,以仅仅或主要达成提供关于生理或病理状态或关于先天性异常的信息的目的,或确定安全性和与潜在接受者的相容性,或监测治疗措施。

[0022] 在某些实施方案中,以侧向流动形式进行免疫测定。侧向流动测试是一种免疫测定形式,其中测试样品以色谱方式沿吸湿性或非吸湿性多孔固体基材流动。侧向流动测试可以竞争性或夹心形式测定来操作。优选侧向流动装置是一次性单次使用测试装置。将样品施加在测试装置的施加区,并且穿过基材,在所述基材处它遇到已用抗体或抗原预处理的管线或区域。如本文所用的术语"测试区"是指侧向流动测试条带上的分立位置,其被探询以产生与目标分析物的存在或数量相关的信号。可检测信号可目视读取或通过将一次性测试装置插入诸如反射计、荧光计或透射光度计的分析仪器中获得。这个清单不意图是限制性的。可在不进行预处理下将样品施加于施加区,或可在施加之前与一种或多种测定试剂预混合。在后述情况下,抗体可提供在独立于一次性测试装置的容器中。

[0023] 本发明的抗体可散布性固定于一次性测试装置内的表面,以使所述抗体在样品接触所述表面时溶入所述样品中。在夹心式测定形式中,这个散布性结合的抗体可结合样品中它的同源抗原,接着在抗原由非散布性地结合在检测区处的第二抗体结合时固定在所述检测区处。在竞争性形式中,样品中它的同源抗原可与作为测定试剂提供的标记的抗原竞争结合散布性结合的抗体。

[0024] 本发明的试剂盒还可包括用以使可检测信号与IGFBP7的浓度相关联的校正。举例来说,校正曲线可提供在由接受一次性测试装置的分析仪器读取的电子存储装置(诸如ROM芯片、闪存驱动器、RFID标签等)上。或者,校正曲线可提供在光学读取的编码标签(诸如2维条形码)上,或通过网络连接来传送。分析仪器可接着使用这个校正曲线来将由测定获得的可检测信号关联成IGFBP7浓度。

[0025] 在某一实施方案中,使用本发明的抗体对执行的测定方法提供与生物样品中人IGFBP7的存在或数量相关的信号,其中所述测定方法中IGFBP7的最小可检测浓度是20ng/mL或小于20ng/mL,更优选是10ng/mL或小于10ng/mL、5ng/mL或小于5ng/mL、1ng/mL或小于

1ng/mL,并且最优选是0.1ng/mL或小于0.1ng/mL。

[0026] 在相关方面,本发明提供用于测定生物样品中人IGFBP7的存在或数量的方法,所述方法包括:

[0027] 用与人IGFBP7一起形成夹心式复合物的第一单克隆抗体和第二单克隆抗体对所述生物样品进行免疫测定,其中所述免疫测定提供与所述生物样品中结合在所述夹心式复合物中的人IGFBP7的存在或数量相关的可检测信号;以及

[0028] 使所述可检测信号与所述生物样品中人IGFBP7的存在或数量相关联。优选地,免疫测定中IGFBP7的最小可检测浓度是20ng/mL或小于20ng/mL,更优选是10ng/mL或小于10ng/mL、5ng/mL或小于5ng/mL、1ng/mL或小于1ng/mL,并且最优选是0.1ng/mL或小于0.1ng/mL。

[0029] 在特别优选的实施方案中,第一单克隆抗体结合由SEQ ID NO:1组成的多肽,并且第二单克隆抗体结合由SEQ ID NO:2组成的多肽,在各情况下亲和力都是至少10^8M^{-1}。

[0030] 优选测定方法包括进行检测人IGFBP7的免疫测定。此类免疫测定可包括使所述体液样品与检测标志物的抗体接触,以及检测与该抗体的结合。优选地,体液样品选自由尿、唾液、血液、血清和血浆组成的组,并且最优选是尿。

[0031] 关于本发明的抗体,本发明也在其它方面涉及编码此类抗体的核酸和表达此类抗体的抗体表达性细胞系。

[0032] 本公开的一个或多个实施方案的细节阐述于附图和以下描述中。本公开的其它特征、目标和优势将根据描述和附图以及根据权利要求而显而易知。

[0033] 发明详述

[0034] 定义

[0035] 如本文所用,术语“胰岛素样生长因子结合蛋白7”和“IGFBP7”是指一种或多种存在于生物样品中的源于胰岛素样生长因子结合蛋白7前体(Swiss-Prot Q16270(SEQ ID NO:5))的多肽

[0036]
```
          10         20         30         40         50         60
MERPSLRALL LGAAGLLLLL LPLSSSSSSD TCGPCEPASC PPLPPLGCLL GETRDACGCC

          70         80         90        100        110        120
PMCARGEGEP CGGGGAGRGY CAPGMECVKS RKRRKGKAGA AAGGPGVSGV CVCKSRYPVC

         130        140        150        160        170        180
GSDGTTYPSG CQLRAASQRA ESRGEKAITQ VSKGTCEQGP SIVTPPKDIW NVTGAQVYLS

         190        200        210        220        230        240
CEVIGIPTPV LIWNKVKRGH YGVQRTELLP GDRDNLAIQT RGGPEKHEVT GWVLVSPLSK

         250        260        270        280
EDAGEYECHA SNSQGQASAS AKITVVDALH EIPVKKGEGA EL
```

[0037] 以下结构域已在胰岛素样生长因子结合蛋白7中加以鉴定:

[0038] 残基 长度 结构域身份

[0039] 1-26 26 信号肽

[0040] 27-282 256 胰岛素样生长因子结合蛋白7

[0041] 除非本文另外明确指示,否则所用术语的定义是用于药物科学领域中的标准定义。除非上下文另外明确规定,否则如说明书和随附权利要求中所用,单数形式“一个/一种

(a/an)”和“该/所述”包括复数个指示物。因此，举例来说，提及“一种药物载体”包括两种或更多种此类载体的混合物等。

[0042] 如本文所用的术语“受试者”是指人或非人生物体。因此，本文所述的方法和组合物可适用于人疾病与兽医学疾病两者。此外，尽管受试者优选是活生物体，但本文所述的本发明也可用于死后分析中。优选受试者是人，并且最优选是“患者”，其如本文所用是指接受针对疾病或病状的医学护理的活人。这包括无确定疾病，正被探究病理征象的人士。

[0043] 优选地，测量样品中的分析物。此类样品可从受试者获得，或可从意图向受试者提供的生物材料获得。举例来说，样品可从被评估以达成可能向受试者中移植的肾获得，并且分析物测量结果用于评估肾的先前存在的损害。优选样品是体液样品。

[0044] 如本文所用的术语“体液样品”是指出于诊断、预测、分类或评估目标受试者(诸如患者或移植供体)的目的而获得的体液样品。在某些实施方案中，此类样品可出于确定正在进行的病状的后果或治疗方案对病状的作用的目的而获得。优选体液样品包括血液、血清、血浆、脑脊髓液、尿、唾液、痰和胸腔积液。此外，本领域技术人员将认识到某些体液样品将在分级分离或纯化程序之后更易于分析，所述程序例如将全血分成血清或血浆组分。

[0045] 如本文所用的术语“诊断”是指熟练技术人员可估计和/或确定患者是否正罹患给定疾病或病状的概率(“可能性”)所采用的方法。在本发明的情况下，“诊断”包括使用针对本发明的肾损伤标志物的测定(最优选是免疫测定)的结果，任选与其它临床特征一起，以达成对从其获得样品并加以测定的受试者的急性肾损伤或ARF的诊断(也就是发生或不发生)。此类诊断被“确定”不意图暗示诊断是100％准确的。许多生物标志物指示多种病状。熟练临床医师在信息空白的情况下不使用生物标志物结果，而是测试结果连同其它临床征候一起被用于达成诊断。因此，相对于在预定诊断阈值的另一侧的测量水平，在预定诊断阈值的一侧的测量的生物标志物水平指示在受试者中发生疾病的可能性更大。

[0046] 类似地，预后风险预示给定过程或后果将发生的概率(“可能性”)。转而与发病(例如肾功能恶化、未来ARF、或死亡)概率增加相关联的预后指标的水平或水平变化被认为是“指示患者中不利后果的可能性增加”。

[0047] 如本文所用的术语“侧向流动”是指试剂在纵向上流过基本上扁平的多孔材料。如果材料的厚度是长度和宽度尺寸的至多10％，那么此类多孔材料是“基本上扁平的”。

[0048] 如本文相对于装置的第一区域所用的术语“下游区域”是指在流体已到达所述第一区域之后接受该流体流的区域。

[0049] 如本文所用的术语“样品施加区”是指测定装置的向其中引入目标流体样品以达成测定其组分的目的的部分。

[0050] 标志物测定

[0051] 一般来说，免疫测定涉及使含有或怀疑含有目标生物标志物的样品与至少一种特异性结合所述生物标志物的抗体接触。接着产生信号，其指示通过样品中多肽与抗体结合形成的复合物的存在或数量。接着使信号与样品中生物标志物的存在或数量相关联。用于检测和分析生物标志物的众多方法和装置为熟练技术人员所熟知。参见例如美国专利6,143,576;6,113,855;6,019,944;5,985,579;5,947,124;5,939,272;5,922,615;5,885,527;5,851,776;5,824,799;5,679,526;5,525,524;和5,480,792,以及The Immunoassay Handbook,David Wild编Stockton Press,New York,1994,其各自据此以引用的方式以包

括所有表格、附图和权利要求的方式整体并入本文。

[0052] 本领域中已知的测定装置和方法可在各种夹心式、竞争性或非竞争性测定形式中利用标记的分子以产生与目标生物标志物的存在或数量相关的信号。合适的测定形式也包括色谱、质谱和蛋白质“印迹”方法。另外，诸如生物传感器和光学免疫测定的某些方法和装置可用于确定分析物的存在或数量而无需标记的分子。参见例如美国专利5,631,171；和5,955,377，其各自据此以引用的方式以包括所有表格、附图和权利要求的方式整体并入本文。本领域技术人员也认识到包括但不限于Beckman ACCESS®、Abbott AXSYM®、Roche ELECSYS®、Dade Behring STRATUS® 系统的机器人仪器在能够进行免疫测定的免疫测定分析器之中。但可利用任何合适的免疫测定，例如酶联免疫测定(ELISA)、放射免疫测定(RIA)、竞争性结合测定等。

[0053] 抗体或其它多肽可固定于多种固体载体上以用于测定中。可用于使特异性结合成员固定的固相包括在固相结合测定中以固相形式开发和/或使用的那些。合适的固相的实例包括膜过滤器、基于纤维素的纸、珠粒(包括聚合、乳胶和顺磁性粒子)、玻璃、硅片、微粒、纳米粒子、TentaGels、AgroGels、PEGA凝胶、SPOCC凝胶和多孔板。测定条带可通过于固体载体上以阵列形式涂布一种或多种抗体来制备。这个条带可接着浸渍至测试样品中，接着通过洗涤和检测步骤快速处理以产生可测量信号，诸如色斑。可通过直接缀合于测定装置表面，或通过间接结合来使抗体或其它多肽结合于测定装置的特定区域。在后述情况的实例中，抗体或其它多肽可固定在粒子或其它固体载体上，并且将该固体载体固定于装置表面。

[0054] 生物测定需要检测方法，并且一种最常见的结果定量方法是使可检测标记缀合于对所研究的生物系统中的一种组分具有亲和力的蛋白质或核酸。可检测标记可包括自身可检测的分子(例如荧光部分、电化学标记、金属螯合物等)以及可通过产生可检测反应产物来间接检测的分子(例如酶，诸如辣根过氧化物酶、碱性磷酸酶等)或通过自身可检测的特异性结合分子来间接检测的分子(例如生物素、地高辛、麦芽糖、寡聚组氨酸、2,4-二硝基苯、苯基砷酸盐、ssDNA、dsDNA等)。

[0055] 制备固相和可检测标记缀合物常常包括使用化学交联剂。交联试剂含有至少两个反应性基团，并且通常被分为同官能性交联剂(含有相同反应性基团)和异官能性交联剂(含有不相同反应性基团)。通过胺、硫氢基偶联，或非特异性反应的同双官能性交联剂可从许多商业来源获得。马来酰亚胺、烷基和芳基卤化物、α-卤代酰基和吡啶基二硫化物是硫醇反应性基团。马来酰亚胺、烷基和芳基卤化物以及α-卤代酰基与硫氢基反应以形成硫醇醚键，而吡啶基二硫化物与硫氢基反应以产生混合的二硫化物。吡啶基二硫化物产物是可裂解的。亚胺酯也极其适用于蛋白质-蛋白质交联。各自组合不同属性以达成成功缀合的多种异双官能性交联剂可商购获得。

[0056] 在某些方面，本发明提供用于分析所述标志物的试剂盒。试剂盒包括用于分析至少一种测试样品的试剂，其包括至少一种特异性结合标志物的抗体。试剂盒也可包括用于进行一种或多种本文所述的诊断和/或预后关联的装置和说明书。优选试剂盒将包括用于进行针对分析物的夹心式测定的抗体对，或用于进行针对分析物的竞争性测定的标记的物质。优选地，抗体对包含缀合于固相的第一抗体和缀合于可检测标记的第二抗体，其中所述第一和第二抗体各自结合肾损伤标志物。最优选地，各抗体是单克隆抗体。用于使用试剂盒以及进行关联的说明书可呈标签形式，所述标签是指在试剂盒的制造、运输、销售或使用期

间的任何时间附着于或另外随附于试剂盒的任何书面或记录材料。举例来说，术语标签涵盖宣传散页和小册、包装材料、说明书、音频或视频卡带、计算机磁盘以及直接书面压印在试剂盒上。

[0057] 在某些实施方案中，使用单次使用的一次性测试装置进行标志物测定。此类测试装置常常采用侧向流动装置形式，所述装置目前由于普遍使用非处方妊娠测试而熟知。通常，这些测定装置具有延长的基底层，在所述基底层上可在样品添加区与评估区之间进行区分。在典型使用中，将样品施加于样品添加区，沿平行于基底层延伸的液体运输路径流动，接着流入评估区中。捕获试剂存在于评估区中，并且可通过用以检测与捕获的分析物缔合的可见部分的多种方案来检测捕获的分析物。举例来说，测定可产生视觉信号，诸如变色、荧光、发光等，此时指示生物样品中存在或不存在分析物。

[0058] 样品添加区可例如以壳体中的开放腔室形式；以吸附垫形式；等提供。样品添加区可为是圆形、长方形、正方形等的各种构型的入口，或所述区域可为装置中的凹槽。

[0059] 过滤器元件可于样品添加区中，于样品添加区上，或邻近于样品添加区放置以从样品过滤颗粒，诸如以从血液移除或阻滞血细胞以使血浆可进一步穿过装置。滤液可接着移动至与过滤器流体连通的多孔构件中。用于移除或阻滞存在于血液中的细胞物质的合适的过滤器在本领域中是熟知的。参见例如美国专利4,477,575；5,166,051；6,391,265；和7,125,493，其各自据此以引用的方式整体并入本文。许多合适的材料为熟练技术人员所知，并且可包括玻璃纤维、合成树脂纤维、各种类型的膜(包括其中孔径从约65至约15μm变化的不对称膜过滤器)以及此类材料的组合。此外，过滤器元件可包括一种或多种化学物质以促进红血细胞与血浆分离。此类化学物质的实例是凝血酶、凝集素、阳离子聚合物、针对一种或多种红血细胞表面抗原的抗体等。促进红血细胞与血浆分离的此类化学物质可通过共价手段、非特异性吸附等提供于过滤器元件中。

[0060] 在某些实施方案中，标记区位于样品接受区的下游，并且含有散布性定位的结合目标分析物或与目标分析物竞争与结合物质结合的标记的试剂。或者，如果使标记的试剂与在向样品接受区施加之前的样品预混合，那么可除去标记区。检测区安置在标记区的下游，并且含有结合目标分析物的固定的捕获试剂。

[0061] 用于本发明中的膜的最优孔直径是约10至约50μm。膜的厚度通常是约1密耳至约15密耳，通常在5或10密耳的范围内，但可多达200密耳以及更厚。膜可背衬有通常水不渗透层，诸如Mylar®聚酯薄膜(DuPont Teijin Films)。当采用时，通常通过粘合剂，诸如3M 444双侧粘合带使背衬紧固于膜。通常，水不渗透背衬用于低厚度膜。可使用广泛多种聚合物，前提是它们不与测定组分非特异性结合，并且不干扰样品的流动。说明性聚合物包括聚乙烯、聚丙烯、聚苯乙烯等。或者，膜可为自行支撑性的。也可使用其它非吸湿性膜，诸如聚氯乙烯、聚乙酸乙烯酯、乙酸乙烯酯和氯乙烯的共聚物、聚酰胺、聚碳酸酯、聚苯乙烯等。在各种实施方案中，标记区材料可用包括封闭剂和稳定剂的溶液预处理。封闭剂包括牛血清白蛋白(BSA)、甲基化BSA、酪蛋白、脱脂奶粉。装置也可包括其它组分，包括例如缓冲剂、HAMA抑制剂、清洁剂、盐(例如钙、镁、钾等的氯化物和/或硫酸盐)和蛋白质组分(例如血清白蛋白、明胶、乳蛋白等)。这个清单不意图是限制性的。

[0062] 装置还可包括各种对照位置，其被读取以确定测试装置已被适当操作。举例来说，可提供与测定检测区分开的程序对照区以验证样品流动如所预期。对照区优选是在空间上

不同的区域，在所述区域处可产生指示适当试剂流动的信号。程序对照区可含有用于分析物测定中的过量标记的抗体可结合的目标分析物或其片段。在操作中，标记的试剂结合对照区，即使当目标分析物不存在于测试样品中时也是如此。使用对照线是有帮助的，因为在对照线中出现信号指示可读取测试结果所处的时间，即使对于阴性结果也是如此。因此，当预期信号出现在对照线中时，可指示捕获区中存在或不存在信号。装置还可包括阴性对照区。这个对照区的目的在于警告使用者测试装置未正常工作。当正常工作时，应无信号或标志可见于阴性对照区中。

[0063] 此类测定装置的外部罩壳或壳体可采用各种形式。通常，它将包括伸长的罩壳，并且可具有多个互相配合的部件。在一个特别优选的实施方案中，壳体包括顶盖和底部支撑物。顶盖含有施加孔口和观察口。在一个优选实施方案中，壳体由例如塑料材料的水分不渗透固体材料制得。预期多种可商购获得的塑料，包括但不限于聚乙烯、尼龙、聚氯乙烯、聚丙烯、聚苯乙烯、聚乙烯、聚碳酸酯、聚硫烷、聚酯、聚氨酯和环氧树脂可用于构建壳体。壳体可通过常规方法，诸如本领域中熟知并使用的标准模制技术来制备。壳体可通过模制技术产生，所述技术包括但不限于注射模制、压缩模制、转移模制、吹气模制、挤压模制、泡沫模制和热成形模制。以上提及的模制技术在本领域中是熟知的，并且因此不在本文中详细讨论。参见例如Processes And Materials Of Manufacture,第3版,R.A.Lindsberg(1983)Allyn and Baron第393-431页。

[0064] 必要时，可接着用适合于标记的仪器评估所采用的可检测标记的比色、发光或荧光强度。举例来说，荧光计可用于检测荧光标记；反射计可用于检测吸光标记等。可通过使测量的响应关联于样品流体中分析物的量来确定样品中目标分析物的浓度。

[0065] 测定关联

[0066] 如本文关于在测定中测量生物标志物所用的术语“关联”和“相关”是指基于从所述测定获得的信号来确定样品中生物标志物的存在，或更优选是数量。常常，这采用将由参与测定的一种物质上的可检测标记产生的信号与可用于将所述信号换算成生物标志物的浓度或阈值量的预定标准曲线进行比较的形式。

[0067] 如本文关于使用生物标志物进行诊断或预测所用的术语“关联”和“相关”是指将患者中生物标志物的存在或数量与它在已知罹患给定病状或已知处于给定病状的风险下的人士中；或在已知无给定病状的人士中的存在或数量进行比较。常常，这采用将呈生物标志物浓度形式的测定结果与被选择以指示疾病的发生或不发生或某一未来后果的可能性的预定阈值进行比较的形式。

[0068] 选择诊断阈值尤其涉及考虑疾病的概率，在不同测试阈值下真诊断和假诊断的分布，以及基于诊断估计治疗(或未能治疗)的结果。举例来说，当考虑施用具有高度有效性，并且具有低风险水平的特定疗法时，需要的测试是少许的，因为临床医师可接受实质性诊断不确定性。另一方面，在其中治疗选项的有效性较小并且更具风险的情况下，临床医师常常需要较高程度的诊断确定性。因此，成本/效益分析涉及选择诊断阈值。

[0069] 可以多种方式确定合适的阈值。举例来说，一种推荐用于使用心脏肌钙蛋白诊断急性心肌梗塞的诊断阈值是见于正常群体中的浓度的第97.5百分位数。另一方法可在于考察来自同一患者的连续样品，其中先前“基线”结果用于监测生物标志物水平的时序变化。

[0070] 群体研究也可用于选择判定阈值。接受者操作特征(“ROC”)产生于在二次世界大

战期间被产生用于分析雷达图像的信号检测理论的领域，并且ROC分析常常用于选择能够最佳区分“患病”子群体与“非患病”子群体的阈值。当人测试阳性，但实际上不患有疾病时，出现在这个情况下的假阳性。另一方面，当人测试阴性，从而表明他们是健康的，而此时他们实际上确实患有疾病时，出现假阴性。为绘制ROC曲线，随着判定阈值连续变化确定真阳性率(TPR)和假阳性率(FPR)。因为TPR与灵敏性等效，并且FPR等同于1-特异性，所以ROC图有时称为灵敏性对(1-特异性)图。完美测试将具有ROC曲线下面积1.0；随机测试将具有面积0.5。选择阈值以提供可接受水平的特异性和灵敏性。

[0071] 在这个情形下，“患病”意指群体具有一种特征(存在疾病或病状或发生某一后果)，并且“非患病”意指群体缺乏所述特征。尽管单一判定阈值是此类方法的最简单应用，但可使用多个判定阈值。举例来说，在第一阈值以下，可以相对较高置信度指定不存在疾病，并且在第二阈值以上，也可以相对较高置信度指定存在疾病。在两个阈值之间可被视为不确定。这实际上仅意图是示例性的。

[0072] 除阈值比较之外，用于使测定结果关联于患者分类(发生或不发生疾病、某一后果的可能性等)的其它方法包括决策树、规则集、贝叶斯方法(Bayesian method)和神经网络方法。这些方法可产生代表受试者属于多个分类中的一个分类所处的程度的概率值。

[0073] 测试准确性的量度可如Fischer等，Intensive Care Med.29:1043-51，2003中所述获得，并且用于确定给定生物标志物的有效性。这些量度包括灵敏性和特异性、预测值、似然比、诊断优势比和ROC曲线面积。ROC图的曲线下面积(“AUC”)等同于分类器对随机选择的阳性实例的分级将高于随机选择的阴性实例的概率。ROC曲线下面积可被认为等效于如果考虑的两个组具有连续数据，那么检验在所述组中获得的评分之间的中值差异的曼-惠特尼U检验(Mann-Whitney U test)，或等效于威尔卡森秩检验(Wilcoxon test of rank)。

[0074] 如上所讨论，合适的测试可展现关于这些各种量度的以下结果中的一个或多个：特异性大于0.5，优选是至少0.6，更优选是至少0.7，更优选是至少0.8，甚至更优选是至少0.9，并且最优选是至少0.95，伴有相应灵敏性大于0.2，优选大于0.3，更优选大于0.4，更优选是至少0.5，甚至更优选是0.6，更优选大于0.7，更优选大于0.8，更优选大于0.9，并且最优选大于0.95；灵敏性大于0.5，优选是至少0.6，更优选是至少0.7，更优选是至少0.8，甚至更优选是至少0.9，并且最优选是至少0.95，伴有相应特异性大于0.2，优选大于0.3，更优选大于0.4，更优选是至少0.5，甚至更优选是0.6，更优选大于0.7，更优选大于0.8，更优选大于0.9，并且最优选大于0.95；至少75％灵敏性与至少75％特异性组合；ROC曲线面积大于0.5，优选是至少0.6，更优选是0.7，更优选是至少0.8，甚至更优选是至少0.9，并且最优选是至少0.95；优势比不同于1，优选是至少约2或大于2或约0.5或小于0.5，更优选是至少约3或大于3或约0.33或小于0.33，更优选是至少约4或大于4或约0.25或小于0.25，甚至更优选是至少约5或大于5或约0.2或小于0.2，并且最优选是至少约10或大于10或约0.1或小于0.1；阳性似然比(计算为灵敏性/(1-特异性))大于1，是至少2，更优选是至少3，更优选是至少5，并且最优选是至少10；和或阴性似然比(计算为(1-灵敏性)/特异性)小于1，小于或等于0.5，更优选小于或等于0.3，并且最优选小于或等于0.1。

[0075] 抗体

[0076] 如本文所用的术语“抗体”是指由一种或多种免疫球蛋白基因或其片段获得，以所述免疫球蛋白基因或其片段为模型，或基本上由所述免疫球蛋白基因或其片段编码，能够

特异性结合抗原或表位的肽或多肽。参见例如Fundamental Immunology,第3版,W.E.Paul编,Raven Press,N.Y.(1993);Wilson(1994;J.Immunol.Methods 175:267-273;Yarmush(1992)J.Biochem.Biophys.Methods 25:85-97。术语抗体包括抗原结合部分,即保留结合抗原的能力的"抗原结合位点"(例如片段、子序列、互补决定区(CDR)),包括(i)Fab片段,即由VL、VH、CL和CH1结构域组成的单价片段;(ii)F(ab')2片段,即包含两个由在铰链区处的二硫桥连接的Fab片段的二价片段;(iii)由VH和CH1结构域组成的Fd片段;(iv)由抗体的单臂的VL和VH结构域组成的Fv片段,(v)dAb片段(Ward等,(1989)Nature 341:544-546),其由VH结构域组成;以及(vi)分离的互补决定区(CDR)。单链抗体也以引用的方式包括在术语"抗体"中。

[0077] 如本文所用,"抗体可变结构域"是指抗体分子的轻链和重链的包括互补决定区(CDR;即CDR1、CDR2和CDR3)和框架区(FR)的氨基酸序列的部分。V_H是指重链的可变结构域。V_L是指轻链的可变结构域。根据用于本发明中的方法,对CDR和FR指定的氨基酸位置可根据Kabat(Sequences of Proteins of Immunological Interest(National Institutes of Health,Bethesda,Md.,1987和1991))来确定。抗体或抗原结合片段的氨基酸编号也根据Kabat的编号。

[0078] "分离的"抗体是已被鉴定并与它的天然环境的组分分离和/或从所述组分回收的抗体。它的天然环境的污染物组分是将干扰抗体的诊断或治疗用途的物质,并且可包括酶、激素和其它蛋白质或非蛋白质溶质。在优选实施方案中,抗体将被纯化(1)以获得大于95重量%的抗体,如通过洛利(Lowry)方法所测定,并且最优选超过99重量%,(2)以达到足以通过使用旋转杯测序仪获得N末端或内部氨基酸序列的至少15个残基的程度,或(3)以达到使用考马斯(Coomassie)蓝或优选银染色在还原性或非还原性条件下,通过SDS-PAGE确定的均质性。分离的抗体包括重组细胞内的原位抗体,因为将不存在抗体的天然环境的至少一种组分。通常,然而,分离的抗体将通过至少一个纯化步骤来制备。

[0079] 通常,抗体可包含重链和/或轻链可变结构域,其包含与具有已知结合特征的亲本抗体的重链或轻链可变结构域的氨基酸序列具有至少75%,更优选至少80%,更优选至少85%,更优选至少90%,并且最优选至少95%氨基酸序列同一性或类似性的氨基酸序列。关于这个序列的同一性或类似性在本文中定义为在比对序列以及必要时引入空位以获得最大序列同一性百分比之后,候选序列中与物种依赖性抗体残基同一(即相同残基)或类似(即来自基于共同侧链性质的同一组的氨基酸残基)的氨基酸残基的百分比。在可变结构域外部对抗体序列的N末端、C末端或内部延伸、缺失或插入都不应解释为影响序列同一性或类似性。天然存在的残基基于共同侧链性质而分成各组:

[0080] (1)疏水性:正亮氨酸、met、ala、val、leu、ile;

[0081] (2)中性亲水性:cys、ser、thr、asn、gln;

[0082] (3)酸性:asp、glu;

[0083] (4)碱性:his、lys、arg;

[0084] (5)影响链定向的残基:gly、pro;以及

[0085] (6)芳族:trp、tyr、phe。

[0086] 尽管保守性取代常常是优选的,但也涵盖非保守性取代(其必须将这些类别中的一个的成员交换成另一类别的成员)。

[0087] 优选治疗性抗体是IgG抗体。如本文所用的术语“IgG”意指属于基本上由认定的免疫球蛋白γ基因编码的抗体的类别的多肽。在人中，这个类别包含IgG1、IgG2、IgG3和IgG4。在小鼠中，这个类别包含IgG1、IgG2a、IgG2b、IgG3。IgG类别的抗体中的已知Ig结构域是VH、Cγ1、Cγ2、Cγ3、VL和CL。IgG由于若干实际原因而成为治疗性抗体的优选类别。IgG抗体是稳定的，易于纯化的，并且能够储存在对于药物供应链来说切实可行的条件下。在体内，它们具有长久生物半衰期，所述半衰期不仅仅随它们的尺寸而变，而且也是它们与所谓Fc受体(或FcRn)相互作用的结果。这个受体似乎保护IgG免遭细胞内分解代谢，并且使它再循环回血浆。

[0088] 抗体是结合特定抗原的免疫蛋白质。在包括人和小鼠的大多数哺乳动物中，抗体由成对重多肽链和轻多肽链构建。在抗体之间，轻链和重链可变区显示显著序列多样性，并且负责结合靶标抗原。各链由个别免疫球蛋白(Ig)结构域组成，并且因此通用术语免疫球蛋白用于此类蛋白质。

[0089] 术语“特异性结合”不意图指示抗体仅结合它的预期靶标，因为如上所指示，抗体结合显示所述抗体所结合的表位的任何多肽。相反，如果抗体对它的预期靶标的亲和力在相较于它对不显示适当表位的非靶标分子的亲和力时大近似5倍，那么它进行“特异性结合”。优选地，抗体对靶标分子的亲和力比它对非靶标分子的亲和力大至少约5倍，优选10倍，更优选25倍，甚至更优选50倍，并且最优选100倍或大于100倍。在优选实施方案中，优选抗体结合的亲和力是至少约$10^{7}M^{-1}$，并且优选在约$10^{8}M^{-1}$至约$10^{9}M^{-1}$、约$10^{9}M^{-1}$至约$10^{10}M^{-1}$、或约$10^{10}M^{-1}$至约$10^{12}M^{-1}$之间。

[0090] 亲和力计算为$K_d = k_{解离}/k_{缔合}$($k_{解离}$是解离速率常数，$K_{缔合}$是缔合速率常数，并且K_d是平衡常数)。可通过在各种浓度(c)下测量标记的配体的结合分数(r)来在平衡状态下确定亲和力。使用斯卡查德(Scatchard)等式：r/c＝K(n-r)将数据绘图，其中r＝在平衡状态下结合的配体的摩尔数/受体的摩尔数；c＝在平衡状态下游离配体浓度；K＝平衡缔合常数；并且n＝每个受体分子的配体结合位点数。通过图解分析，将r/c于Y轴上相对于r于X轴上绘图，由此产生斯卡查德图。通过斯卡查德分析进行抗体亲和力测量在本领域中是熟知的。参见例如van Erp等，J.Immunoassay 12:425-43,1991；Nelson和Griswold,Comput.Methods Programs Biomed.27:65-8,1988。

[0091] 本发明的抗体可通过表位作图来进一步表征，以便可选择在本文所述的免疫测定中具有最大临床效用的抗体和表位。术语“表位”是指能够特异性结合抗体的抗原决定簇。表位通常由各组化学活性表面分子，诸如氨基酸或糖侧链组成，并且通常具有特定三维结构特征以及特定电荷特征。构象表位和非构象表位的区别在于在变性溶剂存在下，与前者而非与后者的结合丧失。优选地，靶向存在于靶标分子上，但部分或完全不存在于非靶标分子上的表位。

[0092] 在一些实施方案中，抗体骨架可为来自不同物种的序列的混合物。因此，如果抗体是抗体，那么此类抗体可为嵌合抗体和/或人源化抗体。一般来说，“嵌合抗体”与“人源化抗体”两者均指组合有来自超过一种物种的区域的抗体。举例来说，“嵌合抗体”在传统上包含来自小鼠(或在一些情况下大鼠)的可变区和来自人的恒定区。“人源化抗体”通常是指可变结构域框架区已替换成见于人抗体中的序列的非人抗体。通常，在人源化抗体中，除CDR之外的整个抗体由人来源的多核苷酸编码，或除在它的CDR内之外与此类抗体相同。将其中一

些或全部由起源于非人生物体中的核酸编码的CDR移植入人抗体可变区的β折叠框架中以产生特异性由移入的CDR决定的抗体。此类抗体的产生描述于例如WO 92/11018;Jones,1986,Nature321:522-525;Verhoeyen等,1988,Science 239:1534-1536中。使所选接受体框架残基“回复突变”成相应供体残基常常为重新获得在初始移植构建体中丧失的亲和力所需(美国专利号5,530,101;美国专利号5,585,089;美国专利号5,693,761;美国专利号5,693,762;美国专利号6,180,370;美国专利号5,859,205;美国专利号5,821,337;美国专利号6,054,297;美国专利号6,407,213)。人源化抗体最优也将包含至少一部分免疫球蛋白恒定区,通常是人免疫球蛋白的一部分恒定区,并且因此将通常包含人Fc区。也可使用具有遗传工程改造的免疫系统的小鼠来产生人源化抗体。Roque等,2004,Biotechnol.Prog.20:639-654。多种用于使非人抗体人源化和再成形的技术和方法在本领域中是熟知的(参见Tsurushita和Vasquez,2004,Humanization of Monoclonal Antibodies,Molecular Biology of B Cells,533-545,Elsevier Science(USA)以及其中引用的参考文献)。人源化方法包括但不限于Jones等,1986,Nature 321:522-525;Riechmann等,1988;Nature332:323-329;Verhoeyen等,1988,Science,239:1534-1536;Queen等,1989,Proc Natl Acad Sci,USA 86:10029-33;He等,1998,J.Immunol.160:1029-1035;Carter等,1992,Proc Natl Acad Sci USA89:4285-9;Presta等,1997,Cancer Res.57(20):4593-9;Gorman等,1991,Proc.Natl.Acad.Sci.USA 88:4181-4185;O’Connor等,1998,Protein Eng 11:321-8中所述的方法。降低非人抗体可变区的免疫原性的人源化或其它方法可包括表面重塑方法,如例如于Roguska等,1994,Proc.Natl.Acad.Sci.USA91:969-973中所述。在一个实施方案中,亲本抗体已加以亲和力成熟,如本领域中所知。基于结构的方法可用于人源化和亲和力成熟,例如如U.S.序列号11/004,590中所述。基于选择的方法可用于使抗体可变区人源化和/或亲和力成熟,包括但不限于Wu等,1999,J.Mol.Biol.294:151-162;Baca等,1997,J.Biol.Chem.272(16):10678-10684;Rosok等,1996,J.Biol.Chem.271(37):22611-22618;Rader等,1998,Proc.Natl.Acad.Sci.USA 95:8910-8915;Krauss等,2003,Protein Engineering 16(10):753-759中所述的方法。其它人源化方法可涉及移植CDR的仅仅各部分,包括但不限于U.S.序列号09/810,502;Tan等,2002,J.Immunol.169:1119-1125;De Pascalis等,2002,J.Immunol.169:3076-3084中所述的方法。

[0093] 在一个实施方案中,抗体是完全人抗体。“完全人抗体”或“全面人抗体”是指具有源于人染色体的抗体的基因序列的人抗体。完全人抗体可例如使用转基因小鼠(Bruggemann等,1997,Curr Opin Biotechnol 8:455-458)或与选择方法联用的人抗体文库(Griffiths等,1998,Curr Opin Biotechnol 9:102-108)获得。

[0094] 抗体的产生

[0095] 可使用本领域中已知的广泛多种技术来产生单克隆抗体制剂,所述技术包括使用杂交瘤、重组和噬菌体展示技术或其组合。举例来说,可使用杂交瘤技术来产生单克隆抗体,所述技术包括本领域中已知以及例如Harlow等,ANTIBODIES:A LABORATORY MANUAL,(Cold Spring Harbor Laboratory Press,第2版1988);Hammerling,等,MONOCLONAL ANTIBODIES AND T-CELL HYBRIDOMAS,第563-681页(Elsevier,N.Y.,1981)(其两者均以引用的方式整体并入本文)中教导的那些。如本文所用的术语“单克隆抗体”不限于通过杂交瘤技术产生的抗体。术语“单克隆抗体”是指源于单一克隆,包括任何真核、原核或噬菌体克

隆的抗体，并且不涉及它被产生所采用的方法。

[0096] 源于除大鼠和小鼠以外的动物的单克隆抗体提供独特优势。与信号转导和疾病相关的许多蛋白质靶标在小鼠、大鼠和人之间是高度保守的，并且因此可被小鼠或大鼠宿主识别为自身抗原，从而使得它们具有较小免疫原性。当使用兔作为宿主动物时，这个问题可被避免。参见例如Rossi等,Am.J.Clin.Pathol.,124,295-302,2005。

[0097] 用于使用杂交瘤技术产生和筛选特定抗体的方法在本领域中是常规以及熟知的。在非限制性实例中，小鼠可用目标抗原或表达此类抗原的细胞免疫。一旦检测到免疫应答，例如在小鼠血清中检测到对抗原具有特异性的抗体，即收获小鼠脾，并且分离脾细胞。接着通过熟知技术使脾细胞融合于任何合适的骨髓瘤细胞。通过限制性稀释来选择和克隆杂交瘤。接着通过本领域中已知的方法测定杂交瘤克隆中分泌能够结合抗原的抗体的细胞。可通过用阳性杂交瘤克隆腹膜内接种小鼠来产生通常含有高水平的抗体的腹水液。

[0098] 可用于抗体产生方法中的佐剂包括但不限于蛋白质佐剂；细菌佐剂，例如完整细菌(BCG、短小棒状杆菌(Corynebacterium parvum)、明尼苏达沙门氏菌(Salmonella minnesota))和细菌组分，包括细胞壁骨架、海藻糖二霉菌酸酯、单磷酰基脂质A、结核菌的甲醇可提取残余物(MER)、完全或不完全弗氏佐剂(Freund's adjuvant)；病毒佐剂；化学佐剂，例如氢氧化铝、碘乙酸盐和半丁二酸胆固醇酯；或裸露DNA佐剂。可用于本发明方法中的其它佐剂包括霍乱毒素、副痘蛋白质、MF-59(Chiron Corporation；也参见Bieg等(1999)“GAD65And Insulin B Chain Peptide(9-23)Are Not Primary Autoantigens In The Type 1Diabetes Syndrome Of The BB Rat,”Autoimmunity,31(1):15-24,其以引用的方式并入本文)、MPL®(Corixa Corporation；也参见Lodmell等(2000)“DNA Vaccination Of Mice Against Rabies Virus:Effects Of The Route Of Vaccination And The Adjuvant Monophosphoryl Lipid A(MPL),”Vaccine,18:1059-1066；Johnson等(1999)“3-O-Desacyl Monophosphoryl Lipid A Derivatives:Synthesis And Immunostimulant Activities,”Journal of Medicinal Chemistry,42:4640-4649；Baldridge等(1999)“Monophosphoryl Lipid A(MPL)Formulations For The Next Generation Of Vaccines,”Methods,19:103-107,其全部以引用的方式并入本文)、RC-529佐剂(Corixa Corporation；来自Corixa的氨基烷基葡萄胺糖苷4-磷酸酯(AGP)化学文库的先导化合物，也参见www.corixa.com)和DETOX™佐剂(CorixaCorporation；DETOX™佐剂包括MPL®佐剂(单磷酰基脂质A)和分支杆菌细胞壁骨架；也参见Eton等(1998)“Active Immunotherapy With Ultraviolet B-Irradiated Autologous Whole Melanoma Cells Plus DETOX In Patients With Metastatic Melanoma,”Clin.Cancer Res.4(3):619-627；以及Gupta等(1995)“Adjuvants For Human Vaccines—Current Status,Problems And Future Prospects,”Vaccine,13(14):1263-1276,其两者均以引用的方式并入本文)。

[0099] 众多出版物讨论使用噬菌体展示技术产生和筛选结合所选分析物的多肽文库。参见例如Cwirla等,Proc.Natl.Acad.Sci.USA 87,6378-82,1990；Devlin等,Science 249,404-6,1990；Scott和Smith,Science 249,386-88,1990；以及Ladner等,美国专利号5,571,698。噬菌体展示方法的基本概念是在编码待筛选的多肽的DNA与所述多肽之间建立实体关联。这个实体关联由噬菌体粒子提供，所述粒子将多肽展示为封闭编码所述多肽的噬菌体基因组的衣壳的一部分。在多肽与它们的遗传物质之间建立实体关联允许同时大量筛选极

其大量的携带不同多肽的噬菌体。展示对靶标具有亲和力的多肽的噬菌体结合所述靶标，并且这些噬菌体通过针对所述靶标的亲和力筛选而加以富集。由这些噬菌体展示的多肽的身份可由它们的相应基因组确定。使用这些方法，被鉴定为对所需靶标具有结合亲和力的多肽可接着通过常规手段大批合成。参见例如美国专利号6,057,098，其据此以包括所有表格、附图和权利要求的方式整体并入本文。

[0100] 可接着通过以下方式选择通过这些方法产生的抗体：首先筛选关于纯化的目标多肽的亲和力和特异性，以及如果需要，那么将结果与抗体关于需要从结合排除的多肽的亲和力和特异性进行比较。筛选程序可涉及将纯化的多肽固定在微量滴定板的单独孔中。接着将含有一种潜在抗体或各组抗体的溶液放置至相应微量滴定孔中，并且孵育约30分钟至2小时。接着洗涤微量滴定孔，并且添加标记的二级抗体(例如如果产生的抗体是小鼠抗体，那么是缀合于碱性磷酸酶的抗小鼠抗体)至各孔中并孵育约30分钟，接着洗涤。添加底物至各孔中，并且当存在针对固定的多肽的抗体时，将出现显色反应。

[0101] 可接着在所选测定设计中进一步分析如此鉴定的抗体的亲和力和特异性。在开发针对靶标蛋白质的免疫测定时，纯化的靶标蛋白质充当使用已选抗体以其判断免疫测定的灵敏性和特异性的标准物。因为各种抗体的结合亲和力可不同；某些抗体对(例如在夹心式测定中)可在空间上彼此干扰等，所以抗体的测定性能可为比抗体的绝对亲和力和特异性更重要的量度。

[0102] 也可使用不能表达功能性内源性免疫球蛋白，但可表达人免疫球蛋白基因的转基因小鼠来产生抗体。举例来说，可随机或通过同源性重组将人重链和轻链免疫球蛋白基因复合体引入小鼠胚胎干细胞中。或者，除人重链和轻链基因之外，也可将人可变区、恒定区和多样性区域引入小鼠胚胎干细胞中。可单独或与通过同源性重组引入人免疫球蛋白基因座同时致使小鼠重链和轻链免疫球蛋白基因成为非功能性的。具体地，纯合性缺失JH区会防止内源性抗体产生。扩增修饰的胚胎干细胞，并且将其显微注射至胚泡中以产生嵌合小鼠。接着使嵌合小鼠交配以产生表达人抗体的纯合性后代。使用常规方法，用所选抗原，例如本发明多肽的全部或一部分使转基因小鼠免疫。可使用常规杂交瘤技术从免疫的转基因小鼠获得针对抗原的单克隆抗体。由转基因小鼠具有的人免疫球蛋白转基因在B细胞分化期间重排，并且随后经受类别转换和体细胞突变。因此，使用此类技术，有可能产生治疗适用的IgG、IgA、IgM和IgE抗体。对于用于产生人抗体的这个技术的概述，参见Lonberg等(1995)“Human Antibodies From Transgenic Mice,”Int.Rev.Immunol.13:65-93，其以引用的方式整体并入本文。对于用于产生人抗体和人单克隆抗体的这个技术以及用于产生此类抗体的方案的详细讨论，参见例如国际公布号WO 98/24893、WO 96/34096和WO 96/33735；以及美国专利号5,413,923、5,625,126、5,633,425、5,569,825、5,661,016、5,545,806、5,814,318和5,939,598，其以引用的方式整体并入本文。此外，诸如Abgenix,Inc.(Freemont,Calif.)和Medarex(Princeton,N.J.)的公司可被雇佣以使用与上述技术类似的技术提供针对所选抗原的人抗体。

[0103] 抗体的重组表达

[0104] 一旦已获得编码本发明的抗体的核酸序列，即可通过重组DNA技术，使用本领域中熟知的技术来产生用于产生所述抗体的载体。为本领域技术人员所熟知的方法可用于构建含有抗体编码序列和适当转录和翻译控制信号的表达载体。这些方法包括例如体外重组

DNA技术、合成技术和体内遗传重组。(参见例如Sambrook等,1990,MOLECULAR CLONING,A LABORATORY MANUAL,第2版,Cold Spring Harbor Laboratory,Cold Spring Harbor,N.Y.以及Ausubel等编,1998,CURRENT PROTOCOLS IN MOLECULAR BIOLOGY,John Wiley&Sons,NY中所述的技术)。

[0105] 可通过常规技术(例如电穿孔、脂质体转染和磷酸钙沉淀)将包含抗体的核苷酸序列的表达载体转移至宿主细胞中,并且接着通过常规技术培养转染的细胞以产生本发明的抗体。在特定实施方案中,抗体的表达由组成型、诱导型或组织特异型启动子调控。

[0106] 用于表达本发明的重组抗体的宿主细胞可为细菌细胞,诸如大肠杆菌(Escherichia coli),或优选是真核细胞,尤其对于表达完整重组免疫球蛋白分子。具体地,诸如中国仓鼠卵巢细胞(CHO)的哺乳动物细胞与诸如来自人巨细胞病毒的主要中期早期基因启动子元件的载体联合是免疫球蛋白的有效表达系统(Foecking等(1986)"Powerful And Versatile Enhancer-Promoter Unit For Mammalian Expression Vectors."Gene 45:101-105;Cockett等(1990)"High Level Expression Of Tissue Inhibitor Of Metalloproteinases In Chinese Hamster Ovary Cells Using Glutamine Synthetase Gene Amplification,"Biotechnology 8:662-667)。

[0107] 多种宿主表达载体系统可用于表达本发明的抗体。此类宿主表达系统代表可产生以及随后纯化抗体的编码序列所采用的载体,而且也代表在用适当核苷酸编码序列转化或转染时可原位表达本发明的抗体的细胞。这些包括但不限于微生物,诸如用含有免疫球蛋白编码序列的重组噬菌体DNA、质粒DNA或粘粒DNA表达载体转化的细菌(例如大肠杆菌和枯草芽孢杆菌(B.subtilis));用含有免疫球蛋白编码序列的重组酵母表达载体转化的酵母(例如毕赤酵母);用含有免疫球蛋白编码序列的重组病毒表达载体(例如杆状病毒)感染的昆虫细胞系统;用含有免疫球蛋白编码序列的重组病毒表达载体(例如花椰菜花叶病毒(CaMV)和烟草花叶病毒(TMV))感染或用含有免疫球蛋白编码序列的重组质粒表达载体(例如Ti质粒)转化的植物细胞系统;或具有含有源于哺乳动物细胞的基因组的启动子(例如金属硫蛋白启动子)或源于哺乳动物病毒的启动子(例如腺病毒晚期启动子;痘苗病毒7.5K启动子)的重组表达构建体的哺乳动物细胞系统(例如COS、CHO、BHK、293、293T、3T3细胞、淋巴细胞(参见美国专利号5,807,715)、Per C.6细胞(由Crucell开发的大鼠视网膜细胞))。

[0108] 在细菌系统中,视所表达的抗体的预定用途而定,可方便地选择许多表达载体。举例来说,当将产生用于产生抗体的药物组合物的大量此类蛋白质时,引导高水平的易于纯化的融合蛋白产物的表达的载体可为合乎需要的。此类载体包括但不限于大肠杆菌表达载体pUR278(Ruther等(1983)"Easy Identification Of cDNA Clones,"EMBO J.2:1791-1794),其中抗体编码序列可与lac Z编码区同框个别地连接至载体中以便产生融合蛋白;pIN载体(Inouye等(1985)"Up-Promoter Mutations In The Lpp Gene Of Escherichia coli,"Nucleic Acids Res.13:3101-3110;Van Heeke等(1989)"Expression Of Human Asparagine Synthetase In Escherichia coli,"J.Biol.Chem.24:5503-5509);等。pGEX载体也可用于以与谷胱甘肽S转移酶(GST)的融合蛋白形式表达外来多肽。一般来说,此类融合蛋白是可溶的,并且可易于通过吸附以及结合基质谷胱甘肽-琼脂糖珠粒,随后在游离谷胱甘肽存在下洗脱来从溶解的细胞纯化。pGEX载体被设计以包括凝血酶或因子Xa蛋白酶裂解位点以使克隆的靶标基因产物可从GST部分释放。

[0109] 在昆虫系统中，苜蓿银纹夜蛾(Autographa californica)核多角体病毒(AcNPV)被用作用以表达外来基因的载体。所述病毒生长在草地贪夜蛾(Spodoptera frugiperda)细胞中。抗体编码序列可被个别地克隆至病毒的非必需区域(例如多角体蛋白基因)中，并且置于AcNPV启动子(例如多角体蛋白启动子)的控制下。

[0110] 在哺乳动物宿主细胞中，可利用许多基于病毒的表达系统。在其中腺病毒被用作表达载体的情况下，可使目标抗体编码序列连接于腺病毒转录/转译控制复合物，例如晚期启动子和三联前导序列。这个嵌合基因可接着通过体外或体内重组插入腺病毒基因组中。插入在病毒基因组的非必需区域(例如区域E1或E3)中将产生在受感染宿主中具有活力并能够表达免疫球蛋白分子的重组病毒。(参见例如Logan等(1984)“Adenovirus Tripartite Leader Sequence Enhances Translation Of mRNAs Late After Infection,” Proc.Natl.Acad.Sci.(U.S.A.)81:3655-3659)。特定起始信号也可为高效翻译插入的抗体编码序列所需。这些信号包括ATG起始密码子和邻近序列。此外，起始密码子必须与所需编码序列的阅读框同相以确保翻译整个插入物。这些外源性翻译控制信号和起始密码子可具有多种天然来源与合成来源两者。表达效率可通过纳入适当转录增强子元件、转录终止子等来增强(参见Bitter等(1987)“Expression And Secretion Vectors For Yeast,” Methods in Enzymol.153:516-544)。

[0111] 此外，可选择调节插入序列的表达，或以所需特定方式修饰以及加工基因产物的宿主细胞株。对蛋白质产物的此类修饰(例如糖基化)和加工(例如裂解)对于蛋白质的功能可为重要的。不同宿主细胞具有用于翻译后加工和修饰蛋白质和基因产物的特征性和特定性机理。可选择适当细胞系或宿主系统以确保对表达的外来蛋白质的正确修饰和加工。为此，可使用具有用于适当加工初级转录物、糖基化以及使基因产物磷酸化的细胞机构的真核宿主细胞。此类哺乳动物宿主细胞包括但不限于CHO、VERY、BHK、Hela、COS、MDCK、293、293T、3T3、WI38、BT483、Hs578T、HTB2、BT20以及T47D、CRL7030和Hs578Bst。

[0112] 对于长期高产率产生重组蛋白，稳定表达是优选的。举例来说，可工程改造稳定表达本发明的抗体的细胞系。胜于使用含有病毒复制起点的表达载体，宿主细胞可用由适当表达控制元件(例如启动子、增强子、序列、转录终止子、聚腺苷酸化位点等)和可选择标记控制的DNA转化。在引入外来DNA之后，可使工程改造的细胞在富集培养基中生长1-2天，接着变换为选择性培养基。重组质粒中的可选择标记赋予对选择的抗性，并且允许细胞将质粒稳定整合至它们的染色体中，并且生长以形成焦点，所述焦点转而可被克隆以及扩增至细胞系中。这个方法可有利地用于工程改造表达本发明的抗体的细胞系。此类工程改造的细胞系可特别适用于筛选和评估直接或间接与本发明的抗体相互作用的化合物。

[0113] 可使用许多选择系统，包括但不限于单纯疱疹病毒胸苷激酶(Wigler等(1977)“Transfer Of Purified Herpes Virus Thymidine Kinase Gene To Cultured Mouse Cells,”Cell 11:223-232)、次黄嘌呤-鸟嘌呤磷酸核糖基转移酶(Szybalska等(1962)“Genetics Of Human Cess Line.IV.DNA-Mediated Heritable Transformation Of A Biochemical Trait,”Proc.Natl.Acad.Sci.(U.S.A.)48:2026-2034)和腺嘌呤磷酸核糖基转移酶(Lowy等(1980)“Isolation Of Transforming DNA:Cloning The Hamster Aprt Gene,”Cell 22:817-823)基因分别可用于tk-、hgprt-或aprt-细胞中。此外，抗代谢物抗性可被用作针对以下基因进行选择的基础：dhfr，其赋予对甲氨蝶呤的抗性(Wigler等(1980)

"Transformation Of Mammalian Cells With An Amplfiable Dominant-Acting Gene," Proc.Natl.Acad.Sci.(U.S.A.)77:3567-3570;O'Hare等(1981)"Transformation Of Mouse Fibroblasts To Methotrexate Resistance By ARecombinant Plasmid Expressing A Prokaryotic Dihydrofolate Reductase,"Proc.Natl.Acad.Sci.(U.S.A.) 78:1527-1531);gpt,其赋予对霉酚酸的抗性(Mulligan等(1981)"Selection For Animal Cells That Express The Escherichia coli Gene Coding For Xanthine-Guanine Phosphoribosyltransferase,"Proc.Natl.Acad.Sci.(U.S.A.)78:2072-2076);neo,其赋予对氨基糖苷G-418的抗性(Tachibana等(1991)"Altered Reactivity Of Immunoglobutin Produced By Human-Human Hybridoma Cells Transfected By pSV2-Neo Gene,"Cytotechnology 6(3):219-226;Tolstoshev(1993)"Gene Therapy,Concepts, Current Trials And Future Directions,"Ann.Rev.Pharmacol.Toxicol.32:573-596; Mulligan(1993)"The Basic Science Of Gene Therapy,"Science 260:926-932;以及Morgan等(1993)"Human gene therapy,"Ann.Rev.Biochem.62:191-217)。重组DNA技术的本领域中通常已知的可使用的方法描述于以下中:Ausubel等(编),1993,CURRENT PROTOCOLS IN MOLECULAR BIOLOGY,John Wiley&Sons,NY;Kriegler,1990,GENE TRANSFER AND EXPRESSION,A LABORATORY MANUAL,Stockton Press,NY;以及第12和13章,Dracopoli等(编),1994,CURRENT PROTOCOLS IN HUMAN GENETICS,John Wiley&Sons,NY.;Colbere-Garapin等(1981)"A New Dominant Hybrid Selective Marker For Higher Eukaryotic Cells,"J.Mol.Biol.150:1-14;以及hygro,其赋予对潮霉素的抗性(Santerre等(1984) "Expression Of Prokaryotic Genes For Hygromycin B And G418Resistance As Dominant-Selection Markers In Mouse L Cells,"Gene30:147-156)。

[0114] 本发明的抗体的表达水平可通过载体扩增来增加(对于综述,参见Bebbington和Hentschel,"The Use Of Vectors Based On Gene Amplification For The Expression Of Cloned Genes In Mammaian Cells,"DNACLONING,第3卷(Academic Press,New York, 1987))。当表达抗体的载体系统中的标记可扩增时,增加存在于宿主细胞的培养物中的抑制剂的水平将使标记基因的拷贝数增加。因为扩增的区域伴有抗体的核苷酸序列,所以抗体的产生也将增加(Crouse等(1983)"Expression And Amplification Of Engineered Mouse Dihydrofolate Reductase Minigenes,"Mol.Cell.Biol.3:257-266)。

[0115] 宿主细胞可用本发明的两种表达载体共转染,其中第一载体编码重链源性多肽,并且第二载体编码轻链源性多肽。两种载体可含有使得重链和轻链多肽能够相等表达的相同可选择标记。或者,可使用编码重链多肽与轻链多肽两者的单一载体。在此类情况下,轻链应被放置在重链之前以避免毒性游离重链过量(Proudfoot(1986)"Expression And Amplification Of Engineered Mouse Dihydrofolate Reductase Minigenes,"Nature 322:562-565;Kohler(1980)"Immunoglobulin Chain Loss In Hybridoma Lines," Proc.Natl.Acad.Sci.(U.S.A.)77:2197-2199)。重链和轻链的编码序列可包括cDNA或基因组DNA。

[0116] 一旦本发明的抗体已被重组表达,它即可通过本领域中已知用于纯化抗体的任何方法纯化,例如通过色谱法(例如离子交换、亲和力(特别是在蛋白A之后通过对特定抗原的亲和力)和尺寸分级柱色谱法)、离心、溶解度差异,或通过用于纯化蛋白质的任何其它标准

技术。

实施例

[0117] 实施例1:在兔中产生单克隆抗体

[0118] 通过用抗原/佐剂乳液皮下注射(SQ)来使雌性新西兰兔免疫。初级免疫用完全弗氏佐剂进行,并且不完全弗氏佐剂用于所有随后加强。每三周以每只兔250μg蛋白质抗原SQ注射兔(两个部位(臀部和肩胛骨)交替)。在第二次加强之后七天从边缘耳静脉获取测试放血。通过间接ELISA测定来测试这个测试放血(免疫血清)以确定兔的免疫应答是否足以产生单克隆抗体。向最佳应答性兔给与最终SQ加强,并且四天后通过放血来安乐死。通过心脏穿刺收集全血。通过关于靶标抗原的间接ELISA鉴定产生目标抗体的B细胞,并且分离免疫球蛋白基因。将重链和轻链克隆至单独哺乳动物表达载体中,转染至HEK细胞中(瞬时转染),并且收获含有兔单克隆抗体的组织培养上清液。

[0119] 实施例2:在小鼠中产生单克隆抗体

[0120] 通过根据标准操作程序用抗原/佐剂乳液腹膜内注射(IP)来使雌性BALB/c小鼠(60天龄)免疫。初级免疫用完全弗氏佐剂进行,并且不完全弗氏佐剂用于所有随后加强。每3周以每只小鼠25μg抗原IP注射小鼠(每只小鼠总体积125μL)。在第二次加强之后7至10天,通过隐静脉穿刺进行测试放血。通过间接ELISA测定来测试这个测试放血(免疫血清)以确定小鼠的免疫应答是否足以融合。通过侧向尾部静脉向2只最佳应答性小鼠给与每只小鼠于无菌盐水中的10μg抗原的最终静脉内加强。在IV加强之后4天,使小鼠安乐死,并且收获脾。从脾分离的淋巴细胞在融合过程中用于使用Kohler,G.;Milstein,C.(1975)."Continuous cultures of fused cells secreting antibody of predefined specificity".Nature 256(5517):495-497的方法产生杂交瘤。使用PEG1500融合过程产生杂交瘤。

[0121] 实施例3:用患者样品筛选抗体(基于微量滴定的ELISA方法)

[0122] 材料:

[0123] 96孔高结合ELISA板-Costar 3590(Corning)

[0124] ELISA涂布缓冲液:PBS

[0125] ELISA洗涤缓冲液:具有0.02%吐温-20(Tween-20)的PBS

[0126] ELISA封闭缓冲液(Thermo Pierce,目录号N502)

[0127] ELISA试剂稀释剂:200mM Tris、1%BSA(BioFx)、0.05%吐温-20,pH 8.1

[0128] 中和亲和素-HRP缀合物(Thermo Pierce,目录号31001)

[0129] 1步超敏TMB底物(R&D systems,目录号34028)

[0130] 终止溶液:2N硫酸

[0131] 捕获抗体

[0132] 生物素缀合的检测抗体

[0133] 重组人IGFBP7(Peprotech,目录号410-02)

[0134] EXLx405板洗涤器(Biotek)

[0135] 多扫描FC板读取器(Fisher Scientific)

[0136] 测试程序

[0137] 将纯化的重组IGFBP7分析物加标至试剂稀释剂中，并且连续稀释以产生一组涵盖一定范围的浓度的标准样品。在室温水浴中解冻患者样品的冷冻的单次使用的等分试样10分钟，接着用试剂稀释剂稀释至所需水平。

[0138] 添加于涂布缓冲液中制备的100μL 5μg/mL捕获抗体溶液至96孔高结合ELISA板上的各孔中，并且在室温(22℃至25℃)下孵育过夜。抽吸各孔，并且使用自动洗涤机用300μL洗涤缓冲液洗涤三次。接着添加250μL ELISA封闭缓冲液至各孔中。在室温下孵育2小时之后，重复上述抽吸/洗涤步骤。

[0139] 添加100μL标准物或患者样品至制备的板的各孔中，并且在室温下在水平轨道振荡器上孵育。在孵育2小时之后，如上所述将板洗涤。接着添加于试剂稀释剂中制备的100μL 0.1μg/mL检测抗体溶液至各孔中。在室温下孵育1小时之后，再次将板洗涤。于试剂稀释剂中制备0.1μg/mL中和亲和素-HRP缀合物溶液，并且添加100μL这种溶液至各孔中。在室温下将板孵育1小时并洗涤。添加100μL 1步超敏TMB底物至各孔中，在室温下避光孵育10分钟，随后添加50μL终止溶液。用设置成450nm波长的微板读取器测量各孔中的光密度。

[0140] 实施例4:用患者样品筛选抗体(侧向流动条带测试方法)

[0141] 材料：

[0142] 硝化纤维素膜

[0143] 背衬卡

[0144] 样品垫

[0145] 芯吸垫

[0146] 膜封闭缓冲液:10mM磷酸钠、0.1％蔗糖、0.1％BSA、0.2％PVP-40,pH 8.0

[0147] 样品垫封闭缓冲液:5mM硼酸盐、0.1％吐温-20、0.25％PVP-40、0.5％BSA,pH 8.5

[0148] 操作缓冲液J:500mM Tris、0.2％10G、0.35％吐温-20、0.25％PVP-40,pH 8.5

[0149] 荧光缀合抗体

[0150] 测试线抗体

[0151] 山羊抗小鼠阳性对照抗体

[0152] 重组人IGFBP7

[0153] 条带组件

[0154] 使用AD3050抽吸分配系统用测试线抗体将硝化纤维素膜分条，用膜封闭缓冲液封闭，并且在37℃下干燥30分钟。在干燥器中固化过夜之后，将分条并封闭的硝化纤维素膜层压于具有芯吸垫和用样品垫封闭缓冲液预处理的样品垫的背衬卡上。将卡切割成5mm宽的测试条带，接着将其放置至柱筒中。

[0155] 样品制备

[0156] 将纯化的重组IGFBP7分析物加标至操作缓冲液J中，并且连续稀释以产生一组涵盖一定范围的浓度的标准样品。在室温水浴中解冻患者样品的冷冻的单次使用的等分试样10分钟，接着用操作缓冲液J稀释至所需水平。

[0157] 测试程序

[0158] 将10μL含荧光缀合抗体(0.025μg/μL)的PBS添加至100μL样品中。接着将100μL这种溶液装载至柱筒上的输入口中。在t＝20分钟时，使用荧光读取器和相关软件读取结果。

[0159] 实施例5:肽作图

[0160] 材料:96孔高结合微量滴定板、中和亲和素、生物素化肽、未缀合抗体、小鼠IgG、兔IgG、山羊IgG、HRP缀合于抗小鼠IgG的HRP缀合物、抗兔IgG HRP缀合物、抗山羊IgG HRP缀合物、TMB底物、2N硫酸用于表位作图实验。

[0161] 将中和亲和素固定在96孔高结合微量滴定板的个别孔中。洗涤各板以移除未反应的中和亲和素,随后进行封闭步骤。将生物素化肽溶解于水性缓冲液中以达到10μg/mL的浓度。添加50μL肽溶液至中和亲和素涂布的微量滴定板的各孔中。在室温下孵育这些板1小时,接着洗涤以移除未结合肽。将未缀合的小鼠和兔抗体稀释至5μg/mL,并且以100μL/孔添加至板中。将抗小鼠IgG(在小鼠抗IGFBP7的情况下)或抗兔IgG(在兔抗IGFBP7的情况下)作为阴性对照添加至相邻孔中。在室温下将板孵育1小时并洗涤。将HRP缀合的抗小鼠IgG(在小鼠抗IGFBP7和小鼠IgG阴性对照的情况下)和HRP缀合的抗兔IgG(在兔抗IGFBP7和兔IgG阴性对照的情况下)稀释至0.2μg/mL,并且添加100μL至板的各孔中。在室温下将这些板孵育20分钟并洗涤。添加100μL/孔的TMB底物,并且在避免暴露于光照下将板孵育20分钟。添加50μL/孔的终止溶液(2N硫酸)至各孔和各板中以终止反应。在设置成在450nm下测量光密度的分光光度性96孔微板读取器上读取吸光度。

[0162] 实施例6:丙氨酸扫描肽作图

[0163] 丙氨酸扫描是一种广泛使用的诱变方法,其中通过定点诱变在所选位置处使靶标蛋白质中的残基系统地取代成丙氨酸,表达并测定功能。用丙氨酸残基取代会消除侧链相互作用而不改变主链构象或引入立体或静电效应。使用自动化诱变方案,靶标多肽中每个残基被变为丙氨酸,并且可确定构成各抗体结合结构域的关键残基。

[0164] 实施例7:结果

[0165] 使用丙氨酸扫描和肽作图的组合结果,独特的IGFBP7单克隆抗体得以鉴定并基于分析性能加以选择。

[0166]

抗体	肽扫描序列	机敏序列(Astute Sequence)(总区域)
7G2.1	$_{210}$PGDRD$_{214}$ (SEQ ID NO: 6)	$_{201}$YGVQRTELLPGDRDNL$_{216}$ (SEQ ID NO: 6)
6D2.1	$_{206}$TELLPGDR$_{213}$ (SEQ ID NO: 3)	$_{191}$LIWNKVKRGHYGVQRT$_{206}$ (SEQ ID NO: 7)
1C9E4.1	$_{36}$EPASC$_{40}$ (SEQ ID NO: 4)	$_{25}$SSSSSDTCGPCEPASCPPLP$_{44}$ SEQ ID NO: 8)

[0167] 实施例8:测序数据

[0168] 抗体IC9E4.1的同种型被确定为鼠类IgG1/κ抗体。获得来自单克隆细胞系的cDNA以通过标准方法测序。重链可变区和轻链可变区的序列如下:

[0169] $V_{轻}$(SEQ ID NO:9)

[0170]
```
DVVMTQTPLT LSVTIGQPAS ISCKSSQSLL YSNGETYLHW LLQRPGQSPK 50
RLIYLVSKLD SGVPDRFTGS GSRTDFTLKI SRVEAEDLGV YYCAQGTHFP 100
HTFGGGTKLE
```

[0171] $V_{重}$(SEQ ID NO:10)

[0172]
```
QIQLVQSGPE LKKPGETVKI SCKASGYSFT DYSIHWVKQA PGKGLKWMGL 50
INTETGEPIY VDDFKGRFAF SLETSARTAY LQINNLKNED TATYFCARAY 100
YWAYWGQGTL V
```

[0173] 抗体1D6

[0174] 抗体1D6的同种型被确定为鼠类IgG1/κ抗体。通过表位作图，确定1D6抗体结合IGFBP7的构象表位。获得来自单克隆细胞系的cDNA以通过标准方法测序。重链可变区和轻链可变区的序列如下：

[0175] $V_{轻}$(SEQ ID NO:11)

QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMHWYQQKSG TSPKRWIYDT 50

[0176] SELASGVPAR FSGSGSGTSY SLTISSMEAE DAATYYCQQW SSSPFTFGSG 100

TKLEIKR

[0177] $V_{重}$(SEQ ID NO:12)

QIQLVQSGPE LKKPGETVKI SCKASGYTFK KYGMNWVKQA PGKGLKWMGW 50

[0178] INTYTGEPIY ADDFKGRFAF SLETSASTAY LQISNLKNED TATYFCAREE

YGPFYAMDYW GQGTSVTVSS

[0179] 尽管本发明已足够详细地被描述和例示以供本领域技术人员进行和使用本发明，但在不脱离本发明的精神和范围下，各种替代方案、修改和改进应为显而易知的。本文提供的实施例代表优选实施方案，是示例性的，并且不意图作为对本发明的范围的限制。其中修改和其它用途将为本领域技术人员所想到。这些修改涵盖在本发明的精神内，并且由权利要求的范围所限定。

[0180] 除非另外陈述，否则在本文中使用“或”意指“和/或”。类似地，“包含(comprise/comprises/comprising/include/includes)”和“包括(including)”可互换，并且不意图具有限制性。

[0181] 应进一步了解当对各种实施方案的描述使用术语“包含”时，本领域技术人员将了解在一些特定情况下，实施方案可使用措辞“基本上由…组成”或“由…组成”加以替代地描述。

[0182] 除非另外定义，否则本文所用的所有技术和科学术语都具有与为本公开所属领域中的普通技术人员通常理解相同的含义。尽管与本文所述的方法和试剂类似或等效的任何方法和试剂都可用于实施公开的方法和组合物，但现时描述示例性方法和材料。

[0183] 本文提及的所有出版物都出于描述和公开出版物中所述的可能与本文描述关联使用的方法的目的以引用的方式全部并入本文。说明书中提及的所有专利和出版物都指示在本公开的提交日期之前，本发明所属领域中的普通技术人员的水平。本文中没有内容应解释为承认本发明者由于先前公开而无权先于此类公开。

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[0186] 其它实施方案阐述在以下权利要求内。