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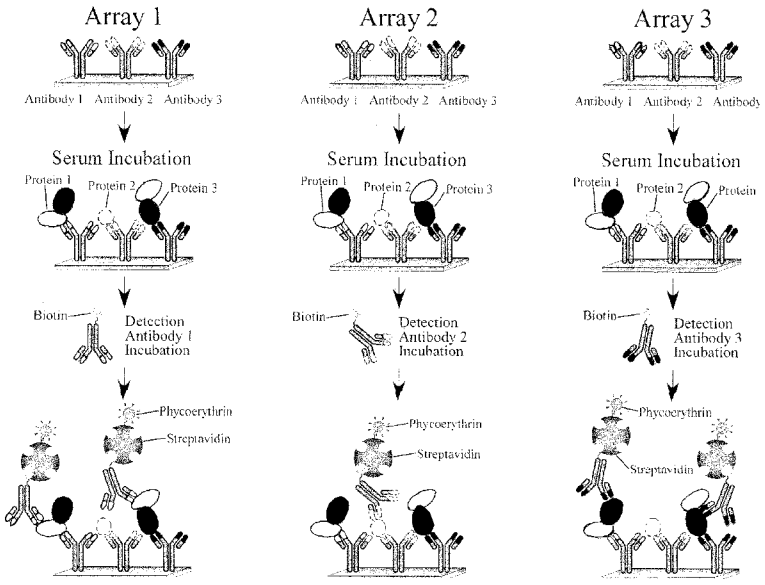
(54) **Title:** METHODS FOR DETECTING MOLECULAR COMPLEXES



(57) **Abstract:** The invention includes methods for analyzing copies of an antibody microarray for a protein complexed with another molecule. In this method, one biological sample is divided and then incubated with copies of an antibody microarray. Each capture antibody is paired with a detection antibody, and only one detection antibody is incubated with each copy of the antibody microarray. The invention further includes methods to compare protein complexes under different conditions, methods of detecting an autoimmune response, and methods of detecting interaction between a protein and a small molecule.

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METHODS FOR DETECTING MOLECULAR COMPLEXES

CROSS-REFERENCE TO RELATED APPLICATION

[001] This application claims benefit of provisional application Serial No. 60/813,838, filed June 15, 2006, entitled A METHOD FOR MAPPING MOLECULAR COMPLEXES, the entire contents of which are incorporated herein in their entirety.

FIELD OF THE INVENTION

[002] This invention relates to the field of molecular biology and medicine and specifically to assays for detecting molecular interactions.

BACKGROUND OF THE INVENTION

[003] Proteins continuously interact with one another in a highly regulated fashion to determine cell fate, such as proliferation, differentiation, or death. Protein-protein interactions represent an enormous and diverse group of targets for therapeutic intervention. Complicated protein-protein interaction can also be regulated through post-translational modification (glycosylation, phosphorylation, etc). In addition to post-translational modification and alternative splicing, the formation of protein-protein complexes contributes to both the exceedingly large size of and the dizzying complexity of the proteome. Not only can proteins exist as single entities whose functions are determined by any number of post-translational modification mechanisms, but proteins can also exist in any number of complexes with other proteins. Indeed, the study of protein-protein interactions has become increasingly important and many researchers are devising new ways to study protein-protein interactions and their associated functions (Russel, RB et al. 2004. Curr Opin Struct Biol. 14:313-324.; Zhu, H. et al. 2002. Curr Opin Cell Biol. 14:173-179).

[004] Protein-protein interactions have been implicated in cell signaling (Gastel, M. 2006. Handb Exp Pharmacol. 172:93-109), and the scientific community has long been interested in studying the signaling pathways that control cellular functions. To this end, they have devised approaches to study both the genes which code for the signaling pathways and the proteins which comprise the signaling pathways. Because of post translational modifications, gene function is regulated not only by its genomic code, but also by the proteins interacting with the gene product. To further decipher which proteins interact to determine functionality of the proteins of interest, a variety of approaches have been devised to investigate protein-protein interactions. The most common methodologies of examining protein complexes are yeast two- or three- hybrid screens, affinity tagging coupled with mass spectrometry, and protein array methodologies (Zhu, H. et al. 2002.

Curr Opin Cell Biol. 14:173-179). Although these techniques are useful, there is currently no high throughput methodology to study protein-protein interactions.

[005] As protein-protein complexes are involved in all cell signaling pathways, protein-protein complexes are also implicated in all phenotypes. Unique protein-protein interactions have been discovered to be associated not only with certain organ systems, but also with particular diseases, including cancer (Fry, DC et al. 2005. J Mol Med. 83:955-963.), CNS diseases (Rabiner, CA et al. 2005. Neuroscientist 11:148-160), diseases of the platelet and associated cardiovascular diseases (Macaulay, IC et al. 2005. J Clin Invest 115:3370-3377), and others (Houtman JD et al. 2005. FEBS 272:5426-5435).

[006] If a protein elicits an antibody-based immune response, antibodies (immunoglobin or "Ig") against those proteins can be generated. Such proteins are known as "self" proteins or "auto-antigens," and the antibodies generated against auto-antigens are called "auto-antibodies." For example, it is known that auto-antigens MUC1 and p53 elicit an auto-immune response, i.e., these proteins elicit the corresponding auto-antibodies in a subject (Finn OJ, Jerome KR, Henderson RA, Pecher G, Domenech N, Magarian-Blander J, Barratt-Boyes SM, Immunol Rev. 1995 Jun;145:61-89). Such auto-antibodies may occur because the auto-antigen is an altered form of a protein, so an individual mounts a limited immune response against that auto-antigen. Auto-antigens indicative of a tumor are known as tumor-associated antigens or "TAAs." In this instance, it is known to detect and measure the presence of the corresponding auto-antibody rather than the auto-antigen. This is a way to identify or detect tumor using the immune response, rather than detecting a protein secreted from a tumor. Detection of the immune response is believed to be a better method than detection of secreted proteins because B-cells will create many auto-antibodies and, thus, a high concentration of auto-antibodies circulate in the bloodstream. Additionally, because antibodies generally are more stable than secreted proteins, there is a consistent concentration of antibodies over a longer period of time. In addition to tumor detection, this method could be used to detect an immune response for any auto-immune disease, e.g., rheumatoid arthritis.

[007] One known method for detecting auto-antibodies includes creating arrays of peptides or proteins (produced using a variety of methods), incubating the arrays with serum, and using anti-immunoglobin to detect the presence of immunoglobulin at any of the protein spots. This method, however, is dependent on creating a universal form of the peptide auto-antigen. That is, recombinant proteins and peptides are spotted on an array but do not necessarily carry the same sequence variations or post-translational modifications as the auto-

antigen in a sample under examination. Auto-antigens from different individuals may have different immunogenic alterations, so the isolation and use of auto-antigen material from one individual does not necessarily provide auto-antigen material that is immunoreactive with auto-antibody for other individuals. For example, an auto-antigen (e.g., protein X) from one subject may include a mutation that has triggered an auto-immune response. A comparable protein X' from another individual may be immunogenic due to a slightly different mutation. Thus, an auto-antibody that binds to protein X' will not necessarily bind to protein X. Using this prior method, an array including auto-antigens from one individual will bind auto-antibodies in only a relatively small subset of the population, e.g., 30% of the population. As such, this method can create a significant number of false negatives. Thus, some researchers have used arrays with bound auto-antigens that were isolated directly from individual samples, but such auto-antigen proteins are difficult to purify completely and difficult to confirm in follow-up studies.

[008] An additional complex of interest is the interaction between a protein and a small molecule (e.g., a drug). A known method for the detection of drug-protein interactions includes mass spectrometry to identify proteins isolated with a drug. Mass spectrometry can be effective, but is expensive, low-throughput, and not quantitative. Drug interactions with specific proteins can be directly tested with such methods if recombinant proteins are available. In many cases, however, proteins may need to be in their native state or interacting with certain other factors in order to bind a drug, making the use of recombinant proteins ineffective. Furthermore, it is otherwise generally difficult or expensive to obtain a wide range of purified proteins.

SUMMARY OF THE INVENTION

[009] The present invention includes various methods for detecting molecular complexes. These methods are high through-put and are based on the use of microarray technology (in particular, using bound anti-protein capture antibodies) to detect a molecular complex in its native form.

[0010] More specifically, the present invention includes a method of analyzing a protein complexed with another molecule, comprising: providing a first microarray slide, wherein the first microarray slide includes a first array that is attached to the microarray slide and a second array that is attached to the first microarray slide and which second array is separated from the first microarray, wherein the first array includes a first capture antibody for specifically binding to a first protein and a second capture antibody for specifically binding to a second protein, wherein the second array includes a duplication of the first

array; providing a first biological sample that may contain the first and second proteins in their native form; incubating an aliquot of the first biological sample on the first and second arrays of the first microarray slide to permit any of the first protein and second protein in the first biological sample to bind to their respective capture antibody; washing off any unbound proteins from the first and second arrays of the first microarray slide; providing a first detection antibody that specifically binds to a first molecule; providing a second detection antibody that specifically binds to a second molecule; incubating the first detection antibody on the first array of the first microarray slide and incubating the second detection antibody on the second array of the first microarray slide to permit the first detection antibody and the second detection antibody to bind to their respective molecules; detecting the presence of any complex including the first molecule in the first array of the first microarray slide; detecting the presence of any complex including the second molecule in the second array of the first microarray slide; and determining whether the first protein is complexed with the second molecule in the first biological sample and whether the second protein is complexed with the first molecule in the first biological sample.

[0011] The method of the present invention also includes the first molecule or second molecule selected from the group consisting of a protein, carbohydrate, lipid, nucleic acid, or small molecule; or the first biological sample is serum (e.g., blood) or a cell lysate from either cell culture or tissue. Additionally, the first protein and the first molecule may be the same and the first capture antibody and the first detection antibody may be specific to different epitopes on the first protein. Similarly, the second protein and the second molecule may be the same and the second capture antibody and the second detection antibody may be specific to different epitopes on the second protein. The method of the present invention further comprises determining the quantity in the first array of any complex that includes the first molecule, or determining the quantity in the second array of any complex that includes the second molecule.

[0012] Various embodiments of the invention include the first detection antibody tagged with a first tag and the second detection antibody tagged with a second tag; and the first and second detection antibodies are biotinylated. Further, one embodiment of the invention includes incubating the first and second arrays with streptavidin-phycoerythrin and scanning the first detection antibody and the second detection antibody for fluorescence. Other detection methods of the present invention include scanning the first detection antibody and the second detection antibody for chemoluminescence or

calorimetrically detecting the presence of the first detection antibody and the second detection antibody.

[0013] Another method of the present invention includes providing a second microarray slide that includes the same first array and the same second array as the first microarray slide; providing a second biological sample that may contain the first and second proteins in their native form; incubating the second biological sample on the first and second arrays of the second microarray slide to permit any of the first protein and second protein in the second biological sample to bind to their respective capture antibody; washing off any unbound proteins from the first and second arrays of the second microarray slide; incubating the first detection antibody on the first array of the second microarray slide and incubating the second detection antibody on the second array of the second microarray slide to permit the first detection antibody and the second detection antibody to bind to their respective molecules; detecting the presence of any complex including the first molecule in the first array of the second microarray slide; detecting the presence of any complex including the second molecule in the second array of the second microarray slide; and determining whether the first protein is complexed with the second molecule in the second biological sample and whether the second protein is complexed with the first molecule in the second biological sample.

[0014] In an additional embodiment the first biological sample originates from a healthy person and the second biological sample originates from a diseased person, the first biological sample is treated with a drug and the second biological sample is not treated with the drug, or the first biological sample is exposed to a hormone and the second biological sample is not exposed to the hormone. In each embodiment, any complexing in the first biological sample is compared with any complexing in the second biological sample.

[0015] Another method of the present invention includes detecting an immune response in a subject, comprising: providing a microarray slide, wherein the microarray slide includes an antibody array that is attached to the microarray slide, wherein the array includes a first capture antibody that specifically binds a first auto-antigen and a second capture antibody that specifically binds a second auto-antigen; providing a biological sample that may contain the first or second auto-antigens in their native form; incubating the biological sample on the array of the microarray slide to permit any of the first and second auto-antigens in the biological sample to be captured by their respective capture antibody; washing off the microarray slide; providing a detection antibody that specifically binds to

an auto-antibody; incubating the detection antibody on the array of the microarray slide to permit the detection antibody to bind to any auto-antibody that is complexed with the first or second auto-antigens; and detecting the presence of any auto-antibody in the array of the microarray slide. Additionally, the subject may be a mammal or, more specifically, a human; the first and second auto-antigens may be different isoforms of the same antigen; and the first and second auto-antigens may be tumor antigens. In other embodiments of the present invention, the method further provides that the presence of any auto-antibody that is complexed with the first or second auto-antigens is indicative of an auto-immune disease or cancer. In other embodiments of the invention, the first and second capture antibodies are specific to different epitopes of an auto-antigen or to different isoforms of an auto-antigen.

[0016] Another method of the present invention includes detecting a protein interaction with a small molecule, comprising: providing a microarray slide; wherein the microarray slide includes an antibody array that is attached to the microarray slide; wherein the array includes a first capture antibody that specifically binds a first protein and a second capture antibody that specifically binds a second protein; providing a biological sample that has been exposed to a small molecule and that may contain the first or second proteins in their native form; incubating the biological sample on the array of the microarray slide to permit any of the proteins in the biological sample to be captured by their respective capture antibody; washing off the microarray slide; providing a detection antibody that specifically binds to the small molecule; incubating the detection antibody on the array of the microarray slide to permit the detection antibody to bind to any small molecule that is complexed with the first or second proteins; and detecting the presence of any small molecule in the array of the microarray slide. Additionally, the small molecule may be a drug.

[0017] A further method of detecting a protein interaction with a small molecule includes: providing a microarray slide, wherein the microarray slide includes an antibody array that is attached to the microarray slide and wherein the array includes a first capture antibody that specifically binds a first protein and a second capture antibody that specifically binds a second protein; providing a biological sample that has been exposed to a small molecule that includes a radioactive label, and which biological sample may contain the first or second proteins in their native form; incubating the biological sample on the array of the microarray slide to permit any of the proteins in the biological sample to be captured by

their respective capture antibody; and detecting any radioactivity at each location of the antibody array of the microarray slide.

[0018] Another method of detecting a protein interaction with a small molecule includes: providing a microarray slide, wherein the microarray slide includes an antibody array that is attached to the microarray slide and wherein the array includes a first capture antibody that specifically binds a first protein and a second capture antibody that specifically binds a second protein; providing a biological sample that has been exposed to a tagged small molecule, and which biological sample may contain the first or second proteins in their native form; incubating the biological sample on the array of the microarray slide to permit any of the proteins in the biological sample to be captured by their respective capture antibody; and detecting the tag of the small molecule at each location of the antibody array of the microarray slide. In a further embodiment, the tag of the small molecule may be a biotin or a FLAG tag.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] Figures 1A and B show antibody-array interaction mapping. Figure 1A is a schematic representation of an experimental strategy. Figure 1B shows molecular-level detail on the detection of hypothetical interactions, and shows an interaction between Protein 1 and Protein 3 and detection of Protein 1-Protein 3 complexes.

[0020] Figure 2 shows representative antibody arrays from a pooled serum sample, detected on separate arrays using the indicated detection antibodies. Some of the relevant capture antibody spots are labeled. Each antibody was spotted in three adjacent spots, and a biotin-labeled BSA positive control appears in the lower right of each array.

[0021] Figures 3A and B show clusters of interaction levels between antibody targets. Each cluster shows the results from a set of 47 arrays, comprising 47 different detection antibodies, using one serum sample pool. For each array, the intensities of the capture antibodies were ranked from highest (48) to lowest (1). Each rank was multiplied by the rank of the interaction when the capture and detection antibodies were switched. The matrix of rank-products was log-transformed, median centered, and clustered. The results from two different serum pools are presented, showing by variation and consistency in the patterns of interactions.

[0022] Figure 4 shows RT-PCR analysis of the expression levels of 11 genes in the pancreatic cancer cell lines. PCR products were analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

[0023] Figure 5 shows CEACAM6 co-immunoprecipitates with IL-1beta in the cell culture system. CEACAM6 was released from BxPC3 cell surface by phospholipase C digestion. The soluble CEACAM6 was mixed with conditioned media collected from different cell lines and immunoprecipitated with anti-CEACAM6 antibody. The immuno-complexes were separated by SDS-PAGE, transferred to PVDF membrane, and blotted with anti-CEACAM6 or anti-IL-1beta antibodies.

[0024] Figures 6A-C show protein and glycan detection in cell culture media using antibody arrays. Figure 6A shows detection of cell culture media on antibody arrays using CA 19-9 (left) and IL-8 (right) antibodies. Some of the capture antibodies are labeled. Figure 6B shows detection of cell culture media from six different cell lines using a MUC1 antibody. Figure 6C shows RT-PCR levels of the MUC1 transcript in pancreatic cancer cell lines.

[0025] Figure 7 shows the preparation of antibody arrays. Antibody arrays are printed onto microscope slides: 48 arrays on each slide (only 9 arrays are shown), with 48 antibodies in each array.

[0026] Figure 8 shows the application of a serum sample to the arrays. Hypothetical complexing for proteins 1, 2, and 3 is shown, which bind proteins to the three antibodies.

[0027] Figure 9 shows probing of the arrays. The first three arrays are probed with antibodies targeting proteins 1, 2, and 3, respectively.

[0028] Figure 10 shows detecting bound antibody. The amount of antibody bound at each spot in each array is detected using the streptavidin-phycoerythrin reagent, followed by scanning for fluorescence.

[0029] Figure 11 shows binding profiles of three detection antibodies. The fluorescence intensities (y-axes) at each antibody spot (x-axes) are shown for the PSA, HC-II, and protein S detection antibodies. All three detection antibodies show signal at the PSA, HC-II, and protein S capture antibodies, indicating a possible complex between these three proteins.

[0030] Figure 12 shows an interaction cluster. For each detection antibody, the level of binding at each capture was ranked (48=highest binding, 1=lowest). Each of those ranks was multiplied by the rank of the reverse sandwich – when the capture and detection antibodies were reversed. Therefore, interactions that were high in both directions had high scores. The scores were logged, median centered, and clustered.

[0031] Figure 13 show a network of interactions found in the serum sample. Arrows indicate binding between proteins.

[0032] Figure 14 shows validation of complexes using protein arrays. Purified proteins are spotted onto slides and incubated with other proteins. Protein-protein interactions are detected using antibodies targeting the protein that was incubated.

[0033] Figure 15 shows binding levels of incubated PSA (solid bars) or TBS buffer (white bars, negative control) at various proteins (x-axis). PSA bound to HC-II, but not the other proteins.

[0034] Figure 16 shows HC-II bound to PSA but not the other proteins.

[0035] Figure 17 shows protein S bound to HC-II but not the other proteins.

[0036] Figure 18 shows a PSA-heparin cofactor II-protein S complex model. Heparin cofactor II is in the core of the complex. As to the PSA and heparin cofactor II interaction: PSA is a serine protease, while heparin cofactor II is a serine protease inhibitor (Serpin), which binds to the serine protease covalently. As to the heparin cofactor II and protein S interaction: heparin cofactor binds to sugar groups, while the protein S is a glycoprotein, the binding may occur through the heparin cofactor II sugar binding domain.

[0037] Figure 19 shows an IP-western blot validation of the complex. Serum was immuno-precipitated with different antibodies, indicated in the different lanes. The precipitates were separated by gel electrophoresis, blotted onto nitrocellulose, and protein with anti-HC-II. HC-II was detected in its own IP and in the IPs from PSA and protein S, indicating it was pulled down with PSA and protein S.

[0038] Figure 20 is a graph showing changes in CRP-kininogen complexing and CRP-bradykinin complexing in induced myocardial ischemia over time.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0039] The preferred embodiments of the present invention may be understood more readily by reference to the following detailed description of preferred embodiments and Examples included hereafter.

[0040] *Definitions*

[0041] As used in the present application, “a” can mean one or more, depending on the context with which is it used.

[0042] "Antibody" refers to a polypeptide comprising a framework region from an immunoglobulin gene or fragments thereof that specifically binds and recognizes an antigen. The term also includes genetically engineered forms such as chimeric antibodies (e.g., humanized murine antibodies) and heteroconjugate antibodies (e.g., bispecific antibodies). A "chimeric antibody" is an antibody molecule in which (a) the constant region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding

site (variable region) is linked to a constant region of a different or altered class, effector function and/or species, or an entirely different molecule which confers new properties to the chimeric antibody, e.g., an enzyme, toxin, hormone, growth factor, drug, etc.; or (b) the variable region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. Typically, the antigen-binding region of an antibody will be most critical in specificity and affinity of binding. An exemplary immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V.sub.L) and variable heavy chain (V.sub.H) refer to these light and heavy chains respectively. The term "antibody" also includes antigen binding forms of antibodies, including fragments with antigen-binding capability (e.g., Fab', $F(ab')_2$, Fab, Fv and rIgG) and recombinant single chain Fv fragments (scFv). Antibodies exist, for example, as intact immunoglobulins or as a number of well-characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab)'.sub.2, a dimer of Fab which itself is a light chain joined to V.sub.H-C.sub.H1 by a disulfide bond. The F(ab)'.sub.2 may be reduced under mild conditions to break the disulfide linkage in the hinge region, thereby converting the F(ab)'.sub.2 dimer into an Fab' monomer. The Fab' monomer is essentially Fab with part of the hinge region (see Fundamental Immunology (Paul ed., 3d ed. 1993). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized de novo either chemically or by using recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies, or those synthesized de novo using recombinant DNA methodologies (e.g., single chain Fv) or those identified using phage display libraries (see, e.g., McCafferty et al., Nature 348:552-554 (1990)).

[0043] "Biological sample" means any fluid or other material derived from the body of a normal or diseased subject, such as blood, serum, plasma, lymph, urine, saliva, tears, cerebrospinal fluid, milk, amniotic fluid, bile, ascites fluid, pus, sputum, stool, urine, and the like. Also included within the meaning of the term "biological sample" is an organ or tissue extract, cultured cells (e.g., primary cultures, explants, and transformed cells), and culture fluid in which any cells or tissue preparation from a subject has been incubated. A biological sample is typically obtained from a eukaryotic organism, most preferably a mammal such as a primate (e.g., chimpanzee or human), cow, dog, cat, rodent (e.g., guinea pig, rat, mouse), rabbit, bird, reptile, or fish.

[0044] The term "cell" is used in its usual biological sense, and does not refer to an entire multicellular organism. The cell, for example, can be in vitro, e.g., in cell culture, or present in a multicellular organism, including, e.g., birds, plants and mammals such as humans, cows, sheep, apes, monkeys, swine, dogs, and cats. The cell can be prokaryotic (e.g., bacterial cell) or eukaryotic (e.g., mammalian or plant cell).

[0045] "Protein", "polypeptide", or "peptide" refer to a polymer in which the monomers are amino acids and are joined together through amide bonds, alternatively referred to as a polypeptide. When the amino acids are .alpha.-amino acids, either the L-optical isomer or the D-optical isomer can be used.

[0046] The term "specific" or "specifically" refers to a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and/or other biomolecules. For example, under designated conditions, a specified antibody preferentially binds to a particular protein and does not bind in a significant amount to other proteins present in a sample.

[0047] The term "tumor cell" refers to a cancerous, pre-cancerous or transformed cell, either in vivo, ex vivo, and in tissue culture, that has spontaneous or induced phenotypic changes that do not necessarily involve the uptake of new genetic material. Although transformation can arise from infection with a transforming virus and incorporation of new genomic nucleic acid, or uptake of exogenous nucleic acid, it can also arise spontaneously or following exposure to a carcinogen, thereby mutating an endogenous gene. The term "tumor" includes at least one tumor cell.

[0048] All references, patents, patent publications, articles, and databases, referred to in this application are incorporated-by-reference in their entirety, as if each were specifically and individually incorporated herein by reference.

[0049] The present invention utilizes antibody microarray technology. Microarrays are orderly arrangements of spatially resolved samples or probes (in the present invention, the probes are antibodies of known specificity to a particular protein).

[0050] The underlying concept of antibody microarray depends on binding between proteins and antibodies specific to proteins. Microarray technology adds automation to the process of resolving proteins of particular identity present in an analyte sample by labeling, preferably with fluorescent labels and subsequent binding to a specific antibody immobilized to a solid support in microarray format. An experiment with a single antibody microarray chip is highly throughput, i.e., the chip can provide simultaneous information on protein levels of many genes. Antibody microarray experiments employ common solid supports such as glass slides, upon which antibodies are deposited at specific locations (addresses).

[0051] Antibody microarray analysis generally involves injecting a fluorescently tagged sample of proteins into a chamber on a microarray slide to bind with antibodies having specific affinity for those proteins; laser excitation at the interface of the array surface and the tagged sample; collection of fluorescence emissions by a lens; optical filtration of the fluorescence emissions; fluorescence detection; and quantification of intensity.

[0052] Antibodies used in connection with the present invention are commercially available or may be synthesized by standard methods known in the art. For preparation of antibodies, e.g., recombinant, monoclonal, or polyclonal antibodies, many techniques known in the art can be used (see, e.g., Kohler & Milstein, Nature 256:495-497 (1975); Kozbor et al., Immunology Today 4: 72 (1983); Cole et al., pp. 77-96 in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss,-Inc. (1985); Coligan, Current Protocols in Immunology (1991); Harlow & Lane, Antibodies, A Laboratory Manual (1988); and Goding, Monoclonal Antibodies: Principles and Practice (2d ed. 1986)). The genes encoding the heavy and light chains of an antibody of interest can be cloned from a cell, e.g., the genes encoding a monoclonal antibody can be cloned from a hybridoma and used to produce a recombinant monoclonal antibody. Gene libraries encoding heavy and light chains of monoclonal antibodies can also be made from hybridoma or plasma cells. Random combinations of the heavy and light chain gene products generate a large pool of antibodies with different antigenic specificity (see, e.g., Kuby, Immunology (3.sup.rd ed. 1997)). Techniques for the production of single chain antibodies or recombinant antibodies (U.S. Pat. No. 4,946,778, U.S. Pat. No. 4,816,567) can be adapted to produce antibodies to polypeptides. Also, transgenic mice, or other organisms such as other

mammals, may be used to express humanized or human antibodies (see, e.g., U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, Marks et al., Bio/Technology 10:779-783 (1992); Lonberg et al., Nature 368:856-859 (1994); Morrison, Nature 368:812-13 (1994); Fishwild et al., Nature Biotechnology 14:845-51 (1996); Neuberger, Nature Biotechnology 14:826 (1996); and Lonberg & Huszar, Intern. Rev. Immunol. 13:65-93 (1995)). Alternatively, phage display technology can be used to identify antibodies and heteromeric Fab fragments that specifically bind to selected antigens (see, e.g., McCafferty et al., Nature 348:552-554 (1990); Marks et al., Biotechnology 10:779-783 (1992)). Antibodies can also be made bispecific, i.e., able to recognize two different antigens (see, e.g., WO 93/08829, Traunecker et al., EMBO J. 10:3655-3659 (1991); and Suresh et al., Methods in Enzymology 121:210 (1986)). Antibodies can also be heteroconjugates, e.g., two covalently joined antibodies, or immunotoxins (see, e.g., U.S. Pat. No. 4,676,980, WO 91/00360; WO 92/200373; and EP 03089).

[0053] Preferably, antibodies employed to practice the present invention bind to a selected target antigen with an affinity (association constant) of greater than or equal to 10^7 M^{-1}. When an antibody is referred to as specific for a particular antigen, it means that the binding reaction is determinative of the presence of the antigen in a heterogeneous population of proteins and other biologics. Thus, under suitable conditions, the specified antibodies bind to a particular protein sequences at least two times the background and more typically more than 10 to 100 times the background.

[0054] One method of the present invention is shown in Figure 1. Multiple antibody arrays are prepared, each array containing multiple antibodies targeting proteins that potentially interact. Figure 1 shows 48 antibody microarrays printed on one microscope slide. The center-to-center spacing of the arrays is 4.5 mm (same as the wells of a 384-well microtiter plate), and each array contains 48 different antibodies spotted in triplicate. The antibodies are printed at a concentration of 0.5 mg/ml in a phosphate-buffered saline (PBS) buffer. Lower concentrations of antibodies, down to about 0.1 mg/ml can also work if the antibody is high affinity, and concentrations up to about 1 mg/ml would also work. A hydrophobic wax line is printed around the borders of each array, so that a different sample can be placed on each array without cross-contamination between arrays.

[0055] Because the present invention uses an antibody microarray, it is high throughput, allowing for the analyses of multiple protein complexes simultaneously. A native, non-denatured biological sample, such as a serum sample, is incubated on each array (a portion

of the same sample is used on each array), e.g., 3 microliters of sample per array. Lysates from cultured or fresh tissue could be used, or bodily fluids such as blood, urine or cerebral spinal fluid would also be applicable. The sample is prepared so that the proteins are not denatured and the protein-protein interactions are not greatly disrupted. After washing away unbound proteins, each array is probed with a different biotinylated detection antibody, andeach detection antibody matched to one of the capture antibodies on the arrays. That is, each array is incubated with one detection antibody. For example, a first detection antibody is incubated with a first copy of the antibody microarray, a second detection antibody is incubated with a second copy of the antibody microarray, and a third detection antibody is incubated with a third copy of the antibody microarray. Because a protein can complex with a molecule other than another protein, a detection antibody also may be used to target a molecule other than a protein, e.g., a carbohydrate, lipid, nucleic acid, or small molecule (e.g., a drug) can be analyzed according to the methods of the present invention.

[0056] Each detection antibody binds wherever its targeted protein was retained, whether at the corresponding capture antibody that targets the same protein, or at another antibody that captured a protein in complex with the targeted protein targeted. The detection antibody could target an epitope of the protein that is different than the epitope targeted by the capture antibody.

[0057] The arrays can be incubated with streptavidin-phycoerythrin and scanned for fluorescence to detect the detection antibody and thereby determine that the target of the detection antibody is complexed with the protein captured by the capture antibody. Moreover, the level of such complexing can be determined by measuring the amount of fluorescence of the detection antibody. One could also use another affinity tag besides biotin, such as digoxigenin, or a different fluorophore besides phycoerythrin, such as Cy3 or Cy5. Alternately, other methods besides fluorescence could be used to detect the tagged and bound antibodies. For example, horseradish peroxidase or alkaline phosphatase could be linked to streptavidin, and the addition of the appropriate substrate for those enzymes would lead to the generation of a detectable signal, as is commonly used for ELISA assays. In this way, one can observe all potential protein-protein interactions within a group of proteins. Since it is a native sample, multi-protein complexes may exist, and some of the observed interactions actually may occur through other proteins.

[0058] In one embodiment, if a protein 1 is complexed with proteins 7, 10, and 12, detection antibody 1 binds to the molecule captured by capture antibodies 1, 7, 10, and 12.

Likewise, detection antibodies 7, 10, and 12 will bind to the proteins bound to capture antibody 1.

[0059] The method of the present invention also can reveal networks of multiple protein complexes, e.g., protein 1 might be complexed with proteins 3, 5, and 7, but also might be complexed with protein 12. In this instance, using the methods of the present invention, one can detect protein 12 binding with protein 1 at capture antibody 1, protein 1 binding with protein 12 at capture antibody 12, but protein 12 will not be detected as binding to proteins 3, 5, and 7 (protein 12 will only be detected as binding to protein 1 at capture antibodies 1 and 12). Molecular complexes formed from many proteins can be examined with the present method, i.e., the detection method is not limited to detecting binary interactions.

[0060] Using the method of the present invention, changes in protein complexes under various conditions can be examined. These analyses may involve examining one biological sample on one microarray slide according to the method described hereinabove, and examining a different biological sample on a second microarray slide according to the methods described hereinabove. The complexing revealed in the two samples then can be compared. Tested conditions might be in cell culture, by treatment with a drug, hormone, or growth factor. Further, using the present invention, changes in protein complexes can be examined as between healthy persons and persons having a disease, such as cancer.

[0061] The invention also would be useful to study interactions within a family of proteins. For example, one could study, using the present method, coagulation pathways relating to blood disorders (such as hemophilia, bleeding disorders, and coagulation disorders), because these processes are regulated by complex networks of protein-protein interactions in the blood. The ability to examine families could yield new interactions related to disease.

[0062] In addition to using the methods of the present invention to detect the presence of protein complexes, the levels of the complexes also can be quantitated.

[0063] Another method of the present invention includes the detection of auto-antigen interactions with auto-antibodies (auto-immune complexes). In this method, a biological sample is incubated on an array of capture antibodies targeting potential auto-antigens. After the auto-antigens have bound the corresponding capture antibodies, the array is washed and probed with a labeled anti-immunoglobulin detection antibody. This anti-Ig detection antibody is used to detect whether any auto-antibodies are complexed with any of the auto-antigens that have been captured by the capture antibodies. This method

depends upon those auto-antigen/auto-antibody complexes that remain complexed in a biological sample. When the biological sample is from an individual, the individual's own auto-antigens are used as the basis to detect (and quantify) the level of auto-antibodies present in the biological sample. In one embodiment of the present method, for each auto-antigen of interest, capture antibodies are prepared for several isoforms of each auto-antigen. These isoforms could be splice variants, cleavage variants, or proteins or peptides resulting from various post-translational modifications of the auto-antigen. By using many different forms of capture antibody against one auto-antigen, one could determine particular forms of the auto-antigen that are more immunogenic. In another embodiment, one could utilize multiple captive antibodies against the same auto-antigen, each capture antibody targeting a different epitope of the auto-antigen, to determine whether certain epitopes are present at a higher level in certain conditions, such as cancer. An array including the capture antibodies for multiple epitopes of an auto-antigen is highly likely to capture an auto-antigen (and consequently any auto-antigen/auto-antibody complexes) in a large cross-section of the population. This method provides a significant advance over known methods which utilize a common or universal auto-antigen to attempt to detect auto-antibodies in biological samples.

[0064] Using the present method, one could use ten captive antibodies against a certain protein, and only one of them may bind to a region of the auto-antigen that was mutated in cancer (not previously known). By looking at the binding of all combinations of capture and detection antibodies for detecting the molecule in both healthy and cancer samples, one could observe that one particular combination using the antibody in question showed much different binding in the cancer samples than in the healthy samples. From that it could be concluded that this antibody must bind a cancer-associated epitope.

[0065] Another method of the present invention includes probing capture antibody arrays with affinity reagents to target other types of molecules. These methods can detect interactions between a protein (or set of proteins) and a small molecule, e.g., a drug. More specifically, this method includes providing an affinity reagent (e.g., an antibody, single-chain antibody, etc) against the drug, peptide, or small molecule of interest. In this method, for example, a biological sample is treated with a drug to be studied. Proteins from the sample are incubated on an array of antibodies that target various proteins that may interact with the drug. The array is then washed and probed with an affinity reagent that targets the drug. The array location of binding of that affinity reagent reveals the

proteins with which the drug interacts. This method could be useful for optimizing doses, or for biological studies.

[0066] In the absence of a suitable affinity reagent, a protein-drug complex can be detected by tagging the drug prior to treatment. For example, the drug could be tagged with biotin and then streptadavin attached to a flourophore could be used to detect the drug. Additionally, a peptide tag, such as a FLAG (Sigma) tag also could be used, or the drug could be synthesized to include a radioactive label.

[0067] The novel detection methods of the present invention are high throughput and can be used to quantitatively measure changes over conditions, or in many different samples. They could be useful in disease monitoring or diagnosis, or to determine whether a drug is efficacious. For example, if a drug was expected to prevent signaling through a certain pathway which was characterized by protein A and protein B forming a complex, one could examine biological samples over the course of treatment with the drug, or at different doses, and determine how the complexing changes at different times (see Example 5 below) or with different doses.

[0068] *Example 1: Analysis of Pancreatic Cancer Samples Using the Method of the Invention*

[0069] The present inventive method was applied to the study of 12 different samples. Each sample was a pool of serum samples from 26-30 different patients. Four pools were from pancreatic cancer patients, four were from patients with benign pancreatic disease, and four were from healthy controls. The use of pools allowed a view of averages in the population without running dozens of samples, which was impractical at this early stage of development. Each of the 12 samples was applied to all of the 48 arrays on one slide, and the 48 arrays were each detected with a different detection antibody (as in Figure 1). Representative images from one of the slides are shown in Figure 2. Some of the detection antibodies bound only at the location of their corresponding capture antibody, such as anti-kininogen. Other detection antibodies bound at the location of not only their corresponding capture antibody, but and also at the location of several other capture antibodies, indicating protein-protein interactions between the target of the detection antibody and the target of the other capture antibodies. Some of these observed protein-protein interactions were previously known, which supports the validity of the present method. For example, haptoglobin was shown to strongly interact with hemoglobin, and vice versa, which is a known interaction. Platelet factor 4 (PF4) is known to interact with IL-8 (56), which was seen on the arrays. The CA 19-9 antibody bound at glycoproteins

known to carry that epitope, such as MUC1, CEACAM6, and CA 125. Other potential interactions had not been seen before and thus can be further studied.

[0070] *Example 2: Validation of Protein-Protein Interactions*

[0071] Newly-observed interactions could be validated using another method, since non-specific cross-reactivity of an antibody could give rise to false-positive results. In one respect, the use of an array contains many internal negative controls. The binding of a detection antibody to its proper target without binding to many of the other antibodies on array gives some indication that the antibody is specific. Further, if an interaction is observed using either antibody in a pair as the capture or detection antibody, or if more than one capture or detection antibody give the same result, the interaction is more likely to be real. Western blots on serum samples are useful to show a general level of cross reactivity of an antibody. Clean, single bands in the western blot of biological samples indicate a detection antibody has low general cross-reactivity. Protein-protein interactions could be validated by binding assays between purified proteins, by immunoprecipitation-western blot, by immunoprecipitation-mass spectrometry, or by the use of additional, different antibodies targeting the same proteins. All these methods have been used to validate some of the newly-observed interactions described above.

[0072] Immunoprecipitation/mass spectrometry was used to validate a CRP-kininogen interaction. Biotinylated anti-CRP was incubated with a serum pool and precipitated using streptavidin-coated beads. The eluent from those beads was analyzed using mass spectrometry, and one of the top identifications after CRP was kininogen. Negative control beads, using normal mouse IgG instead of anti-CRP, showed no kininogen in the identifications. Both the CRP and kininogen antibodies showed single bands in western blots of serum samples (not shown).

[0073] Another newly-observed interaction was validated using protein arrays. Strong interactions were seen between PSA, heparin cofactor II, and protein S (Figures 11, 15-18). Recombinant versions of these proteins were obtained and printed in microarrays, along with some negative control proteins. The individual proteins were incubated on the arrays, and the binding level of the proteins to each spotted protein was detected using a biotinylated antibody followed by streptavidin-phycoerythrin. The PSA and the heparin cofactor II bound each other, but the protein S only bound heparin cofactor II. This validated the direct interaction between PSA and heparin cofactor II and also indicated that the observed interaction between protein S and PSA may be mediated through heparin cofactor II.

[0074] Another interaction, between IL-1beta and CEACAM6, was validated by observing consistent results on the arrays with two different IL-1beta antibodies and by immunoprecipitation-western blot (Figure 5). These validation studies show that the present invention is useful for discovering new protein-protein interactions.

[0075] *Example 3: Interaction Clustering*

[0076] Some of the observed interactions seemed to take place among clusters of proteins, in which all the proteins interacted with each other. The inventor used a cluster method to better visualize the grouping of interactions among the set of antibodies. An interaction between two proteins is measured twice in this method: once using one of the antibodies from the pair as the detection antibody, and again using the other antibody from the pair as the detection antibody. The strongest interactions will show good signal using either antibody as the detection antibody. Therefore, the level of interaction between two proteins was scored by multiplying the two types of measurements. All of the interaction scores were clustered (Figures 3A and 3B) to look at the relative strengths of interactions as well as the higher patterns of interactions. The inventor made a cluster of all 12 serum pools, of which two representatives are shown in Figures 3A and 3B. The values on the diagonal are the sandwich assays, measuring the level of a single protein, and the clusters are symmetric about the diagonal. A fair amount of diversity was seen in the cluster patterns between the 12 serum pools. Some showed tight clusters of interactions (Figure 3A), and others had more diffuse interactions (Figure 3B). Interactions among the group of proteins identified by arrows in Figures 3A and 3B were regularly seen. The cluster in Figure 3A indicates that all members of that cluster interacted with all the other members. The group is less tight in Figure 3B, but some level of interaction is still seen among the members.

[0077] *Example 4: Cell Culture Studies*

[0078] Cell culture experiments were designed to more thoroughly study the interactions among IL-1beta, CEACAM6, MUC1, tenascin C, CRP, 90k, and CA 19-9-containing proteins. Having a cell culture system to study these interactions is useful since it is a less complex system than serum and because the expression of individual proteins can be manipulated. Many different pancreatic cancer cell lines were tested for their expression of these proteins. The cell lines tested are shown in Table 1.

[0079] Table 1

Cell line	Morphology	Derived from metastatic site	Tumorogenic
Hs 766T	Epithelial	Lymph node	Yes, in immunosuppressed mice
HPAF-ll	Epithelial	-	Yes, in athymic mice which resemble the original tumor
PANC-1	Epithelial	-	The cells will grow in soft agar
SW1990	Epithelial	Spleen	Yes, forms tumors in nude mice
BxPC-3	Epithelial	-	Yes, tumor developed within 21 days at 100% frequency in nude mice
Capan-2	Polygonal	-	Yes, in nude mice; forms well differentiated adenocarcinoma
CFPAC-1	Epithelial	-	Yes, in nude mice
Capan-1	Epithelial	Liver	Yes, in nude mice; forms adenocarcinoma
Su.86.86	Epithelial	Liver	Yes, in nude mice
MPanc-96	Epithelial	-	Yes, in SCID mice
HPAC	Epithelial	-	Yes, the cells form tumors in athymic nude mice
AsPC-2	Epithelial	-	?
L3-3	Epithelial	-	Derived from COLO357, highly metastatic
MIAPACA	Epithelial	-	?

[0080] Most of the cell lines tested are highly tumorigenic, and some were derived from metastases. Total RNA was collected from each, and first strand cDNAs were synthesized.

PCR primers were designed to probe the expression of the 11 different genes. The primers were designed to encompass an intron, so that amplification from contaminating genomic DNA would produce a product of the wrong size. Figure 4 shows the expression levels of the tested genes. Some were expressed in all cell lines, and others were more variable. This information should allow for identification of the cell culture media which contains certain groups of proteins, so that the interactions between those proteins can be further studied.

[0081] The protein CEACAM6 is normally membrane bound via a GPI anchor. In order to examine the interactions in the media, phospholipase C was used to cleave the GPI anchor at the juxta-membrane position and release it into the media. The cleavage took place after the normal media had been rinsed away. The CEACAM6 preparation was mixed with the media from several cells lines that either did or did not express IL-1beta. After the media were mixed, CEACAM6 was immunoprecipitated. The eluent was probed by western blot for the presence of both CEACAM6 and IL-1beta, which were predicted to interact by the present invention. CEACAM6 protein was found in all media, and the media that was not spiked with the CEACAM6 preparation showed very minimal levels, meaning that some CEACAM6 normally escapes into the media. The detection with anti-IL-1beta (17 kD) showed that is co-immunoprecipitated with CEACAM6 only when using the Hs766T and Su.86.86 cell lines. Those cell lines express high IL-1beta (Figure 4), and none of the cell lines that showed low IL-1beta showed co-IP in Figure 5. The BxPC3 cell line expressed high IL-1beta showed no band in the co-IP, perhaps because something besides the presence of those two proteins regulated their interaction. This validates the interaction between IL-1beta and CEACAM6 and also establishes cell culture models for studying interactions among the group of proteins observed in Figure 3.

[0082] Also, the detection of proteins expressed (in Figure 4) in the media of these cultures was tested. Several of the media were incubated on antibody arrays and probed with different detection antibodies. Figure 6A shows the detection of many of the glycoproteins in the media using the CA 19-9 detection antibody, and also shows that these glycoproteins bear the sialyl Lewis^{A} epitope in that cell culture. This set of experiments did not make use of the same pair of IL-1beta antibodies used in the experiments of Figures 2 and 3. However, the ability to specifically detect cytokines in cell culture media was confirmed, as shown for IL-8. The abundance of MUC1 in the media (Figure 6B) was closely correlated with the MUC1 transcript levels (Figure 6C). For example, the cell line Su 86.86 showed no expression both in the media and at the

RNA level, and the cell line Capan-2 showed high levels in both. So, for MUC1, media levels could be predicted based on the expression levels. Another interesting result from this experiment was the high binding of the anti-MUC1 antibody at the anti-CA19-9 capture antibody in the media from the Capan-2 and BxPC3 cell lines but in none others. Therefore a proportionally higher level of the CA19-9 epitope is present on MUC1 in those two cell lines. The identification cell lines with secreted MUC1 bearing either high or low CA 19-9 epitopes will be useful to study the functional consequences of the presence of that epitope.

[0083] *Example 5: Testing the Time Dependence of C-Reactive Protein Levels Over Time*

[0084] Methods of the present invention have been used to test the time-dependence of C-reactive protein (CRP) over time. Specifically, a CRP-kininogen complex has been elucidated and a CRP-bradykinin complex also has been determined using the present invention. See the attached Figures.

[0085] CRP is known to be elevated in association with injury and infection (acute-phase reactin), it functions in innate immunity, and is a significant risk factor for heart disease. Kininogen circulates in complex with pre-kallikrein and factor XI; is activated after injury, coagulation; when cleaved by kallikrein, it releases the peptide bradykinin, which is involved in vascular muscular relaxation, edema, and pain; and is rapidly cleaved by circulating peptidases. Cleaved kininogen chains are involved in protease inhibition and angiogenesis inhibition. Recent reports also suggest the involvement of CRP in bradykinin-related pathologies. Angioedema in patients treated with ACE-inhibitor only occurs when CRP is high. In CRP-transgenic mice, the effects of angiotensity and giotensin are greatly enhanced when CRP is high. CRP in kininogen can bind the same receptor on endothelial cells. CRP down-regulates eNOS, and bradykinin up-regulates eNOS.

[0086] The present invention was used to test the time-dependence of CRP-interactions in induced myocardial ischemia. Myocardial ischemia was induced in five patients (three samples each) by inflating a balloon in the left interior descending (LAD) coronary artery, thus occluding blood flow. Peripheral venous blood sampling was performed on the patients at baseline (before myocardial ischemia) and ten minutes and twenty-four hours after the myocardial ischemic event. Using the present method, it was determined that there is a negative correlation between CRP-bradykinin complexing and CRP-kininogen complexing over time (Figure 20).

What is claimed is:

1. A method of analyzing a protein complexed with another molecule, comprising:

providing a first microarray slide;

wherein the first microarray slide includes a first antibody array that is attached to the microarray slide and a second antibody array that is attached to the first microarray slide and which second array is separated from the first array;

wherein the first array includes a first capture antibody for specifically binding a first protein and a second capture antibody for specifically binding a second protein;

wherein the second array includes at least the first and second capture antibodies that are attached to the first microarray slide;

providing a first biological sample that may contain the first and second proteins in their native form;

incubating the first biological sample on the first and second arrays of the first microarray slide to permit any of the first protein and second protein in the first biological sample to be captured by their respective capture antibody;

washing off any uncaptured proteins from the first and second arrays of the first microarray slide;

providing a first detection antibody that specifically binds to a first molecule;

providing a second detection antibody that specifically binds to a second molecule;

incubating the first detection antibody on the second array of the first microarray slide and incubating the second detection antibody on the first array of the first microarray slide to permit the first detection antibody and the second detection antibody to bind to their respective molecules;

detecting the presence of any first molecule in the second array of the first microarray slide;

detecting the presence of any second molecule in the first array of the first microarray slide; and

determining whether the first protein is complexed with the second molecule in the first biological sample and whether the second protein is complexed with the first molecule in the first biological sample.

2. The method of Claim 1 wherein the first molecule is selected from the group consisting of a protein, carbohydrate, lipid, nucleic acid, or small molecule.

3. The method of Claim 1 wherein the second molecule is selected from the group consisting of a protein, carbohydrate, lipid, nucleic acid, or small molecule.

4. The method of Claim 1 wherein the first biological sample is serum.

5. The method of Claim 4 wherein the first biological sample is blood.

6. The method of Claim 1 wherein the first biological sample is at least one cell from a cell culture or tissue.

7. The method of Claim 1 wherein the first protein and the first molecule are the same.

8. The method of Claim 7 wherein the first capture antibody and the first detection antibody are specific to different epitopes on the first protein.

9. The method of Claim 7 wherein the second protein and the second molecule are the same.

10. The method of Claim 9 wherein the second capture antibody and the second detection antibody are specific to different epitopes on the second protein.

11. The method of Claim 1 further comprising determining the quantity in the second array of any complex that includes the first molecule.

12. The method of Claim 1 further comprising determining the quantity in the first array of any complex that includes the second molecule.

13. The method of Claim 1 wherein the first detection antibody is tagged with a first tag and the second detection antibody is tagged with a second tag.

14. The method of Claim 13 wherein the first and second detection antibodies are biotinylated.

15. The method of Claim 14 further comprising incubating the first and second arrays with streptavidin-phycoerythrin.

16. The method of Claim 15 further comprising scanning the first detection antibody and the second detection antibody for fluorescence.

17. The method of Claim 1 further comprising scanning the first detection antibody and the second detection antibody for chemoluminescence.

18. The method of Claim 1 further comprising calorimetrically detecting the presence of the first detection antibody and the second detection antibody.

19. The method of Claim 1 further comprising:

providing a second microarray slide that includes the same first array and the same second array as the first microarray slide;

providing a second biological sample that may contain the first and second proteins in their native form;

incubating the second biological sample on the first and second arrays of the second microarray slide to permit any of the first protein and second protein in the second biological sample to bind to their respective capture antibody;

washing off any unbound proteins from the first and second arrays of the second microarray slide;

incubating the first detection antibody on the second array of the second microarray slide and incubating the second detection antibody on the first array of the second microarray slide to permit the first detection antibody and the second detection antibody to bind to their respective molecules;

detecting the presence of any complex including the first molecule in the second array of the second microarray slide;

detecting the presence of any complex including the second molecule in the first array of the second microarray slide; and

determining whether the first protein is complexed with the second molecule in the second biological sample and whether the second protein is complexed with the first molecule in the second biological sample.

20. The method of Claim 19 wherein the first biological sample originates from a healthy person and the second biological sample originates from a diseased person.

21. The method of Claim 20 further comprising comparing any complexing in the first biological sample with any complexing in the second biological sample.

22. The method of Claim 19 wherein the first biological sample is treated with a drug and the second biological sample is not treated with the drug.

23. The method of Claim 22 further comprising comparing any complexing in the first biological sample with any complexing in the second biological sample.

24. The method of Claim 19 wherein the first biological sample is exposed to a hormone and the second biological sample is not exposed to the hormone.

25. The method of Claim 24 further comprising comparing any complexing in the first biological sample with any complexing in the second biological sample.

26. A method of detecting immune response in a subject, comprising:

providing a microarray slide;

wherein the microarray slide includes an antibody array that is attached to the microarray slide;

wherein the array includes a first capture antibody that specifically binds a first auto-antigen and a second capture antibody that specifically binds a second auto-antigen;

providing a biological sample that may contain the first or second auto-antigens in their native form;

incubating the biological sample on the array of the microarray slide to permit any of the first and second auto-antigens in the biological sample to be captured by their respective capture antibody;

washing off the microarray slide;

providing a detection antibody that specifically binds to an auto-antibody;

incubating the detection antibody on the array of the microarray slide to permit the detection antibody to bind to any auto-antibody that is complexed with the first or second auto-antigens; and

detecting the presence of any auto-antibody in the array of the microarray slide.

27. The method of claim 26 wherein the subject is a mammal.

28. The method of claim 27 wherein the mammal is a human.

29. The method of claim 26 wherein the first and second auto-antigens are different isoforms of the same antigen.

30. The method of claim 26 wherein the first and second auto-antigens are tumor antigens.

31. The method of claim 26 wherein the presence of any auto-antibody that is complexed with the first or second auto-antigens is indicative of an auto-immune disease.

32. The method of claim 26 wherein the presence of any auto-antibody that is complexed with the first or second auto-antigens is indicative of cancer.

33. The method of claim 26 wherein the first capture antibody and second capture antibody are specific to different epitopes of an auto-antigen.

34. The method of claim 26 wherein the first capture antibody and second capture antibody are specific to different isoforms of an auto-antigen.

35. A method of detecting a protein interaction with a small molecule, comprising:

providing a microarray slide;

wherein the microarray slide includes an antibody array that is attached to the microarray slide;

wherein the array includes a first capture antibody that specifically binds a first protein and a second capture antibody that specifically binds a second protein;

providing a biological sample that has been exposed to a small molecule and that may contain the first or second proteins in their native form;

incubating the biological sample on the array of the microarray slide to permit any of the proteins in the biological sample to be captured by their respective capture antibody;

washing off the microarray slide;

providing an affinity reagent that specifically binds to the small molecule;

incubating the affinity reagent on the array of the microarray slide to permit the affinity reagent to bind to any small molecule that is complexed with the first or second proteins; and

detecting the presence of any small molecule in the array of the microarray slide.

36. The method of claim 35 wherein the small molecule is a drug.

37. The method of claim 35 wherein the affinity reagent is a detection antibody.

38. A method of detecting a protein interaction with a small molecule, comprising:

providing a microarray slide;

wherein the microarray slide includes an antibody array that is attached to the microarray slide;

wherein the array includes a first capture antibody that specifically binds a first protein and a second capture antibody that specifically binds a second protein;

providing a biological sample that has been exposed to a small molecule that includes a radioactive label, and which biological sample may contain the first or second proteins in their native form;

incubating the biological sample on the array of the microarray slide to permit any of the proteins in the biological sample to be captured by their respective capture antibody; and

detecting any radioactivity at each location of the antibody array of the microarray slide.

39. A method of detecting a protein interaction with a small molecule, comprising:

providing a microarray slide;

wherein the microarray slide includes an antibody array that is attached to the microarray slide;

wherein the array includes a first capture antibody that specifically binds a first protein and a second capture antibody that specifically binds a second protein;

providing a biological sample that has been exposed to a tagged small molecule, and which biological sample may contain the first or second proteins in their native form;

incubating the biological sample on the array of the microarray slide to permit any of the proteins in the biological sample to be captured by their respective capture antibody; and

detecting the tag of the small molecule at each location of the antibody array of the microarray slide.

40. The method of claim 39, wherein the tag of the small molecule is biotin or FLAG tag.

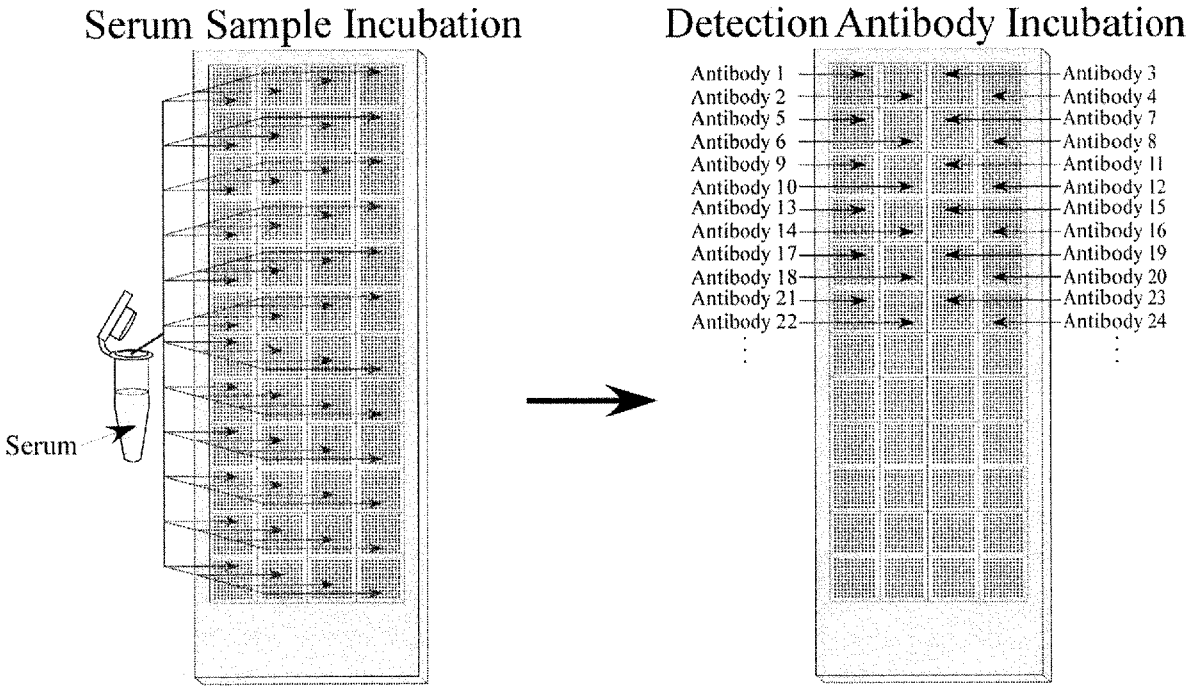
Serum Sample Incubation
Serum
Detection Antibody Incubation
Antibody 1
Antibody 2
Antibody 5
Antibody 6
Antibody 9
Antibody 10
Antibody 13
Antibody 14
Antibody 17
Antibody 18
Antibody 21
Antibody 22
Antibody 3
Antibody 4
Antibody 7
Antibody 8
Antibody 11
Antibody 12
Antibody 15
Antibody 16
Antibody 19
Antibody 20
Antibody 23
Antibody 24

FIGURE 1A

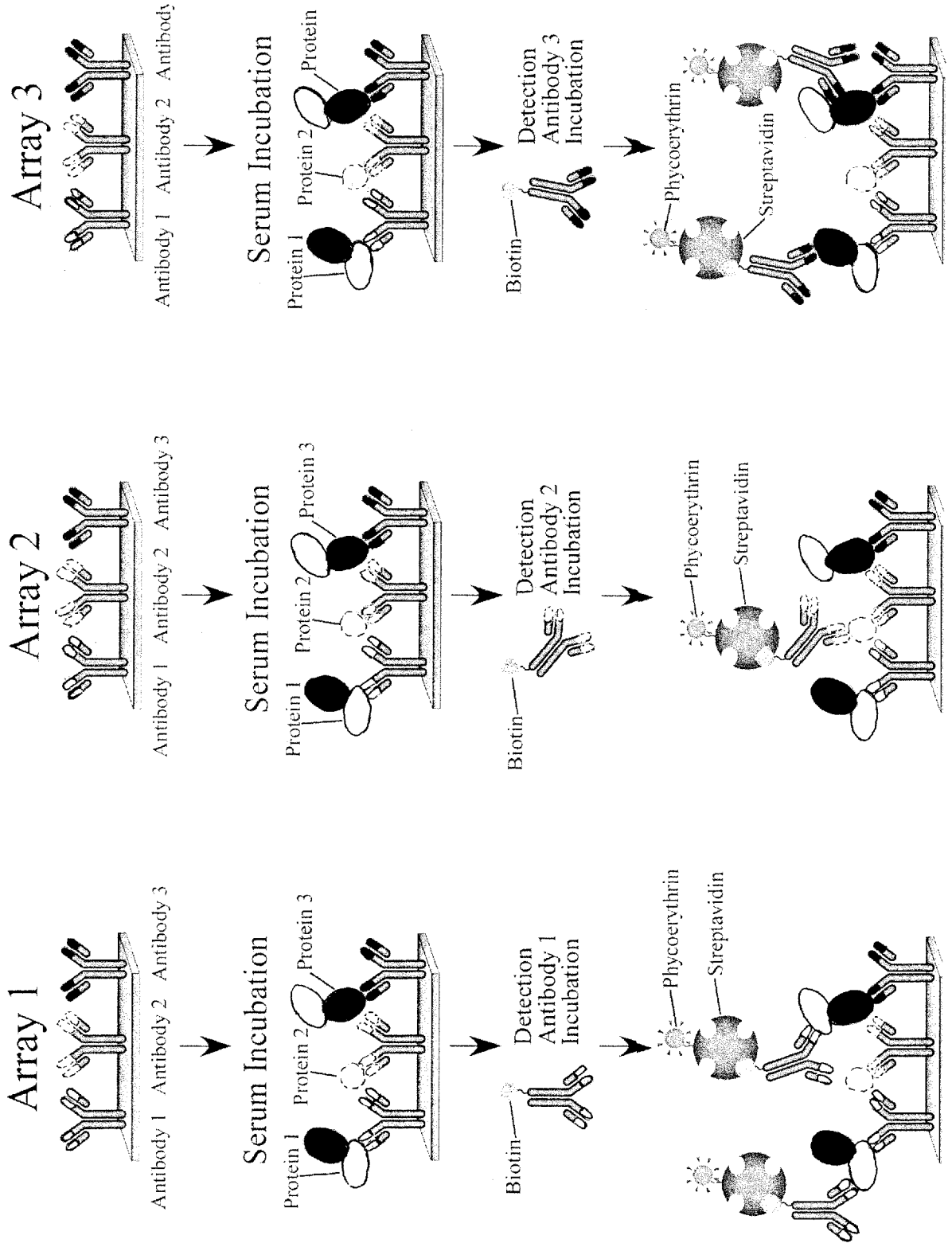
Array 1
Antibody 1
Antibody 2
Antibody 3
Serum Incubation
Protein 1
Protein 2
Protein 3
Detection Antibody 1 Incubation
Biotin
Phycoerythrin
Streptavidin
Array 2
Antibody 1
Antibody 2
Antibody 3
Serum Incubation
Protein 1
Protein 2
Protein 3
Detection Antibody 2 Incubation
Biotin
Phycoerythrin
Streptavidin
Array 3
Antibody 1
Antibody 2
Antibody
Serum Incubation
Protein 1
Protein 2
Protein
Detection Antibody 3 Incubation
Biotin
Phycoerythrin
Streptavidin

FIGURE 1B

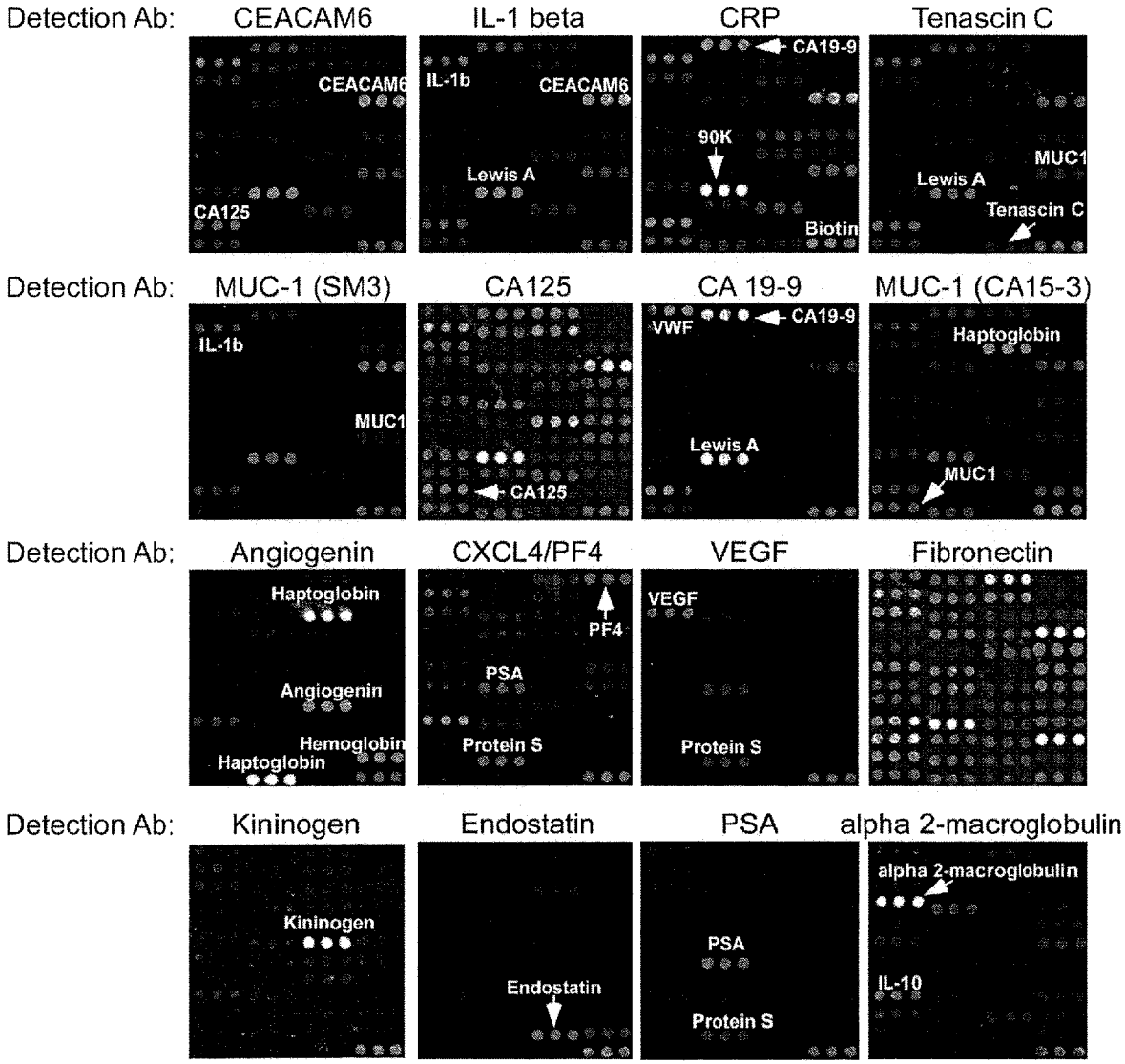
Detection Ab:
CEACAM6
IL-1 beta
CRP
Tenascin C
CEACAM6
CA125
IL-1b
CEACAM6
Lewis A
CA19-9
90K
Biotin
MUC1
Lewis A
Tenascin C
Detection Ab:
MUC-1 (SM3)
CA125
CA 19-9
MUC-1 (CA15-3)
IL-1b
MUC1
CA125
VWF
CA19-9
Lewis A
Haptoglobin
MUC1
Detection Ab:
Angiogenin
CXCL4/PF4
VEGF
Fibronectin
Haptoglobin
Angiogenin
Hemoglobin
Haptoglobin
PF4
PSA
Protein S
VEGF
Protein S
Detection Ab:
Kininogen
Endostatin
PSA
alpha 2-macroglobulin
Kininogen
Endostatin
PSA
Protein S
alpha 2-macroglobulin
IL-10

FIGURE 2

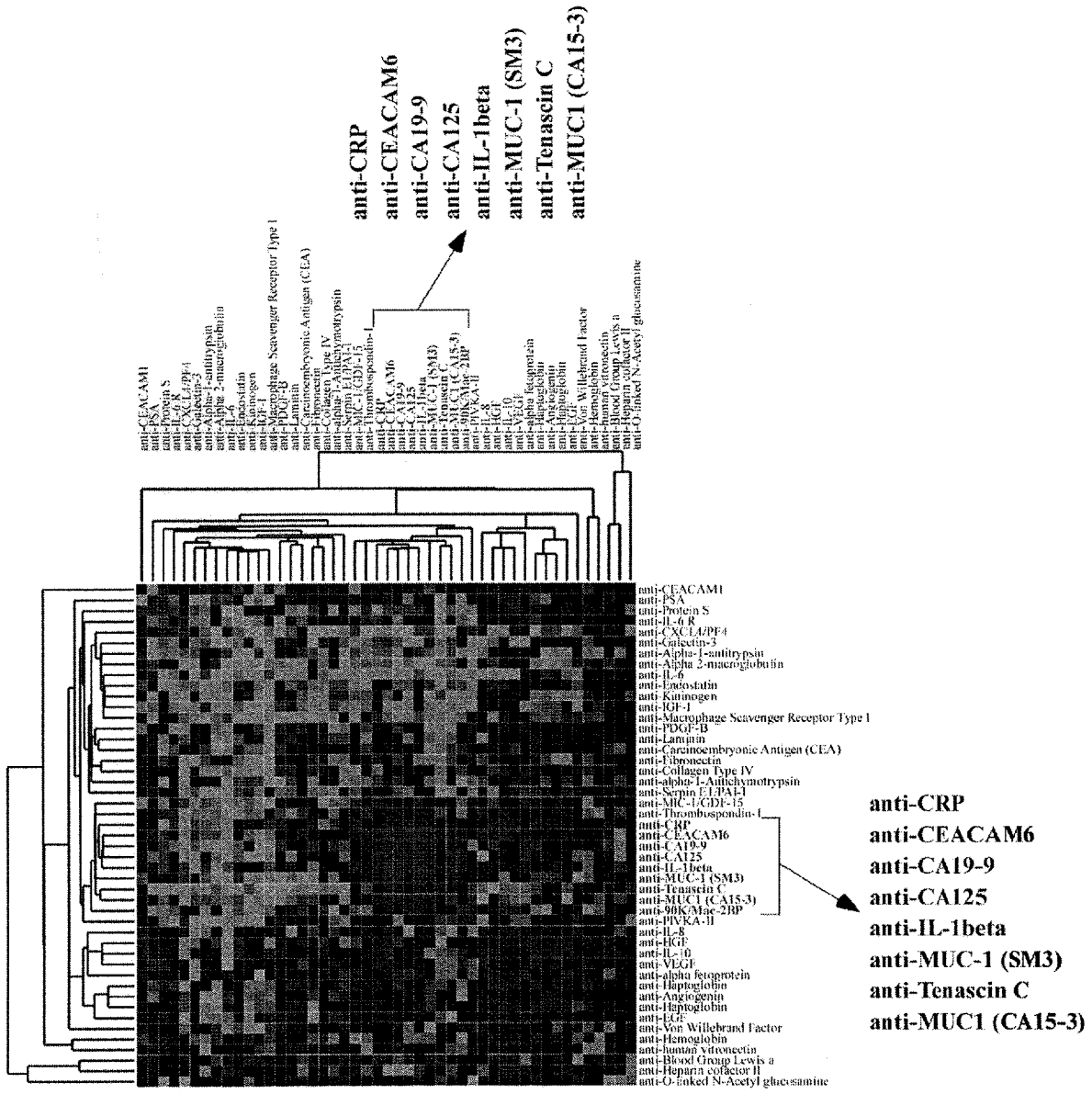
anti-CRP
anti-CEACAM6
anti-CA19-9
anti-CA125
anti-IL-1beta
anti-MUC-1 (SM3)
anti-Tenascin C
anti-MUC1 (CA15-3)
anti-CEACAM1
anti-PSA
anti-Protein S
anti-IL-6 R
anti-CXCL4/PF4
anti-Galectin-3
anti-Alpha-1-antitrypsin
anti-Alpha 2-macroglobulin
anti-IL-6
anti-Endostatin
anti-Kininogen
anti-IGF-1
anti-Macrophage Scavenger Receptor Type 1
anti-PDGF-B
anti-Laminin
anti-Carcinoembryonic Antigen (CEA)
anti-Fibronectin
anti-Collagen Type IV
anti-alpha-1-Antichymotrypsin
anti-Serpin E1/PAI-1
anti-MIC-1/GDF-15
anti-Thrombospondin-1
anti-CRP
anti-CEACAM6
anti-CA19-9
anti-CA125
anti-IL-1beta
anti-MUC-1 (SM3)
anti-Tenascin C
anti-MUC1 (CA15-3)
anti-90K/Mac-2BP
anti-PIVKA-II
anti-IL-8
anti-HGF
anti-IL-10
anti-VEGF
anti-alpha fetoprotein
anti-Haptoglobin
anti-Angiogenin
anti-Haptoglobin
anti-EGF
anti-Von Willebrand Factor
anti-Hemoglobin
anti-human vitronectin
anti-Blood Group Lewis a
anti-Heparin cofactor II
anti-O-linked N-Acetyl glucosamine
anti-CRP
anti-CEACAM6
anti-CA19-9
anti-CA125
anti-IL-1beta
anti-MUC-1 (SM3)
anti-Tenascin C
anti-MUC1 (CA15-3)

FIGURE 3A

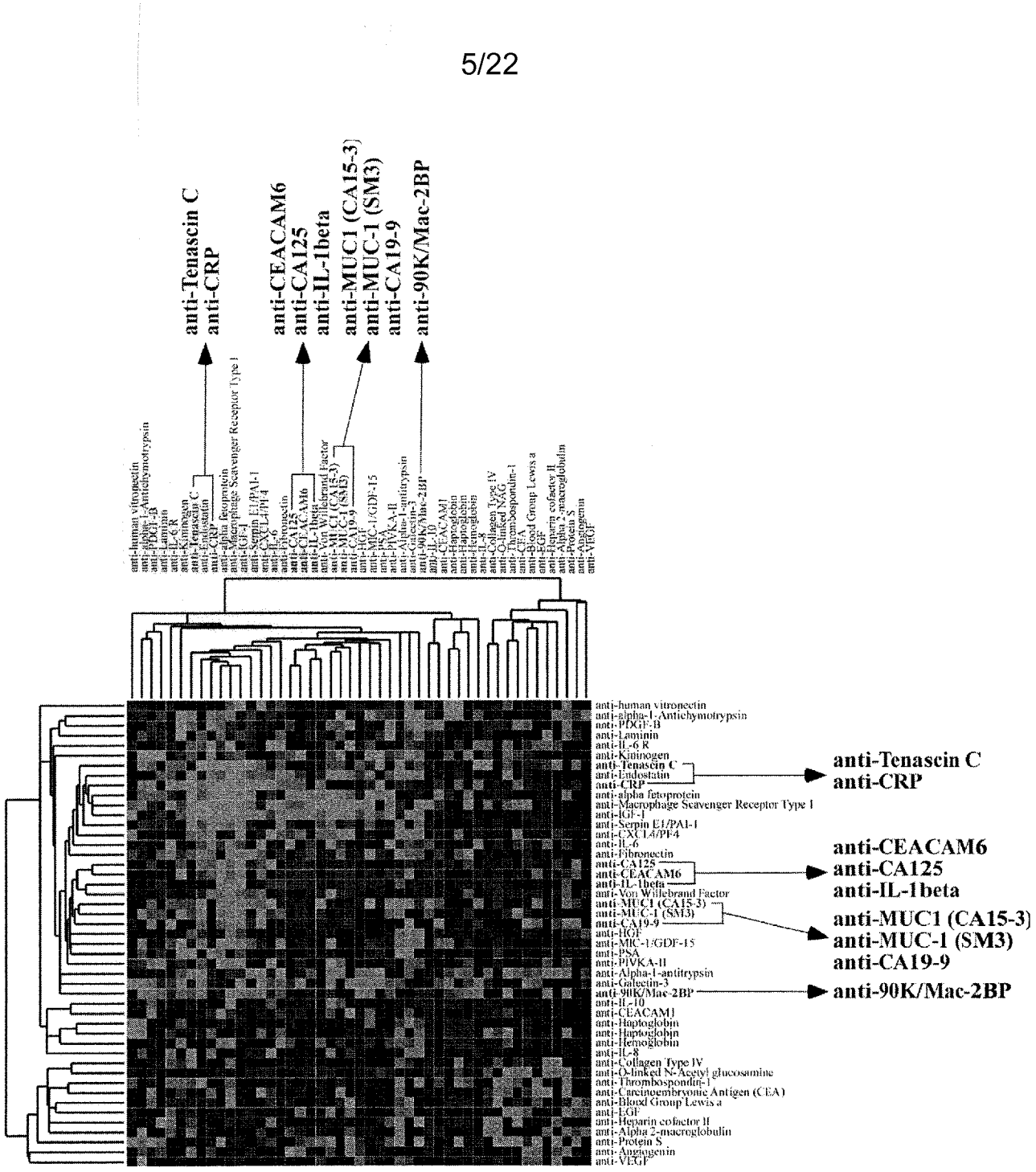
anti-Tenascin C
anti-CRP
anti-CEACAM6
anti-CA125
anti-IL-1beta
anti-MUC1 (CA15-3)
anti-MUC-1 (SM3)
anti-CA19-9
anti-90K/Mac-2BP
anti-human vitronectin
anti-alpha-1-Antichymotrypsin
anti-PDGF-B
anti-Laminin
anti-IL-6 R
anti-Kininogen
anti-Tenascin C
anti-Endostatin
anti-CRP
anti-alpha fetoprotein
anti-Macrophage Scavenger Receptor Type I
anti-IGF-I
anti-Serpin E1/PAI-1
anti-CXCL4/PF4
anti-IL-6
anti-Fibronectin
anti-CA125
anti-CEACAM6
anti-IL-1beta
anti-Von Willebrand Factor
anti-MUC1 (CA15-3)
anti-MUC-1 (SM3)
anti-CA19-9
anti-HGF
anti-MIC-1/GDF-15
anti-PSA
anti-PIVKA-II
anti-Alpha-1-antitrypsin
anti-Galectin-3
anti-90K/Mac-2BP
anti-IL-10
anti-CEACAM1
anti-Haptoglobin
anti-Haptoglobin
anti-Hemoglobin
anti-IL-8
anti-Collagen Type IV
anti-O-linked N-Acetyl glucosamine
anti-Thrombospondin-1
anti-CEA
anti-Blood Group Lewis a
anti-EGF
anti-Heparin cofactor II
anti-Alpha 2-macroglobulin
anti-Protein S
anti-Angiogenin
anti-VEGF
anti-Tenascin C
anti-CRP
anti-CEACAM6
anti-CA125
anti-IL-1beta
anti-MUC1 (CA15-3)
anti-MUC-1 (SM3)
anti-CA19-9
anti-90K/Mac-2BP

FIGURE 3B

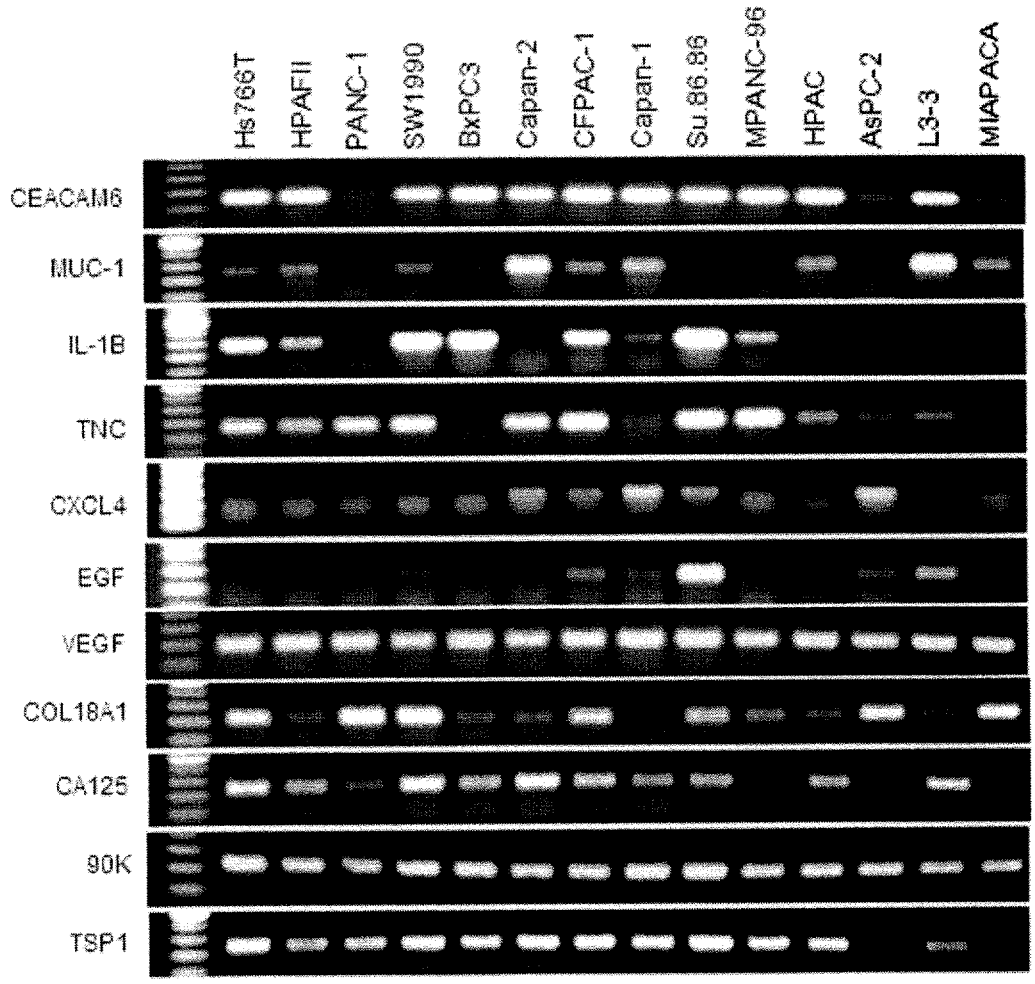
Hs766T
HPAFII
PANC-1
SW1990
BxPC3
Capan-2
CFPAC-1
Capan-1
Su.86.86
MPANC-96
HPAC
AsPC-2
L3-3
MIAPACA
CEACAM6
MUC-1
IL-1B
TNC
CXCL4
EGF
VEGF
COL18A1
CA125
90K
TSP1

FIGURE 4

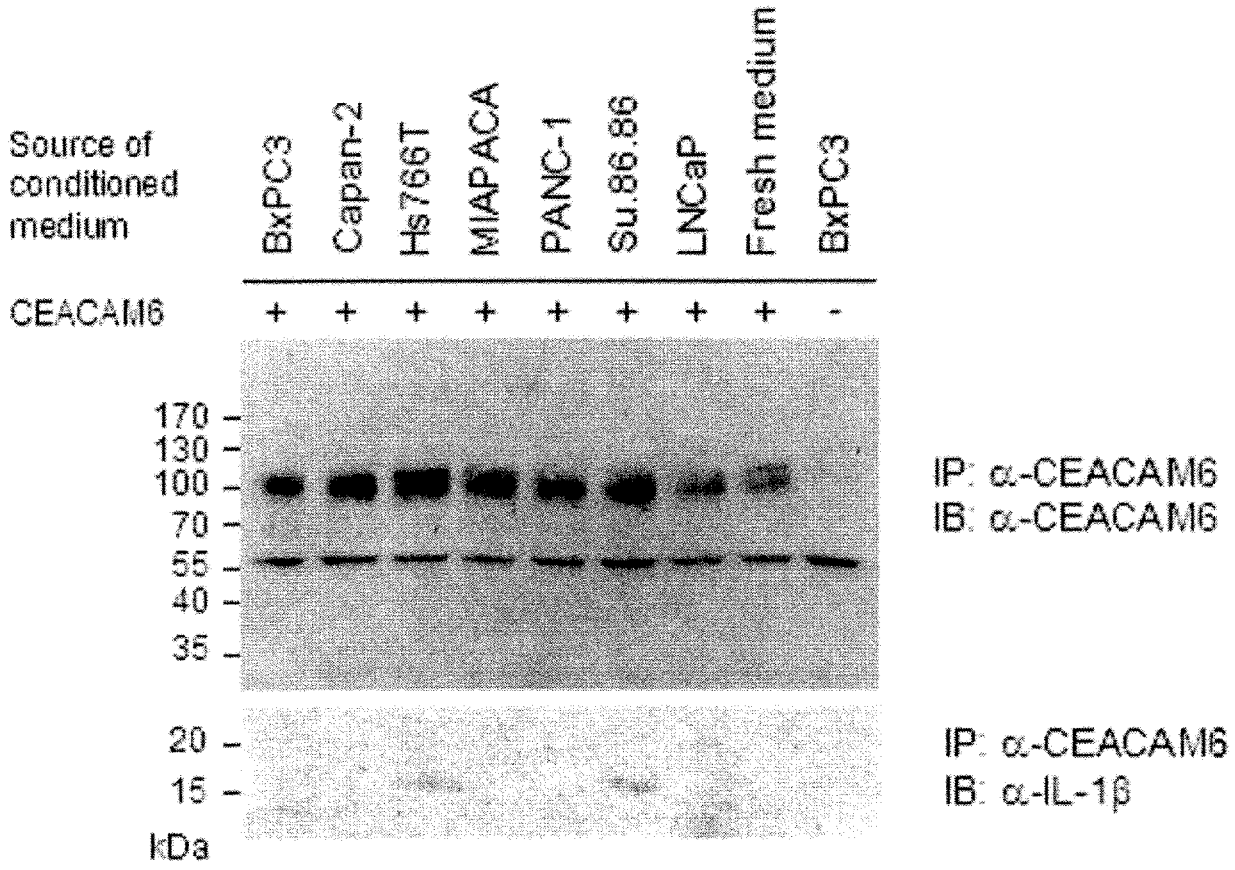
Source of conditioned medium
BxPC3
Capan-2
Hs766T
MIAPACA
PANC-1
Su.86.86
LNCaP
Fresh medium
BxPC3
CEACAM6
+ + + + + + + + -
170
130
100
70
55
40
35
IP: α-CEACAM6
IB: α-CEACAM6
20
15
kDa
IP: α-CEACAM6
IB: α-IL-1β

FIGURE 5

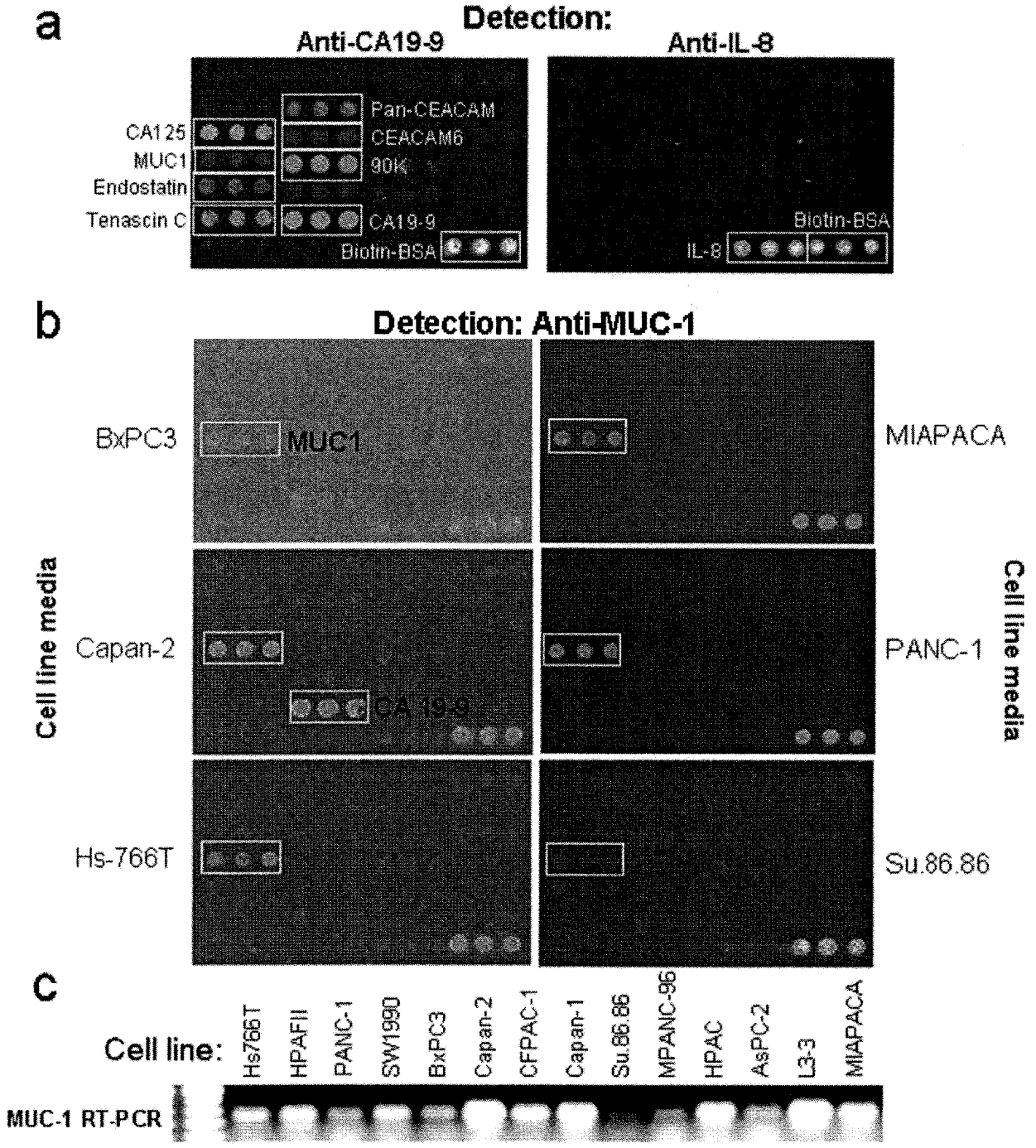
a
Detection:
Anti-CA19-9
Anti-IL-8
CA125
MUC1
Endostatin
Tenascin C
Pan-CEACAM
CEACAM6
90K
CA19-9
Biotin-BSA
IL-8
Biotin-BSA
b
Detection: Anti-MUC-1
BxPC3
MUC1
MIAPACA
Capan-2
CA 19-9
PANC-1
Hs-766T
Su.86.86
Cell line media
Cell line media
c
Cell line:
Hs766T
HPAFII
PANC-1
SW1990
BxPC3
Capan-2
CFPAC-1
Capan-1
Su.86.86
MPANC-96
HPAC
AsPC-2
L3-3
MIAPACA
MUC-1 RT-PCR

FIGURE 6

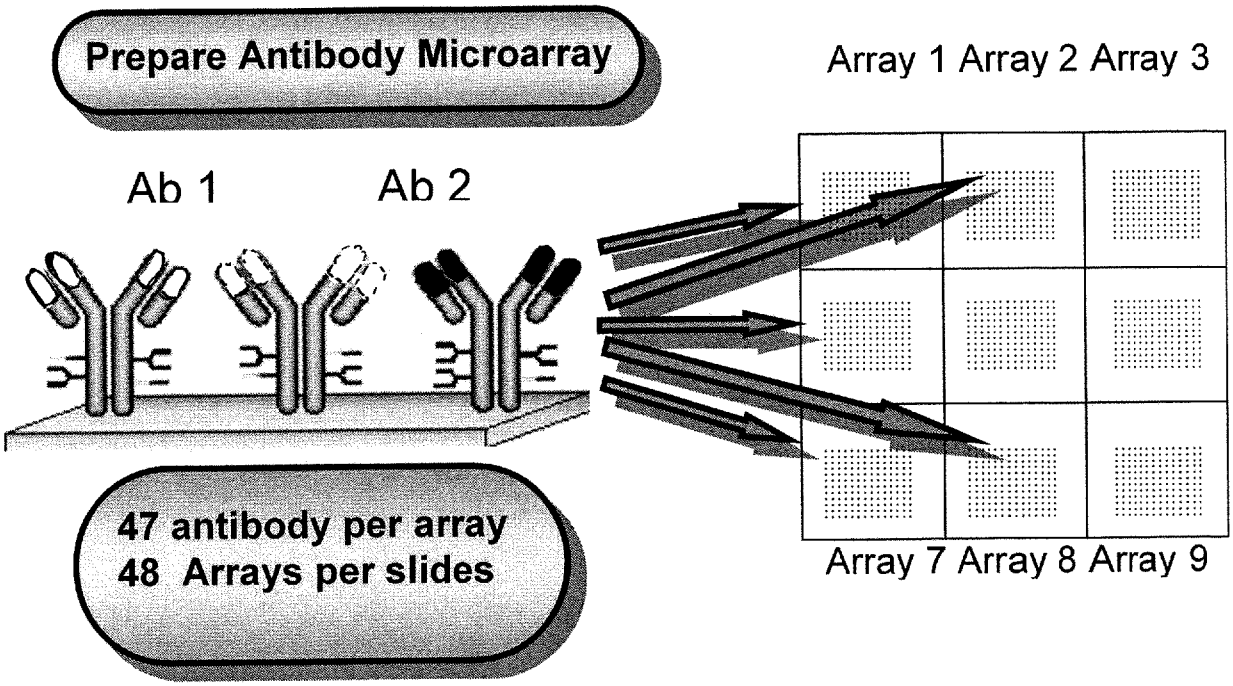
Prepare Antibody Microarray
Ab 1
Ab 2
47 antibody per array
48 Arrays per slides
Array 1 Array 2 Array 3
Array 7 Array 8 Array 9

FIGURE 7

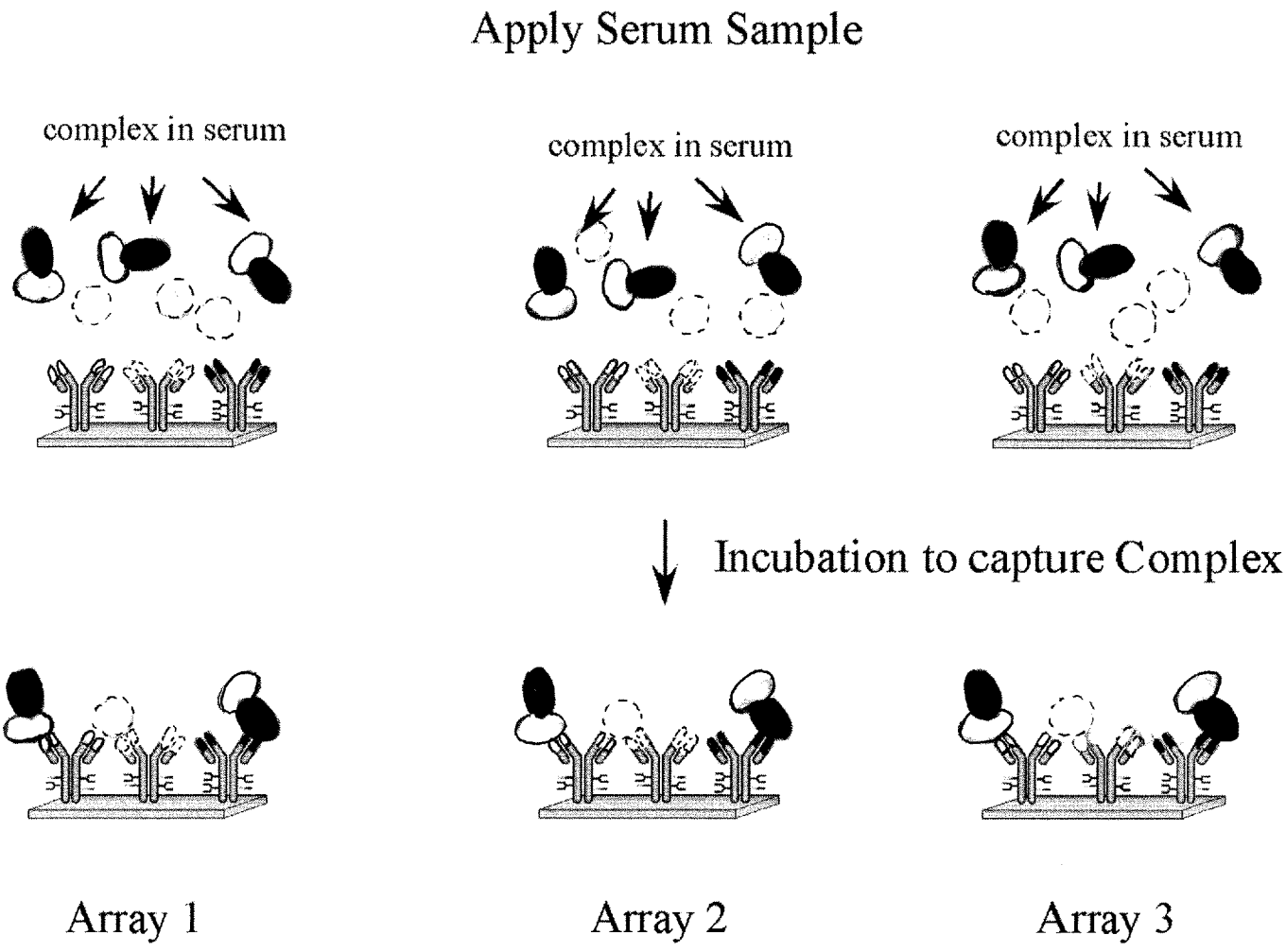
Apply Serum Sample
complex in serum
complex in serum
complex in serum
Incubation to capture Complex
Array 1
Array 2
Array 3

FIGURE 8

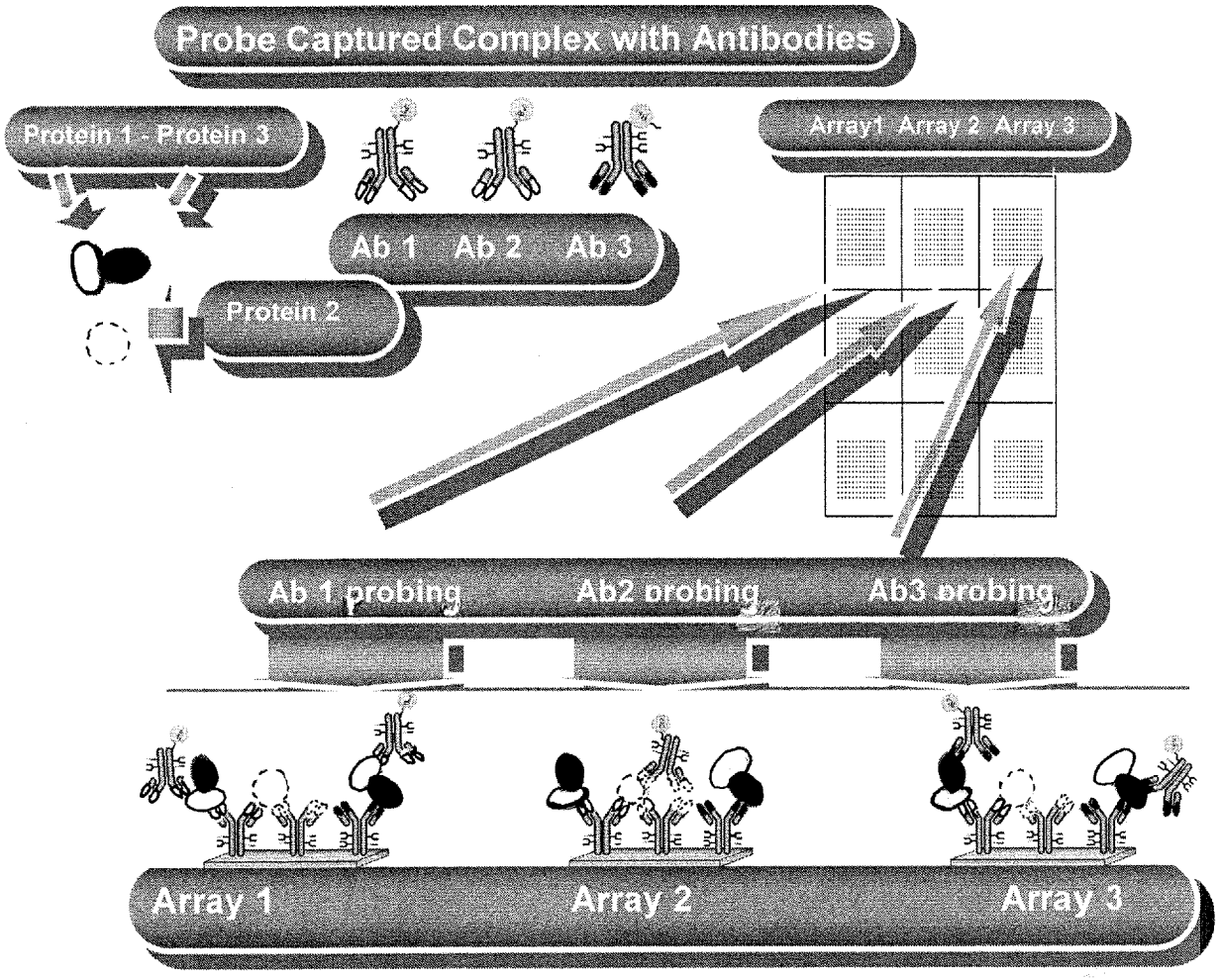
Probe Captured Complex with Antibodies
Protein 1 - Protein 3
Ab 1 Ab 2 Ab 3
Protein 2
Array1 Array 2 Array 3
Ab 1 probing
Ab2 probing
Ab3 probing
Array 1
Array 2
Array 3

FIGURE 9

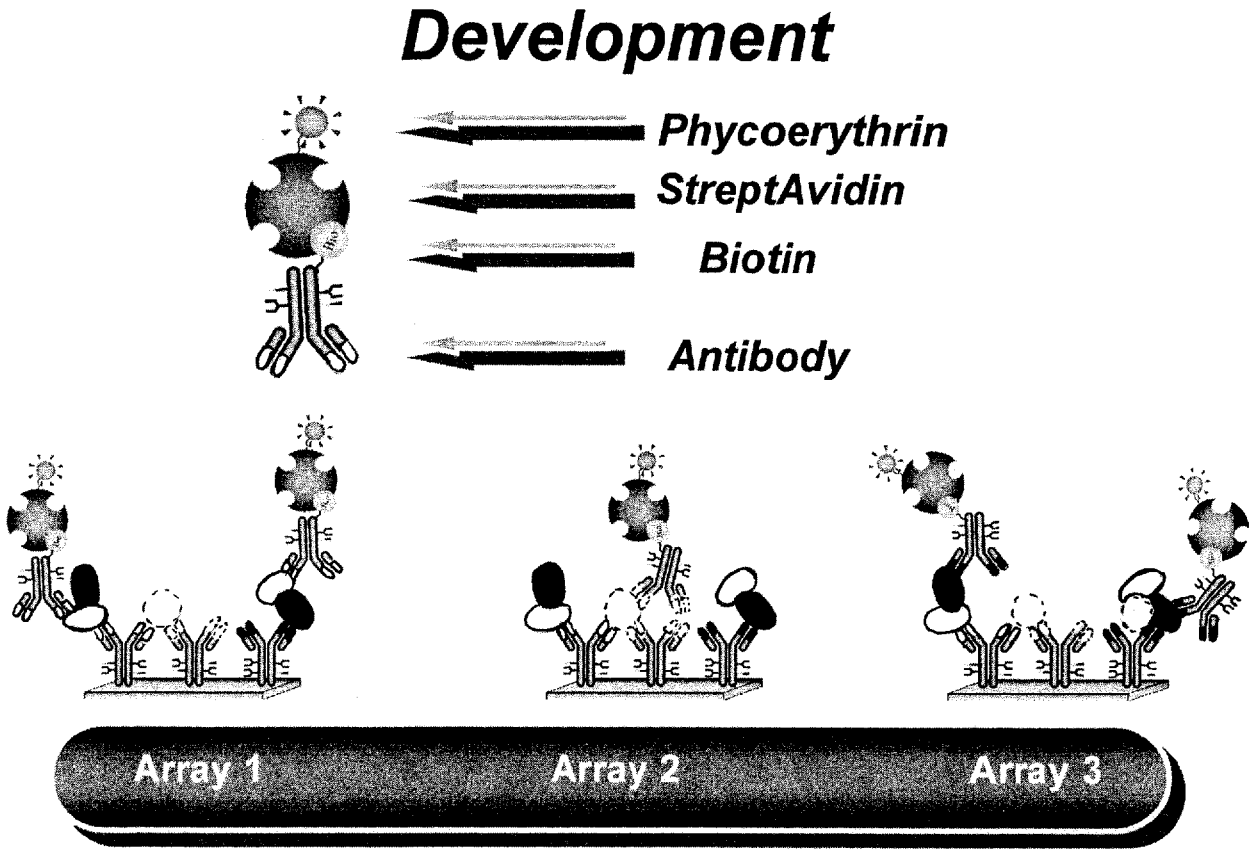
Development
Phycoerythrin
StreptAvidin
Biotin
Antibody
Array 1
Array 2
Array 3

FIGURE 10

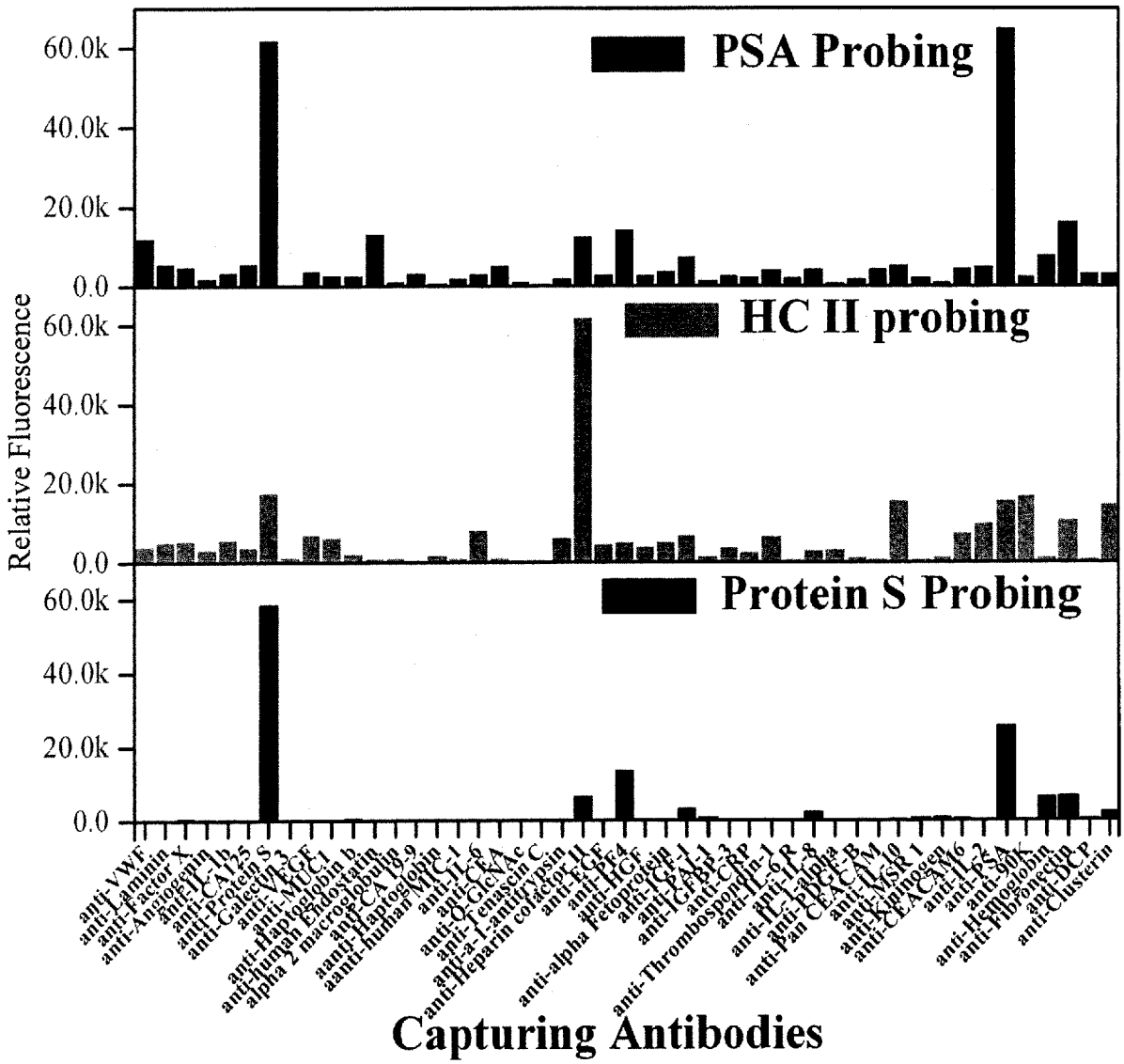
PSA Probing
HC II probing
Protein S Probing
Relative Fluorescence
60.0k
40.0k
20.0k
0.0
anti-VWF
anti-Laminin
anti-Factor X
anti-Angiogenin
anti-IL-1b
anti-CA125
anti-Protein S
anti-Calectrin
anti-VEGF
anti-MUC1
anti-Haptoglobin b
anti-human Endostatin
alpha 2 macroglobulin
aanti-CA 19-9
aanti-Haptoglobin
aanti-human MIC-1
anti-IL-6
anti-CEA
anti-GlcNAc
anti-Tenascin C
anti-a-1-antitrypsin
anti-Heparin cofactor II
anti-FGF
anti-PF4
anti-HGF
anti-alpha Fetoprotein
anti-IGF-1
anti-IGFBP-3
anti-Thrombospondin-1
anti-CRP
anti-IL-6R
anti-IL-8
anti-PDGF-B
anti-Pan CEACAM
anti-IL-10
anti-MSR 1
anti-Kininogen
anti-CEACAM6
anti-IL-2
anti-PSA
anti-90K
anti-Hemoglobin
anti-Fibronectin
anti-DCP
anti-Clusterin
Capturing Antibodies

FIGURE 11

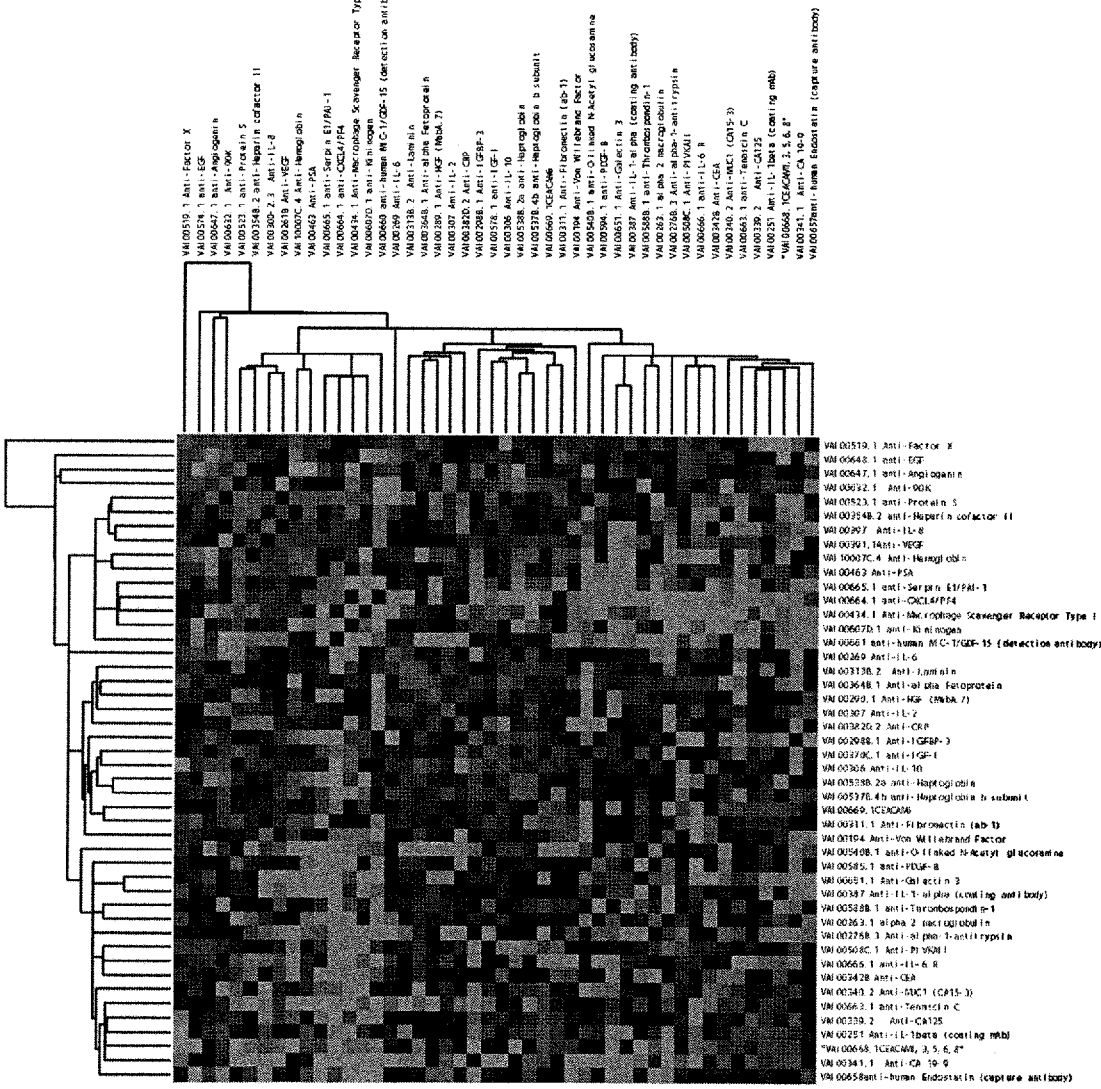

FIGURE 12

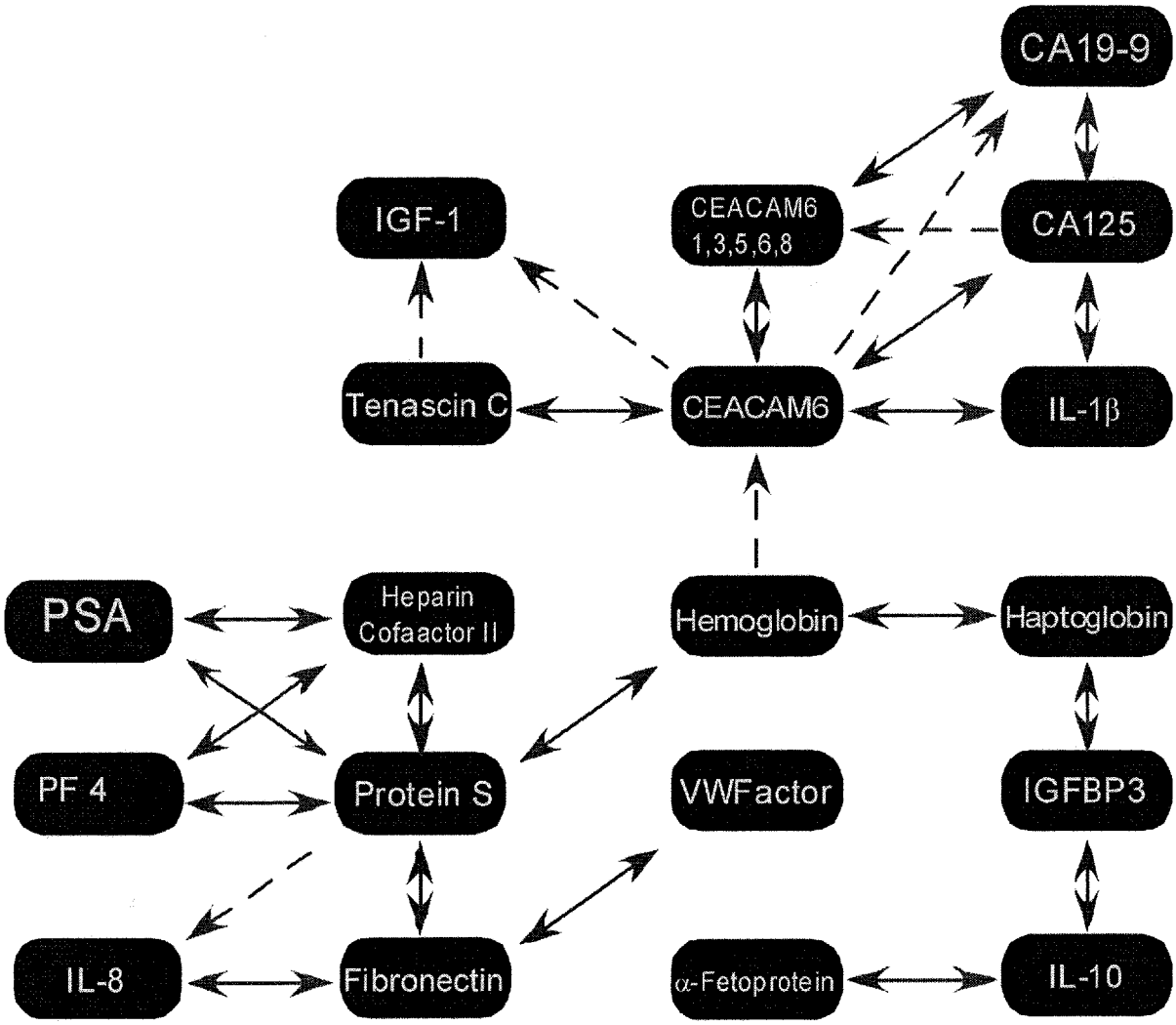
CA19-9
IGF-1
CEACAM6
1,3,5,6,8
CA125
Tenascin C
CEACAM6
IL-1β
PSA
Heparin
Cofaactor II
Hemoglobin
Haptoglobin
PF 4
Protein S
VWFactor
IGFBP3
IL-8
Fibronectin
α-Fetoprotein
IL-10

FIGURE 13

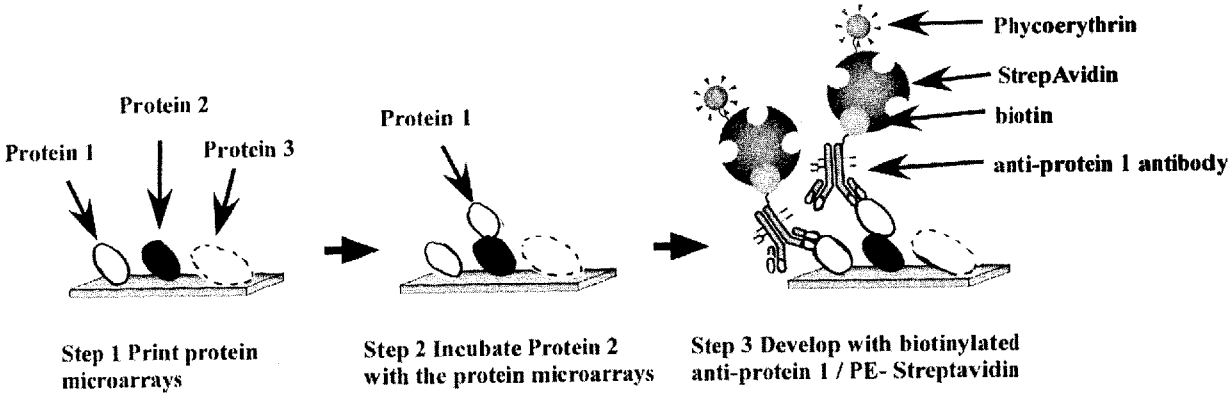
Protein 1
Protein 2
Protein 3
Protein 1
Phycoerythrin
StrepAvidin
biotin
anti-protein 1 antibody
Step 1 Print protein microarrays
Step 2 Incubate Protein 2 with the protein microarrays
Step 3 Develop with biotinylated anti-protein 1 / PE- Streptavidin

FIGURE 14

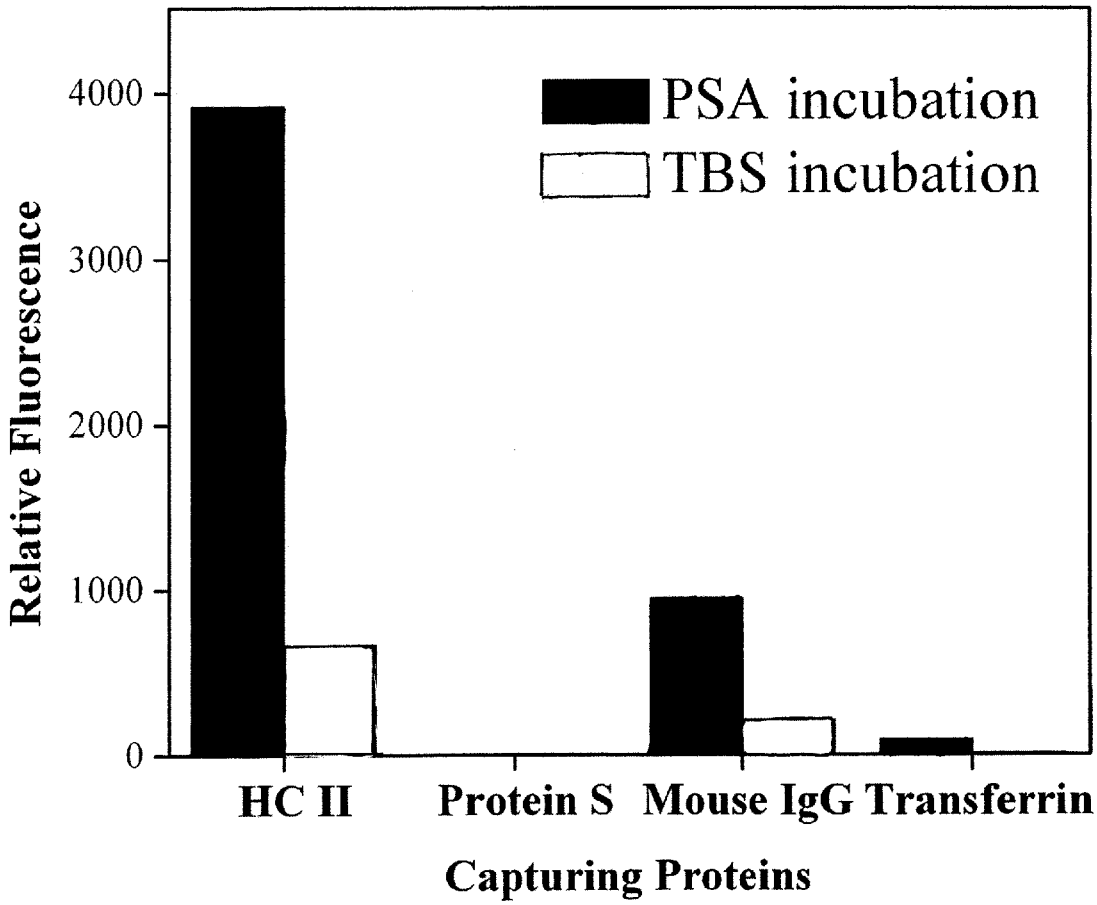
PSA incubation
TBS incubation
Relative Fluorescence
4000
3000
2000
1000
0
HC II
Protein S
Mouse IgG
Transferrin
Capturing Proteins

FIGURE 15

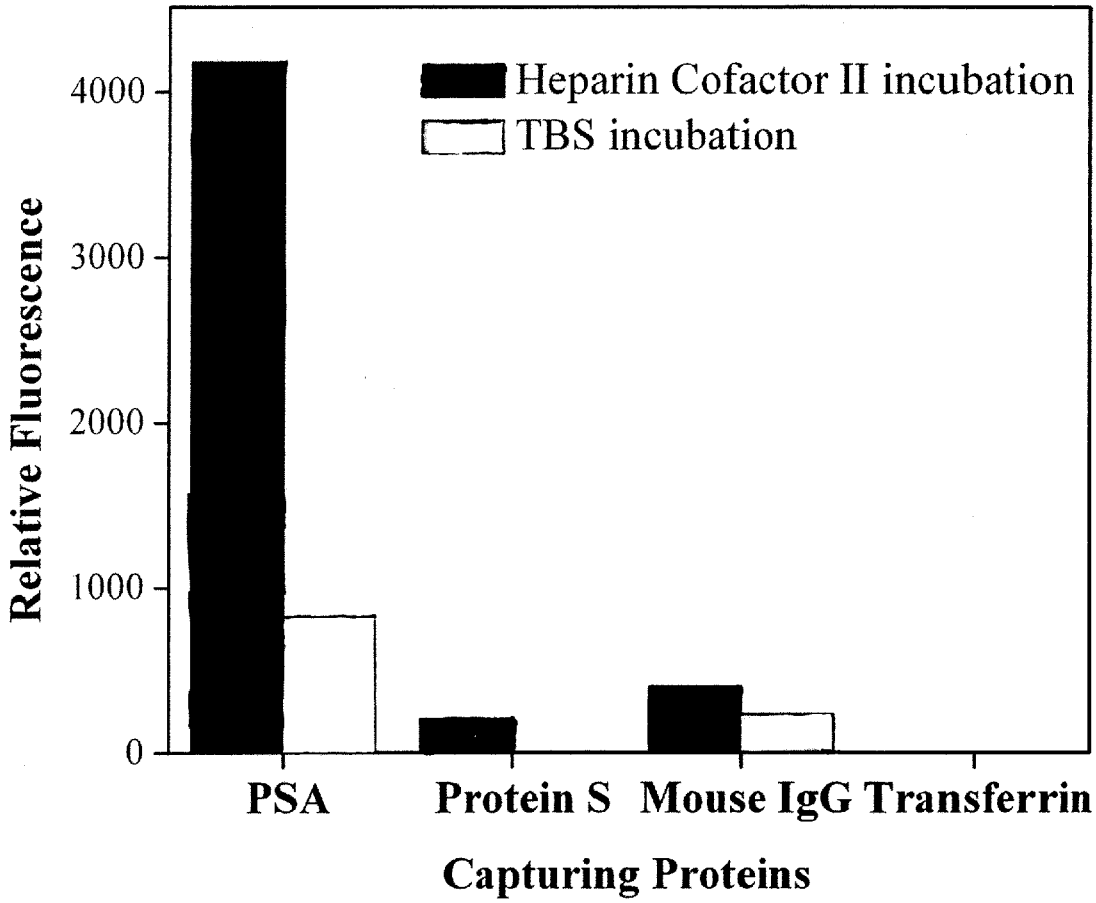
Heparin Cofactor II incubation
TBS incubation
Relative Fluorescence
4000
3000
2000
1000
0
PSA
Protein S
Mouse IgG
Transferrin
Capturing Proteins

FIGURE 16

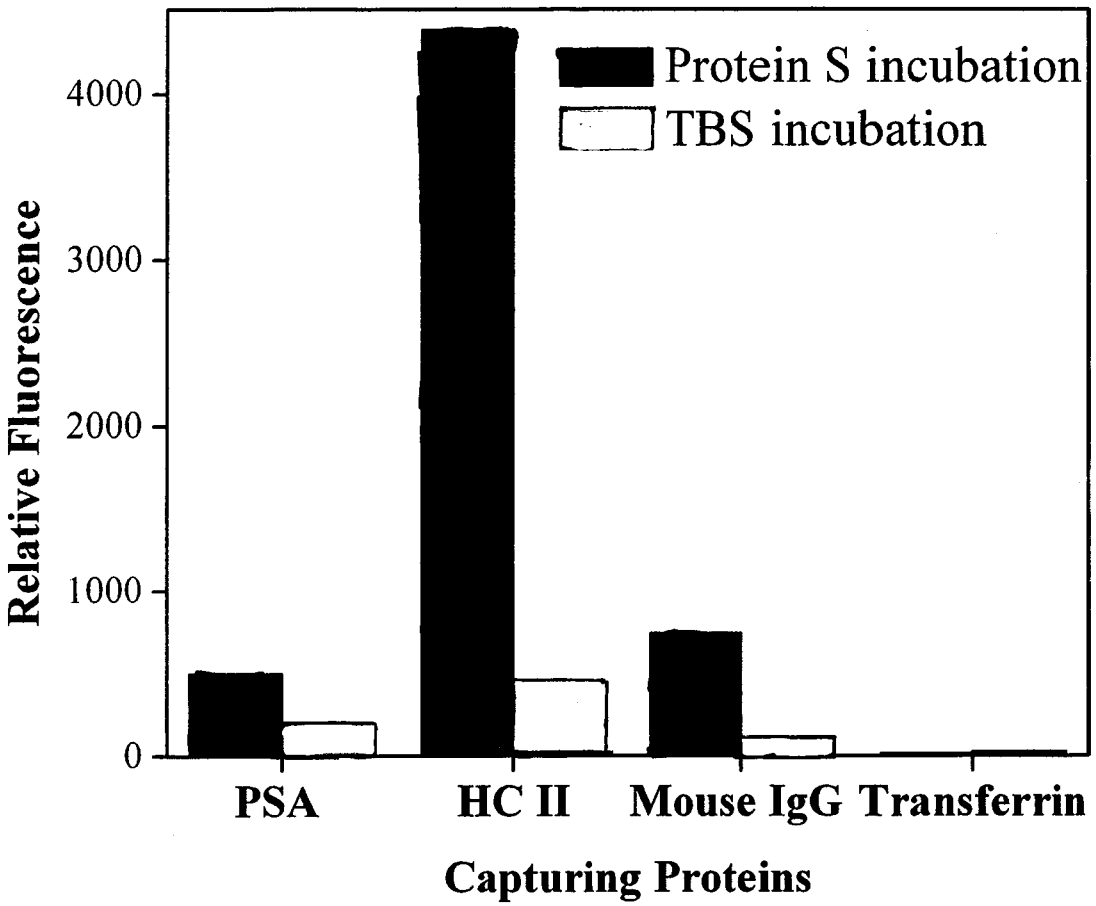
Protein S incubation
TBS incubation
Relative Fluorescence
4000
3000
2000
1000
0
PSA
HC II
Mouse IgG
Transferrin
Capturing Proteins

FIGURE 17

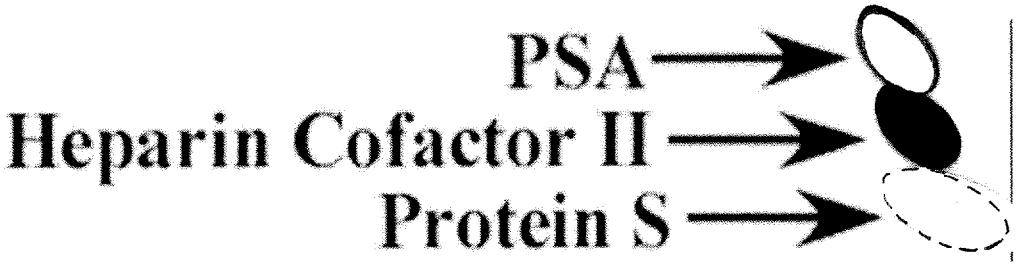

FIGURE 18

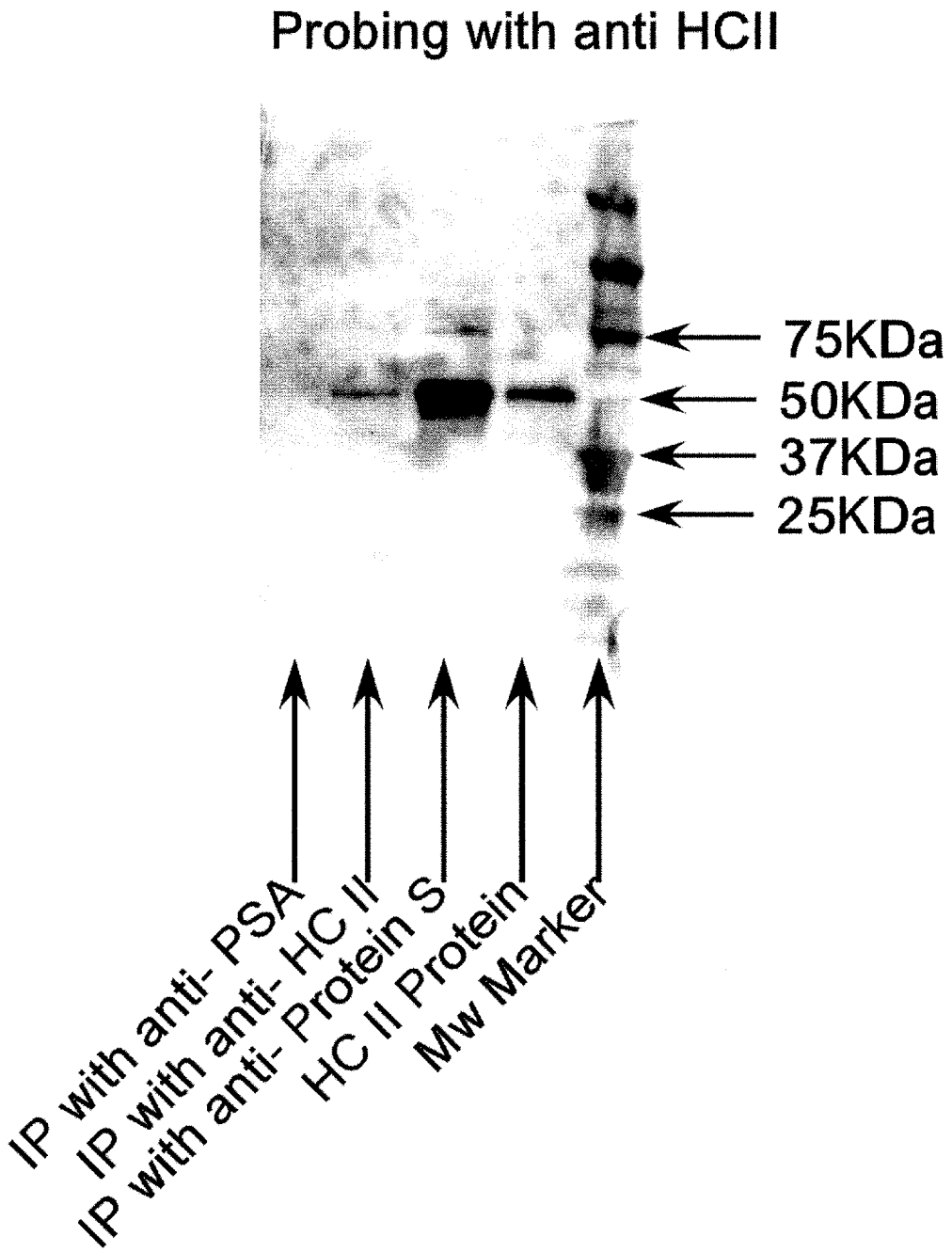
Probing with anti HCII
75KDa
50KDa
37KDa
25KDa
IP with anti- PSA
IP with anti- HC II
IP with anti- Protein S
HC II Protein
Mw Marker

FIGURE 19

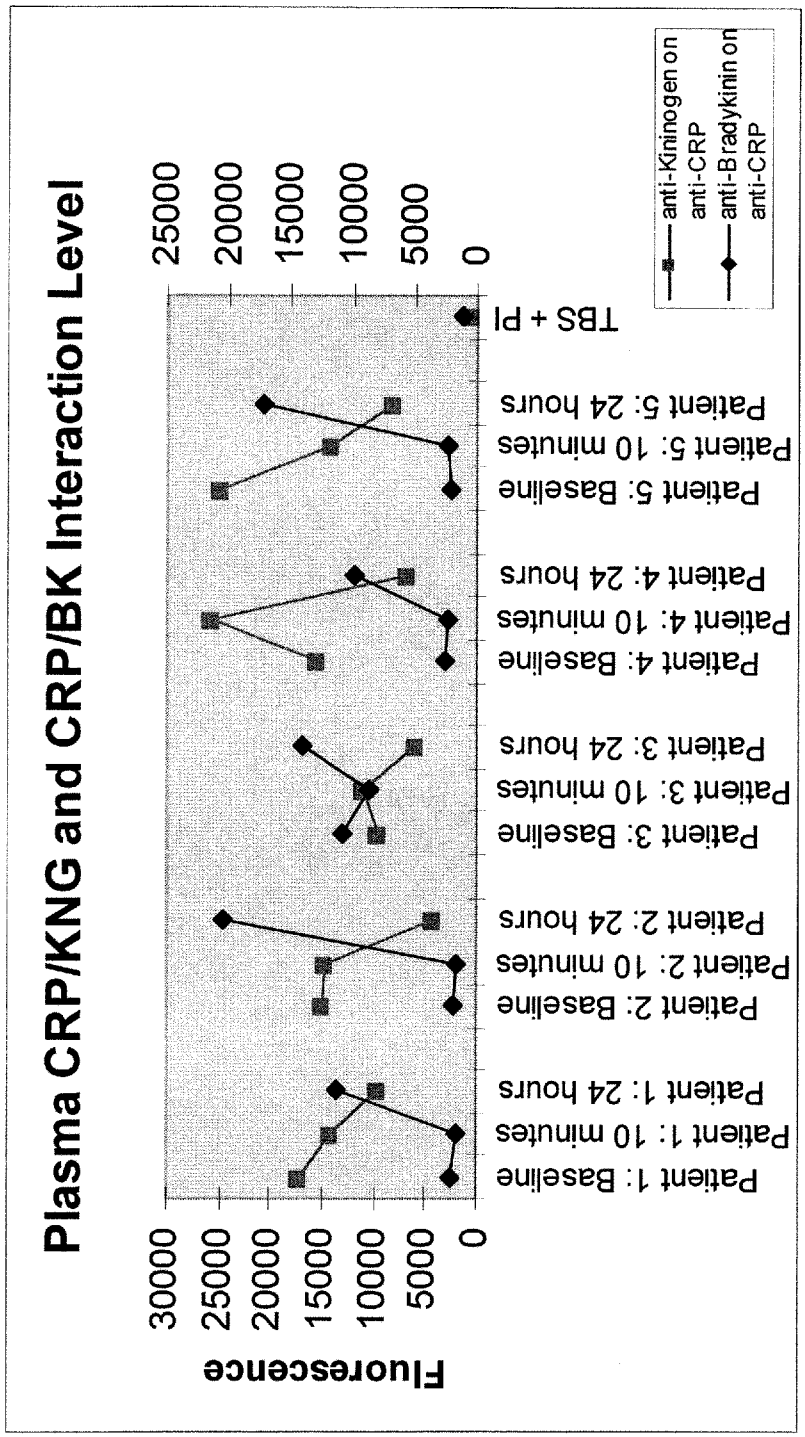
Plasma CRP/KNG and CRP/BK Interaction Level
Fluorescence
30000
25000
20000
15000
10000
5000
0
TBS + PI
Patient 1: Baseline
Patient 1: 10 minutes
Patient 1: 24 hours
Patient 2: Baseline
Patient 2: 10 minutes
Patient 2: 24 hours
Patient 3: Baseline
Patient 3: 10 minutes
Patient 3: 24 hours
Patient 4: Baseline
Patient 4: 10 minutes
Patient 4: 24 hours
Patient 5: Baseline
Patient 5: 10 minutes
Patient 5: 24 hours
anti-Kininogen on anti-CRP
anti-Bradykinin on anti-CRP

FIGURE 20

专利名称(译)	检测分子复合物的方法		
公开(公告)号	EP2044437A2	公开(公告)日	2009-04-08
申请号	EP2007798658	申请日	2007-06-15
[标]申请(专利权)人(译)	范安德尔研究所		
申请(专利权)人(译)	温安洛研究所		
当前申请(专利权)人(译)	温安洛研究所		
[标]发明人	HAAB BRIAN B		
发明人	HAAB, BRIAN, B.		
IPC分类号	G01N33/53 G01N33/68		
CPC分类号	G01N33/543 G01N33/54306 G01N33/6842 G01N33/6845		
优先权	60/813838 2006-06-15 US		
其他公开文献	EP2044437A4		
外部链接	Espacenet		

摘要(译)

本发明包括用于分析抗体微阵列的拷贝用于与另一分子复合的蛋白质的方法。在该方法中，将一个生物样品分开，然后与抗体微阵列的拷贝一起温育。每种捕获抗体与检测抗体配对，并且仅将一种检测抗体与抗体微阵列的每个拷贝一起温育。本发明还包括在不同条件下比较蛋白质复合物的方法，检测自身免疫应答的方法，以及检测蛋白质和小分子之间相互作用的方法。