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(71) Applicant *(for all designated States except US)*: **THE AMERICAN NATIONAL RED CROSS** [US/US]; 2025 E Street, N.W., Washington, DC 20006 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **HAMMOND, David, J.** [GB/US]; 4916 Ripplemead Court, Laytonsville, MD 20882 (US).

(74) Agent: **MEYER, Mercedes, K.**; Drinker Biddle & Reath LLP, 1500 K Street, N.W., Suite 1100, Washington, DC 2005-1209 (US).

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(54) **Title:** METHODS FOR REDUCING THE RANGE IN CONCENTRATIONS OF ANALYTE SPECIES IN A SAMPLE

(57) **Abstract:** The present invention relates to the fields of molecular biology, combinatorial chemistry and biochemistry. Particularly, the present invention describes methods and kits for dynamically reducing the variance between analyte taken from complex mixtures.

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METHODS FOR REDUCING THE RANGE IN CONCENTRATIONS OF ANALYTE SPECIES IN A SAMPLE

CROSS-REFERENCES TO RELATED APPLICATIONS

This is application is a Continuation-in-Part of U.S. Patent Application No. 11/089,128, filed March 23, 2005, which claims the benefit of: U.S. Provisional Application Serial Number 60/559,108, filed April 2, 2004; U.S. Provisional Application Serial Number 60/582,650, filed June 23, 2004; U.S. Provisional Application Serial Number 60/587,585, filed July 12, 2004; U.S. Provisional Application Serial Number 60/643,483, filed January 12, 2005; and European Patent Application Serial Number 04290775.8, filed March 23, 2004.

FIELD OF THE INVENTION

The present invention relates to the fields of combinatorial chemistry, protein chemistry and biochemistry.

BACKGROUND OF THE INVENTION

Proteomics seeks to identify the proteins present in an entire proteome and, through analysis of this information, to identify potential diagnostic and therapeutic entities. Current technologies for resolving protein mixtures include two-dimensional gel electrophoresis and multi-dimensional liquid chromatography. Both of these techniques may be coupled to mass spectrometry. An example of this approach is the resolution and identification of 1,484 proteins in yeast (Washburn *et al., Nat. Biotechnol.* 19(3): 242-2471 (2001)). Another example of methodology that separates and identifies proteins is a modified version of the yeast two-hybrid screening assay developed by Uetz *et al.* (Uetz *et al., Nature* 403(6770): 623-627 (2000)) and Ito *et al.* (Ito *et al., Proc. Natl. Acad. Sci.* USA 98(8): 4569-4574 (2001)), which identified over 4,000 protein-protein interactions in *Saccharomyces cerevisiae*. A quantitative methodology for protein separation and identification is isotope coded affinity tag (ICAT), developed by Aebersold and colleagues (Smolka *et al., Anal. Biochem.* 297(1): 25-312 (2001)). ICAT involves site-

specific, covalent labeling of proteins with isotopically normal or heavy reagents to quantitate levels of protein expression.

Complex protein mixtures may also be separated on libraries of combinatorially-generated ligands. Following exposure of an entity to a combinatorial library, the entity may bind to ligands in the library. Detection of the bound entity may be accomplished when a purified, radiolabeled initial entity is used (Mondorf *et al., J. Peptide Research* 52: 526-536 (1998)). Other methods include detection by an antibody against the entity (Buettner *et al., International Journal of Peptide & Protein Research* 47: 70-83 (1996); Furka *et al., International Journal Peptide Protein Research* 37(6): 487-493 (1991); and Lam *et al.,* (1991) *supra*). Ligands to multiple entities can be detected using beads immobilized on an adhesive in combination with a subtractive screening method. This is referred to as the QuASAR method (International (PCT) Patent Application Publication Number WO 01/40265) and was used to detect ligands that bound to virus and prion protein.

The Bead blot method (Lathrop et al (2007) Analytical Biochemistry 361-65-76 and US Patent 7, 217, 507) was developed to identify ligands and target proteins in mixtures through fractionating proteins on combinatorial libraries and transferring the captured proteins to a membrane. The proteins then are evaluated for a chemical, biochemical or biological property.

FIoNA assay technology (Hammond *et al.* International (PCT) Patent Application Publication Number WO 04/007757) and other combinatorial techniques can identify a ligand-entity interaction. The FIoNA assay technology identifies proteins from mixtures based on chemical, physical, biological, and/or biochemical function and not merely on their ability to bind a ligand within the library. Thus, the goal of FIoNA is to identify a ligand-support that binds a desired property, then to decode the ligand on the appropriate bead, and synthesize the bead in appropriate amounts to purify the one or few proteins with the desired activity using current proteomic methods

The full analysis of analytes in complex biological materials is hindered by the large difference in concentration between individual analytes. In most biological mixtures some analytes are present at high concentration and others only are present at trace levels. As a result, the concentration of analytes may not be adapted to the dynamic

range of a given analytical method. That is to say, the difference in the signal strengths produced by the most abundant and least abundant analyte species in a sample generally is wider than the ability of the analytical method to detect and accurately measure. For example, highly concentrated proteins may saturate the detection system and very low concentrations may be below the sensitivity of the analytical method, as occurs in human serum where the difference in concentration between the most abundant protein (albumin – tens of mg/ml) and the least abundant (e.g., IL-6 – less than 1 pg/ml) may reach factors as high as hundreds of millions.

Two ways are currently used to deal with this issue: the first is to design more effective instruments and the second is to alter the sample for analysis.

One method of altering the sample is to deplete the sample of the more abundant species, thereby making the less abundant species more available for detection. This method involves, for example, the use of linker moieties, such as antibodies or specific dyes, that are directed to particular species in the sample. For example, in the case of plasma, the abundant proteins include albumin, immunoglobulins, fibrinogen, and alpha-1 proteinase inhibitor. Immunoaffinity columns are expensive, seldom totally specific for their target and will remove proteins associated with the target proteins. Moreover, once the most abundant proteins are removed, another set becomes the most abundant, which then creates the need to develop additional affinity columns. In addition, biological samples from different tissues within the same species and tissues from different species may have a completely different set of most abundant proteins. This method also suffers from the fact that elimination of some analyte species also eliminates species that interact with them, such as the immunoglobulins and their bound antigens. Thus some species that may be of interest are depleted. While eliminating proteins of high abundance may help in some instances, this approach does not result in the detection of very low abundance proteins whose concentration is still below the limit of detection of the instrument. Moreover, highly abundant species are represented by several proteins (even several dozen in some situations) and therefore a number of specific methods would have to be designed to address each different abundant species. Therefore, this method does not substantially compress the range of concentrations between the remaining analyte species. Moreover, the proteins are recovered in a more dilute, less stable format than the

starting sample, frequently necessitating the introduction of a concentration step prior to analysis.

Another method is to fractionate the sample, typically by chromatography. This method results in the compartmentalization of classes of analytes into different fractions based on similar biochemical properties. For example, ion exchange chromatography will compartmentalize proteins into fractions based on charge, while size exclusion chromatography compartmentalizes proteins based on size. Therefore, these methods may reduce the concentration range of the analytes, but at the cost of substantially decreasing the diversity of the population of analyte species within each compartment.

SUMMARY OF THE INVENTION

This invention provides a method to compress the range of concentrations between different analyte species in a complex sample while substantially maintaining the diversity of the population of analyte species in the sample. More specifically, the method decreases the concentration of more abundant species while enriching the sample for the less abundant species but does not involve substantially eliminating from the sample analyte species based on physical-chemical characteristics, thereby improving the detection of the analytes in a given sample.

As noted, each analytical technology has a dynamic range of detection. When the amount of an analyte in a sample is below the limit of detection of the system, the analyte cannot be detected. Furthermore, signals from abundant analytes may interfere with the ability to detect less abundant analytes even if the less abundant analytes are within the dynamic range of detection. The methods of this invention allow trace analytes to be concentrated and more easily detected in a sample This allows one to provide an increased number of different analyte molecules to the detector system so as to be above the sensitivity threshold of detection, while, at the same time decreasing the amount of the abundant analtyes submitted for detection, so that there is considerably less saturation of the detection system by abundant analytes and, consequently, reduced interference with the ability to detect less abundant species that are present above the sensitivity threshold. The result is an ability to detect more analyte species in a sample. Using this method, one can detect many more species from serum by mass spectrometry

as compared with the untreated sample. In addition, the sensitivity of detection of a single analyte may be greatly improved by decreasing the amount of interfering substances.

The method of this invention contrasts with other methods of manipulating a sample for detection. For example, depletion of selected abundant species does not significantly decrease the range in concentrations of the wide number of species in a sample. Fractionation decreases the range in concentration of analytes, but does so by substantially decreasing the diversity of the species within the population of the compartment. It also tends to further dilute the analytes.

This invention achieves its result by exposing a complex sample to a selected amount of a library containing many different binding moieties. Both variables – diversity of the library constituents and amount of the library used – can be manipulated to advantage in this invention. By manipulating the diversity of the different binding moieties and their construction to which the sample is exposed, it is possible to bind species throughout the range of concentrations, that is, both abundant and rare species. Also, the larger the number of different binding moieties used, the larger the number of species within the sample population it is possible to capture.

The amount of the library also must be selected so that the binding moieties are close to saturation by at least the more abundant species in the sample. In this way, the relative amounts of abundant and rare species in the sample that are captured will be much closer than their relative concentrations in the original sample. This results in compression of the concentration range, which allows a greater number of signals produced by both abundant and rare species during detection that are within the dynamic range of the selected detection system.

It is an object of this invention to increase significantly the number of species detectable in a sample and, in particular, the discovery of new species within a sample. Certain kinds of libraries of binding moieties are preferred for achieving this end. In particular, one can best achieve this end by using libraries of large numbers of different binding moieties that have not been pre-selected for their ability to bind particular analytes in a sample. Such libraries are referred to herein as "random" libraries. Using such libraries increases the likelihood of capturing species throughout the population without prior discrimination or selection of particular entities. Thus, for example, a

library of antibodies in which each antibody is directed to a known binding partner will select only the species to which each antibody is directed; in contrast, a germline antibody library of the same size does not contain antibodies that bind to pre-selected analytes. Such a library is more likely to select species not known to exist in a sample. One can create random libraries by employing combinatorial chemistry or by randomly assembling chemical moieties. Furthermore, by increasing the diversity of a library, one can increase the number of different analyte species in a sample captured and detected. Examples of random libraries of binding moieties include germ line antibody libraries, phage display libraries of recombinant binding proteins, dye libraries and non-combinatorial libraries in which the binding specificity of the members is not pre-selected, combinatorial libraries of various sorts and portions thereof.

It should also be noted that the amount of concentration compression achieved by treatment of a sample with the library depends upon the relative amounts of binding moieties and analytes in the sample. This result is particularly useful for comparing the relative concentrations of analyte species between two different sample classes. For example, in biomarker discovery, samples taken from organisms having two different phenotypic states (e.g., cancer versus non-cancer) are compared to identify analyte species that are differentially present between the two states. By preserving the concentration differences between rare species, the methods of this invention allow one to find biomarkers among these rare analytes. In one embodiment, the ratio of binding moieties to analyte species in the sample is at most 1:500 and, more preferably, at most 1:50 or at most 1:5.

This invention provides a method to reduce the range of concentrations between different analyte species in a complex sample while substantially maintaining the diversity of the population of analyte species in the sample. In a preferred embodiment of the present invention, a method is provided comprising the steps of (a) providing a first sample comprising a plurality of different analyte species present in the first sample in a first range of concentration; (b) contacting the first sample with an amount of a library comprising at least 100 different binding moieties; (c) capturing amounts of the different analyte species from the first sample with the different binding moieties and removing unbound analyte species; and (d) isolating the captured analyte species from the binding

moieties to produce a second sample comprising a plurality of different analyte species present in the second sample in a second range of concentrations. The amount of the library is selected to capture amounts of the different analyte species so that the second range of concentrations is less than the first range of concentrations.

The first sample comprises at least 100, at least 1,000, at least 10,000, at least 100,000, at least 1,000,000 or at least 10,000,000 different analyte species. In some embodiments, the library comprises at least 1,000, at least 10,000, at least 100,000, at least 1,000,000 or at least 10,000,000 different binding moieties.

Preferably, the binding moieties comprise bio-organic polymers. Preferably, the bio-organic polymers are selected from the group consisting of peptides, oligonucleotides and oligosaccharides. In another embodiment of the present invention, the binding moieties are selected from the group consisting of antibodies.

In a preferred embodiment of the present invention, the binding moieties are bound to a solid support or supports. Preferably, the solid support or supports is a collection of beads or particles. Each bead or particle has attached to it a different binding moiety. Preferably, the beads or particles have a diameter of less than 100 μm. Smaller beads or particles can be formed from larger beads by milling using a method selected from the group comprising crushing, grinding and sonicating. In a preferred embodiment of this method, the particles are coupled to a second solid support to form an array or dipstick.

In another preferred embodiment of the present invention, the solid support or supports is selected from the group consisting of fibers, monoliths, membranes and plastic strips.

In a preferred embodiment of the present invention, the library contacting a first sample is a random library.

Preferably, the different binding moieties are comprised in a complete or incomplete combinatorial library. A preferred combinatorial library is a hexapeptide library.

In one embodiment of the present invention, the second sample has a diversity of analyte species that is substantially the same as the first sample.

Many samples can be used to practice the methods of the invention. In a preferred embodiment of the present invention, the sample is selected from the group consisting of amniotic fluid, blood, cerebrospinal fluid, intra-articular fluid, intraocular fluid, lymphatic fluid, milk, perspiration plasma, saliva, semen, seminal plasma, serum, sputum, synovial fluid, tears, umbilical cord fluid, urine, biopsy homogenate, cell culture fluid, cell extracts, cell homogenate, conditioned medium, fermentation broth, tissue homogenate, environmental samples including soil, water, and derivatives of these.

In one embodiment, the method of the present invention comprises the step of detecting analyte species in the second sample. Preferably, detecting the analytes is done by using a method selected from the group of colorimetric, spectrophotometric, magnetic resonance, mass spectrometric, electrophoretic, chromatographic, enzymatic, and sequence analysis.

Optionally, the method of the present invention further comprises the step of fractionating the analytes in the second sample based on a physical or chemical property or the step of identifying at least one of the isolated analytes. Preferably, fractionating the analytes comprises segregating the analytes using a technique selected from the group consisting of chromatography, electrophoresis, capillary electrophoresis, filtration and precipitation.

In one embodiment of the present invention, the method further comprises the step of contacting a biospecific binding moiety with the second sample and determining whether the biospecific binding moiety has captured an analyte species from the second sample.

Removing non-bound analytes may comprise the step of washing the captured analytes with a wash buffer.

The methods of this invention can be practiced using different analytes. In a preferred embodiment of the present invention, the analytes are selected from the group consisting of protein complexes, viruses, subcellular organelles, polypeptides, nucleic acids, complex carbohydrates, complex lipids, synthetic inorganic compounds and synthetic organic compounds.

The present invention also provides a method for identifying a diagnostic biomarker. In a preferred embodiment, the method comprises the steps of (a) providing a

first set of biosamples from a first set of organisms having a first phenotype; (b) providing a second set of biosamples from a second set of organisms having a second phenotype; (c) performing the method for reducing the range of concentrations between different analyte species in a sample as described herein (Claim 1) on each of the biosamples, thereby creating a third and fourth set of biosamples, respectively; (d) detecting analyte species in each of the third and fourth set of biosamples; and (e) identifying at least one analyte species that is differentially present in the third and fourth set of biosamples, whereby the at least one analyte species is a biomarker for distinguishing the first phenotype from the second phenotype. In a preferred embodiment step (e) of this method comprises identifying a biomarker profile that provides better predictive power than any one of the biomarkers in the profile alone.

The invention further provides a method for reducing the relative amounts of analytes in a sample. In a preferred embodiment of the present invention, the method comprises the steps of (a) providing a first sample comprising a first plurality of different analytes having a first variance in amounts; (b) contacting the first sample with a plurality of different binding moieties, each binding moiety present in a determined amount; (c) capturing a portion of the first different analytes from the first sample with the different binding moieties and removing uncaptured analytes; and (d) isolating the captured analytes from the binding moieties to produce a second sample comprising a second plurality of different analytes having a second variance in amounts. The determined amount of each of the plurality of different binding moieties is selected to capture amounts of the different analytes whereby the second variance in amounts is less than the first variance in amounts.

The present invention also provides kits for detecting a plurality of analytes in a sample. In a preferred embodiment of the present invention, a kit comprises a container comprising a library of at least 100 different binding moieties and instructions for using the library to perform a method of the present invention. Preferably, the binding moieties are coupled to a solid support or supports. The library may also comprise a hexapeptide combinatorial library or a portion thereof, wherein the hexapeptides are attached to particles.

Optionally, the kits of the present invention comprise a binding buffer for capturing analytes with the binding moieties or an elution buffer for eluting captured analytes from the binding moieties. Additional kit embodiments of the present invention include optional functional components that would allow one of ordinary skill in the art to perform any of the method variations described herein

The present invention also provides for libraries comprising binding moieties. In a preferred embodiment of the present invention, a library comprises at least 100 different binding moieties, wherein a plurality of different binding moieties are attached to the same solid support or supports. Preferably, the binding moieties comprise a combinatorial hexapeptide library or a portion thereof.

The present invention also provides a means of combining two or more specific ligand resins directed to binding highly interactive proteins along with a random library to sequester highly interactive proteins during incubation of the library with a sample, thereby improving detection of trace analytes in the sample.

The invention also provides a method for detecting biomarkers comprising: (a) providing a first set of biosamples from a first set of organisms having a first phenotype; (b) providing a second set of biosamples from a second set of organisms having a second phenotype; (c) performing the method of claim 1 on each of the biosamples, thereby creating a third and fourth set of biosamples, respectively, wherein the difference in the concentration of the biomarker analyte between the first and second biosamples is maintained between the third and fourth biosamples; and, (d) detecting analyte species in each of the third and fourth set of biosamples; wherein an analyte species differentially present in the third and fourth set of biosamples is a biomarker. In a preferred embodiment, the analytes are selected from the group consisting of polypeptides, nucleic acids, complex carbohydrates, complex lipids, synthetic inorganic compounds and synthetic organic compounds. In another embodiment, at least one biomarker is identified. In a preferred embodiment, the biomarker is troponin. In another preferred embodiment, the biomarker is CRP. In yet a different embodiment, the biosample is selected from the group consisting of amniotic fluid, blood, cerebrospinal fluid, intraarticular fluid, intraocular fluid, lymphatic fluid, milk, perspiration plasma, saliva, semen, seminal plasma, serum, sputum, synovial fluid, tears, umbilical cord fluid,

urine, biopsy homogenate, cell culture fluid, cell extracts, cell homogenate, conditioned medium, fermentation broth, tissue homogenate and derivatives of these.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts an analysis showing the result of the incubation of a combinatorial ligand library of the invention with plasma. The library was incubated with plasma according to the methods described in Example 1.

Figure 2 is a comparison of plasma with and without removal of IgG prior to incubation with a library. The experiment was conducted as described in Example 2.

Figure 3 depicts the result of an incubation of a combinatorial ligand library of the invention with serum. The experiment was conducted according to Example 3.

Figure 4 is an LDS-PAGE analysis of Protein G column retentate. The panel on the left is stained with Coomassie Blue stain; the panel on the right is the same gel stained with Silver Quest.

Figure 5 is a graphical depiction of blood fractions (based on mass), highlighting the trace nature of a large number of low abundance proteins.

Figure 6 is a graphical depiction of one embodiment of the equalizer bead concept of the present invention.

Figure 7 depicts the increased number of ligands identified with increasing concentrations of a target protein are incubated with the library, demonstrating that differences in target protein concentration between samples are maintained following treatment with the library.

Figure 8 depicts ELISA results demonstrating that treatment of a sample with the library not only retains the concentration differential, but improves detection of the desired analyte by increasing the intensity of the signal.

DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton *et al., Dictionary of Microbiology and Molecular Biology* (2nd ed. 1994); *The Cambridge Dictionary of Science and Technology* (Walker ed., 1988); *The Glossary of Genetics, 5th Ed.*, R. Rieger *et al.* (eds.), Springer Verlag (1991); and Hale & Marham, *The Harper Collins Dictionary of Biology* (1991). As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

"Analyte" refers to any molecular moiety capable of binding to a binding moiety of the present invention in a manner that is not completely disrupted by contact with a wash solution as described herein. "Captured analyte" is any analyte bound by a binding moiety of the present invention after contact with a wash solution.

"Adsorbent" refers to any material capable of binding an analyte (e.g., a target polypeptide). "Chromatographic adsorbent" refers to a material typically used in chromatography. Chromatographic adsorbents include for example, ion exchange materials, metal chelators, hydrophobic interaction adsorbents, hydrophilic interaction adsorbents, dyes, and mixed mode adsorbents (e.g., hydrophobic attraction/electrostatic repulsion adsorbents). "Biospecific adsorbent" refers to an adsorbent comprising a biomolecule, e.g., a nucleotide, a nucleic acid molecule, an amino acid, a polypeptide, a simple sugar, a polysaccharide, a fatty acid, a lipid, a steroid or a conjugate of these (e.g., a glycoprotein, a lipoprotein, a glycolipid). In certain instances the biospecific adsorbent can be a macromolecular structure such as a multiprotein complex, a biological membrane or a virus. Examples of biospecific adsorbents are solid supports coupled to antibodies, receptor proteins, lectins and nucleic acids. Biospecific adsorbents typically have higher specificity for a target analyte than a chromatographic adsorbent.

Binding moieties may exist and interact with analytes detectable using the present invention in any physical state compatible with formation of molecular interactions, including gaseous, aqueous and organic suspensions and emulsions and, most preferably in a liquid state.

"Solid support" refers to any insoluble material including particles (e.g., beads), fibers, monoliths, membranes, filters, plastic strips and the like.

"Protein biochip" refers to a biochip adapted for the capture of polypeptides. Many protein biochips are described in the art.

"Gas phase ion spectrometer" refers to an apparatus that detects gas phase ions. Gas phase ion spectrometers include an ion source that supplies gas phase ions. Gas phase ion spectrometers include, for example, mass spectrometers, ion mobility spectrometers, and total ion current measuring devices. "Gas phase ion spectrometry" refers to the use of a gas phase ion spectrometer to detect gas phase ions.

"Mass spectrometer" refers to a gas phase ion spectrometer that measures a parameter that can be translated into mass-to-charge ratios of gas phase ions. Mass spectrometers generally include an ion source and a mass analyzer. Examples of mass spectrometers are time-of-flight, magnetic sector, quadrapole filter, ion trap, ion cyclotron resonance, electrostatic sector analyzer and hybrids of these. "Mass spectrometry" refers to the use of mass spectrometry to detect gas phase ions.

"Probe" or "mass spectrometer probe" in the context of this invention refers to a device that can be used to introduce ions derived from an analyte into a gas phase ion spectrometer, such as a mass spectrometer. A "probe" will generally comprise a solid substrate (either flexible or rigid) comprising a sample-presenting surface on which an analyte is presented to the source of ionizing energy. "Adsorbent surface" refers to a surface to which an adsorbent is bound. "Chemically selective surface" refers to a surface to which is bound either an adsorbent or a reactive moiety that is capable of binding a binding moiety, e.g., through a reaction forming a covalent or coordinate covalent bond.

"Variance" in the context of the present invention refers to the mathematical variance in the concentrations of analytes in a test sample. A reduction in variance is one that is statistically significant ($p > 0.05$). In simplest terms, the variance is the square of the standard deviation of all analyte concentrations in a test sample that are detected by at least one detection method. A preferred detection method is mass spectrometry, where the amount of a detectable analyte is the area beneath the mass peak identified by the detector.

"Wash buffer" refers to a solution that may be used to wash and remove non-bound material from an adsorbent surface. Wash buffers typically include salts that may or may not buffer pH within a specified range, detergents, and optionally may include other ingredients useful in removing adventitiously associated material from a surface or complex.

"Elution buffer" refers to a solution capable of dissociating a binding moiety and an associated analyte. In some circumstances, an elution buffer is capable of disrupting the interaction between subunits when the subunits are associated in a complex. As with wash buffers, elution buffers may include detergents, salt, organic solvents and the like used separately or as mixtures. Typically, these latter reagents are present at higher concentrations in an elution buffer than in a wash buffer making the elution buffer more disruptive to molecular interactions. Elution buffers may be at extremes of pH, or may contain chaotropes such as Guanidine HCl or urea.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides kits and methods that allow one of ordinary skill in the art to improve detection of analytes by reducing the concentration range of analytes of interest found in a complex mixture. The methods described here have particular advantage over prior art methods using analyte-specific reagents because they allow a reduction in the range of concentrations of analytes in samples that have unknown constituents and that are complex in both number of different analytes present (greater than 10^3) and in dynamic range of concentrations present (on the order of greater than 10^3). Consequently, using the claimed invention allows simultaneous analysis of the "Deep Proteome," which consists of the large number of low abundance proteins present in many fluids from biological sources, including blood, plasma and serum. (See Figure 5). The invention thus has utility in analytical preparation of complex mixtures of molecules, such as biological samples.

Reduction of the range of concentrations of analytes or concentration variance is accomplished by utilizing binding moiety libraries of defined size and diversity, preferably synthesized or coupled onto an inert support. When introduced to a solution containing a diversity of analytes, the binding moieties of the claimed invention will bind

analytes of the solution. Abundant analytes will be present in amounts far in excess of the amount necessary to saturate the capacity of their respective binding moiety; therefore, a high percentage of the total amount of these abundant analytes will remain unbound. Conversely, the lesser amounts of the trace analytes means that these molecules will not saturate all of their available binding moieties; therefore, a greater percentage of the starting amount of the trace analytes will remain bound to their respective binding moieties as compared with the abundant analytes. Non-bound analytes may be removed by washing. When the bound analytes are eluted from the binding moieties there is a decreased relative amount of the abundant analytes in the eluted material relative to the starting material. In contrast, the amount of trace analytes is increased in the eluted material relative to the starting material. This coincident alteration of the relative concentrations of analytes results in an eluted material where many, if not all, analytes present in the solution can be detected in a single analysis, or in fewer analysis steps than would be the case with the starting material. In animal serum for example, albumins are abundant, many complement-associated proteins, hormone-binding proteins are present in intermediate concentrations, while growth factors and cellular markers may be present at minute concentrations. Using the present invention, the range in analyte abundance observed in sera can be reduced, allowing many, if not most or even all analytes of interest to be analyzed.

Preparation of samples using the claimed invention is straightforward. After adsorbing the analytes of interest, the analytes are optionally washed to remove non-bound analytes. Adsorbed analytes are then eluted from the binding moieties using, for example, an elution buffer. The resulting solution contains all analytes of interest free from binding moieties; however, unlike the original complex mixture, the range in analyte concentration present in the resulting solution is relatively small as the concentration of high abundance analytes has been decreased and that of low abundance analytes increased relative to the original complex mixture. This modification in range of concentrations of analytes or concentration range between analytes allows for a larger percentage of the analytes in the resulting solution to be detected without the recalibration of the detection device necessary for direct analysis of complex mixtures having components present at widely different concentrations.

In some embodiments of the invention, analytes bound to binding moieties are eluted directly onto a probe or protein chip suitable for use in a mass spectrometer. To aid in analysis, elution buffers used in these embodiments may include a matrix material suitable for use in a mass spectrometer.

In some embodiments of the invention, analytes bond to binding moieties are digested with a protease such as trypsin. The change in structure due to fragmentation may release the resulting peptides from the binding moieties.

By providing a plurality of binding moieties, each recognizing a single or a low percentage of the analytes of interest present in a complex mixture, the present invention allows the composition of the complex mixture to be detected with minimal or no recalibration of the detection device. This includes detection of species that would otherwise not be detectable because either they were masked by high abundance analytes, or were present at too low a concentration to be detected by the method of analysis. This provides enormous benefits to high-throughput analysis techniques that would otherwise be limited at the detection step by the need for multiple recalibrations, and/or multiple channels, and/or multiple detection steps or expensive and wasteful fractionation techniques necessitated by the large concentration range of the analytes present in many complex mixtures. Moreover, by increasing the relative concentration of low-abundance analytes, the invention allows detection of analytes that are only present in the sample in trace amounts. Using serum as an example, certain analytes such as some hormones are present at only trace amounts in unconcentrated sera. Other analytes, such as albumin, are abundant, being present in amounts ranging from micromolar to millimolar. The present invention concentrates the low abundance analytes relative to the high abundance analytes. Thus, in preparation of the exemplary serum sample using the present invention, the concentration of cytokines is increased relative to the concentration of albumin and other high abundance analytes. By bringing the concentrations of low and high abundance analytes from the sera closer together, the analyte composition can be determined both qualitatively and quantitatively.

By the same approach, the present invention allows determining trace amounts of proteins present in biological samples such as purified therapeutic proteins, where the tolerance for protein impurity is very limited. For example, purified antibodies from cell

culture supernatants may contain traces of different proteins coming from the cells used for the expression of antibodies. These latter should not be present and are generally detected by specific ELISA assays. However, when the concentration of impurities is very low, immunochemical tests are not effective. If the sample to analyze is first treated according to the present invention, trace protein impurities may be significantly concentrated and therefore detected by regular chemical or immunochemical methods.

I. Reducing Relative Analyte Concentrations in a Sample

A. Suitable Test Samples

Test samples of the present invention may be in any form that allows analytes present in the test sample to be contacted with binding moieties of the present invention, as described herein. Suitable test samples include gases, liquids, suspensions, emulsions, and the like. Preferably test solutions are liquids. Test samples may be taken directly from a source and used in the methods of the present invention without any preliminary manipulation. For example, a water sample may be taken directly from an aquifer and treated directly using the methods described herein.

Alternatively, the original sample may be prepared in a variety of ways to enhance its suitability for testing. Such sample preparations include depletion of certain analytes, concentrating, grinding, extracting, percolating and the like. For example, solid samples may be pulverized to a powder, then extracted using an aqueous or organic solvent. The extract from the powder may then be subjected to the methods of the present invention. Gaseous samples may be bubbled or percolated through a solution to dissolve and/or concentrate components of the gas in a liquid prior to subjecting the liquid to methods of the present invention.

Test samples preferably contain at least four analytes of interest, more preferably at least 8, 15, 20, 50, 100, 1000, 100,000, 1,000,000, 10,000,000 or more analytes of interest. In some circumstances, test samples suitable for manipulation using the methods of the present invention may include hundreds or thousands of analytes of interest. Preferably, the concentrations of analytes present in the test sample spans at least an order of magnitude, more preferably at least two, three, four or more orders of magnitude. Once subjected to the methods of the present invention, this concentration range for

analytes detectable by at least one detection method will be decreased by at least a factor of two, more preferably a factor of 10, 20, 50, 100, 1000 or more.

Test samples may be collected using any suitable method. For example, environmental samples may be collected by dipping, picking, scooping, sucking, or trapping. Biological samples may be collected by swabbing, scraping, withdrawing surgically or with a hypodermic needle, and the like. The collection method in each instance is highly dependent upon the sample source and the situation, with many alternative, suitable techniques of collection well known to those of skill in the art.

1. Biological Test Samples

Test samples may be taken from any source that potentially includes analytes of interest including environmental samples such as air, water, soil, extracts and the like. A preferred test sample of the present is a biological sample, preferably a biological fluid. Biological samples that can be manipulated with the present invention include amniotic fluid, blood, cerebrospinal fluid, intra-articular fluid, intraocular fluid, lymphatic fluid, milk, perspiration plasma, saliva, semen, seminal plasma, serum, sputum, synovial fluid, tears, umbilical cord fluid, urine, biopsy homogenate, cell culture fluid, cell extracts, cell homogenate, conditioned medium, fermentation broth, tissue homogenate and derivatives of these. Analytes of interest in biological samples include proteins, lipids, nucleic acids and polysaccharides. More particularly, analytes of interest are cellular metabolites that are normally present in the animal, or are associated with a disease or infectious state such as a cancer, a viral infection, a parasitic infection, a bacterial infection and the like. Particularly interesting analytes are those that are markers for cellular stress. Analytes indicating that the animal is under stress are an early indicator of a number of disease states, including certain mental illnesses, myocardial infarction and infection.

Analytes of interest also include those that are foreign to the animal, but found in tissue(s) of the animal. Particularly interesting analytes in this regard include therapeutic drugs including antibiotics, many of which exist as different enantiomers and toxins that may be produced by infecting organisms, or sequestered in an animal from the environment. Other analytes of interest include plants, food such as egg white, or muscle extracts.

2. Environmental Test Samples

Environmental samples are another class of preferred test samples for use with the present invention. Preferred environmental samples include soil, dust, dander, natural and synthetic fibers, water, plant materials, animal feces and the like. Preferred analytes in environmental samples include natural and synthetic toxins, fertilizers, herbicides and insecticides, and markers for bacterial and viral agents such as structural proteins characteristic of the agent of interest. Particularly preferred analytes sought in environmental test samples are toxins, particularly toxins such as botulinum, ricin, anthrax toxins and the like. Disease-related analytes of interest present in environmental test samples include complete virions as well as characteristic proteins and nucleic acids of botulinus, ebola, HIV, SARS, anthrax, plague, malaria, small pox, prions associated with bovine spongiform encephalopathy, scrapie, variant CJD etc.

Exemplary environmental samples can be obtained from numerous sources including the natural environment, such as a naturally-occurring body of water. The naturally-occurring body of water can be, for example, an ocean, a lake, a sea, a river, a swamp, a pond, a delta, or a bay. The environmental extract can alternatively be an extract from a water treatment center.

Alternatively, the environmental sample can be taken from a man-made environment, such as a building. The building can be any man-made building. Preferably, the building is contaminated with one or more biological pathogens such as small pox, anthrax, or one or more toxic agents, such as sarin, soman, nerve poisons, explosive chemicals, pesticides, VX, and blister agents. Methods for obtaining the environmental sample include dry swabbing the surface of the building, or wet swabbing the building's surface using a suitable solvent known to those of skill in the art.

B. Suitable Binding Moieties

Suitable binding moieties of the present invention include small organic molecules, such as dyes and triazines, and biopolymers such as peptides, proteins, polynucleotides, oligosaccharides or lipids. Binding moieties of the present invention may be molecules having molecular weights of 100 KDa or more, such as antibodies, but preferably are smaller molecules with a molecular weight in the range of 10 KDa, more preferably around 1 KDa, desirably less than 1 KDa for example, or less than 750 Da.

Ideally, binding moieties of the present invention are coupled to an insoluble particulate material. Each insoluble particle preferably carries several copies of the same binding moiety, with each particle type coupling a different binding moiety.

Binding moieties of the present invention may be in solution, suspension, or in any other situation allowing contact of the binding moiety with analyte including mounted on a solid support.

The binding moieties may be part of a "phage display library" where the peptide is presented as part of the phage coat. (See, e.g., Tang, Xiao-Bo, *et al.*; *J. Biochem*; 1997; pp. 686-690; vol. 122, No. 4). Presenting the peptide on the surface of the phage particle allows high-throughput screening of combinatorial libraries of small peptides, a method that is also advantageous for screening combinatorial antibody libraries. A phage display library is formed from bacteriophage that have been recombinantly manipulated to express binding moiety as part of the phage protein coat. Using phage display, libraries of binding moieties may be easily constructed.

Binding moieties may also be soluble combinatorial molecules. Soluble combinatorial molecules preferably comprise a capture moiety that allows the binding moiety to be coupled to a complementary solid support. Soluble binding moiety embodiments are typically contacted to the sample and allowed to bind analyte(s) of interest prior to isolating the resulting complexes by binding or coupling the binding moiety to a solid support. Combinatorial libraries may be composed of building blocks containing chiral atoms such as 19 of the naturally occurring amino acids. Chiral ligands allow chiral isomers to bind to separate binding moieties.

Binding moieties of the present invention may be produced using any technique known to those of skill in the art. For example, binding moieties may be chemically synthesized, harvested from a natural source or, in the case of binding moieties that are bio-organic polymers, produced using recombinant techniques. For this latter reason, peptides having no more than 15, 10, 8, 6 or 4 amino acids are particularly advantageous, as they are easily produced using recombinant or solid phase chemistry techniques. Moreover, chemically synthesized libraries are described, for example, in Fodor *et al.*, *Science* 251: 767-773 (1991) and Houghten *et al.*, *Nature* 354: 84-86 (1991). In the split-couple-recombine solid phase combinatorial synthesis Lam *et al.*, *Nature* 354, 82-84

(1991) such that the diversity of the complement of binding moieties is a result of the number of different amino acids to the power of the length of the binding moiety (number of amino acids in an individual binding moiety).

Nucleic acids are another preferred bio-organic polymer binding moiety. As with peptides, nucleic acids may be produced using synthetic or recombinant techniques well-known to those of skill in the art. Preferable nucleic acid binding moieties of the present invention are at least 4, more preferably 6, 8, 10, 15, or 20 nucleotides in length. Nucleic acid binding moieties include single or double stranded DNA or RNA molecules (e.g., aptamers) that bind to specific molecular targets, such as a complementary nucleic acid, a protein, or a metabolite.

Oligosaccharide binding moieties are also contemplated as part of the invention. Oligosaccharide binding moieties are preferably at least 5 monosaccharide units in length, more preferably 8, 10, 15, 20, 25 or more monosaccharide units in length.

A biopolymer binding moiety can be a lipid. As used herein, the term "lipid" refers to a hydrophobic or amphipathic moiety. Thus, a lipid library of binding moieties is also contemplated for use in the methods and kits of the invention. Suitable lipids include a C14 to C50 aliphatic, aryl, arylalkyl, arylalkenyl, or arylalkynyl moiety, which may include at least one heteroatom selected from the group consisting of nitrogen, sulfur, oxygen, and phosphorus. Other suitable lipids include a phosphoglyceride, a glycosylglyceride, a sphingolipid, a sterol, a phosphatidyl ethanolamine or a phosphatidyl propanolamine. Lipid library of binding moieties are preferably at least 5 units in length, more preferably at least 8, 10, 15, 20, 25, 50 or more units in length.

Small organic molecules are also contemplated as binding moieties of the present invention. Typically, such molecules have properties that allow for ionic, hydrophobic or affinity interactions with the analyte. Small organic binding moieties include chemical groups traditionally used in chromatographic processes such as mono-, di- and tri-methyl amino ethyl groups, mono-, di- and tri-ethyl amino ethyl groups, sulphonyl, phosphoryl, phenyl, carboxymethyl groups and the like. For example, libraries may use benzodiazepines, (see, e.g. Bunin *et al., Proc. Natl. Acad. Sci.* USA 91: 4708-4712 (1994)) and peptoids (e.g. Simon *et al., Proc. Natl. Acad. Sci.* USA 89: 9367-9371 (1992)). In another embodiment, the binding moiety is a dye or a triazine derivative.

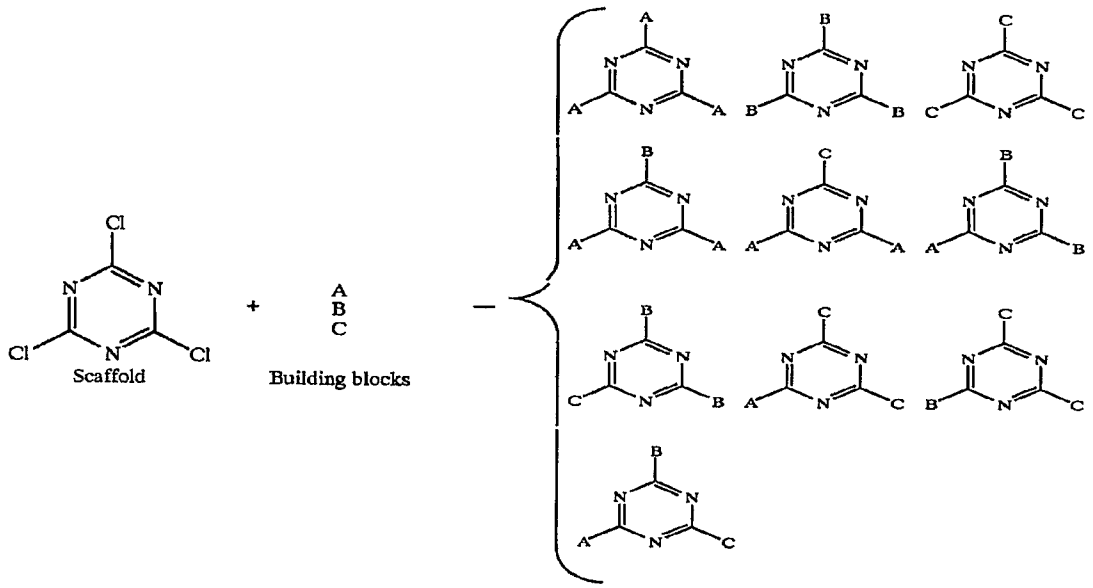
This list is by no means exhaustive, as one of skill in the art will readily recognize thousands of chemical functional groups with ionic, hydrophobic or affinity properties compatible with use as binding moieties of the present invention. The production and use of combinatorial binding moiety libraries is discussed in more detail, below.

Binding moieties may be purchased pre-coupled to the supports, synthesized on the support, or may be indirectly attached or directly immobilized on the support using standard methods (see, for example, Harlow and Lane, *Antibodies*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1988); Biancala *et al., Letters in Peptide Science* 7(291): 297(2000); MacBeath *et al., Science* 289: 1760-1763 (2000); Cass *et al.*, ed., *Proceedings of the Thirteenth American Peptide Symposium*; Leiden, Escom, 975-979 (1994); U.S. Patent Number 5,576,220; Cook *et al., Tetrahedron Letters* 35: 6777-6780 (1994); and Fodor *et al., Science* 251(4995): 767-773 (1991)).

Combinatorial Libraries

In one embodiment of this invention the library of binding moieties is a combinatorial library or portion thereof. A combinatorial chemical library is a collection of compounds generated by either chemical synthesis or biological synthesis, by combining a number of chemical "building blocks" in all possible combinations. For example, a complete linear combinatorial chemical library, such as a polypeptide library, is formed by combining a set of chemical building blocks (amino acids) in every possible way for a given compound length (i.e., the number of amino acids in a polypeptide compound). As an example, if the number of building blocks is 5 and the construct is composed of five members, the number of possible linear combinations is of 5^5 or 3,125 members. In this case the building blocks (A, B, C, D and E) are assembled linearly such as: A-A-A-A-A; A-A-A-A-B; A-A-A-A-C; A-A-A-B-A; A-A-A-B-B; A-A-A-B-C; . . . . .; A-A-B-A-A; A-A-B-A-B; A-A-B-A-C; . . . . .; E-E-E-E-C; E-E-E-E-D; E-E-E-E-E.

Another form of combinatorial library is scaffold-based. These constructs are based on a single central molecule or core, comprising positions that can be substituted by building blocks. An example is given by trichloro-triazine (three substitutable positions) on which several substituents can be attached. If the number of substituents is three, the number of possible combinations is 10. It is also possible to consider the relative positioning of each substituent; in this case the number of combinations is larger.



Combinatorial Library

As a third level it is possible to combine linear combinatorial libraries with scaffold-based libraries where substituents of the latter are combinatorial linear sequences.

Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks. For peptide binding moieties, the length is preferably limited to 15, 10, 8, 6 or 4 amino acids. Nucleic acid binding moieties of the invention have preferred lengths of at least 4, more preferably 6, 8, 10, 15, or at least 20 nucleotides. Oligosaccharides are preferably at least 5 monosaccharide units in length, more preferably 8, 10, 15, 20, 25 or more monosaccharide units.

Combinatorial libraries may be complete or incomplete. Complete combinatorial libraries of biopolymers are those libraries containing a representative of every possible permutation of monomers for a given polymer length and composition. Incomplete libraries are those libraries lacking one or more possible permutation of monomers for a given polymer length.

Peptide binding moieties are a preferred embodiment of the claimed invention. Methods for generating libraries of peptide binding moieties suitable for use in the

claimed invention are well known to those of skill in the art, e.g., the "split, couple, and recombine" method (see, e.g., Furka *et al., Int. J. Peptide Protein Res.*, 37: 487-493 (1991); Houghton *et al., Nature* 354:84-88 (1991); Lam *et al., Nature*, 354: 82-84 (1991); International Patent Application Publication Number WO 92/00091; and U.S. Patent Numbers 5,010,175; 5,133,866; and 5,498,538) or other approaches known in the art. The expression of peptide libraries also is described in Devlin *et al., Science*, 249: 404-406 (1990).

Combinatorial and synthetic chemistry techniques well-known in the art can generate libraries containing millions of members (Lam *et al., Nature* 354: 82-84 (1991) and International (PCT) Patent Application Publication Number WO 92/00091), each having a unique structure. A library of linear hexamer ligands made with 18 of the natural amino acids, for example, contains 34 x 10^6 different structures. When amino acid analogs and isomers are also included, the number of potential structures is practically limitless. Moreover, each member of such a library potentially possesses the capacity to bind to a different molecule. Members of a combinatorial library can be synthesized on or coupled to a solid support, such as a bead, with each bead essentially having millions of copies of a library member on its surface. As different beads may be coupled to different library members and the total number of beads used to couple the library members large, the potential number of different molecules capable of binding to the bead-coupled library members is enormous.

Hammond *et al.*, US Patent No. 7,217,507 describes combinatorial libraries along the following lines. Peptide binding moiety libraries may be synthesized from amino acids that provide increased stability relative to the natural amino acids. For example, cysteine, methionine and tryptophan may be omitted from the library and unnatural amino acids such as 2-naphylalanine and norleucine included. The N-terminal amino acid may be a D-isomer or may be acetylated to provide greater biochemical stability in the presence of amino-peptidases. The binding moiety density must be sufficient to provide sufficient binding for the target molecule, but not so high that the binding moieties interact with themselves rather than the target molecule. A binding moiety density of 0.1 μmole - 500 μmole per gram of dry weight of support is desired and more preferably a binding moiety density of 10 μmole - 100 μmole per gram of support is

desired. A 6-mer peptide library was synthesized onto Toyopearl-AF Amino 650M resin (Tosoh Bioscience,, Montgomeryville, Pennsylvania). The size of the resin beads ranged from 60-130 μm per bead. Initial substitution of the starting resin was achieved by coupling of a mixture of Fmoc-Ala-OH and tBoc-Ala-OH (1:3.8 molar ratio). After coupling, the tBoc protecting group was removed with neat TFA. The resulting deprotected amino groups were then acetylated. Peptide chains were assembled via the remaining Fmoc-Ala-OH sites on the resin bead. Standard Fmoc synthetic strategies were employed. In one embodiment, a typical experiment, six grams of Fmoc-Ala-(Ac-Ala-) Toyopearl Resin was deprotected with 20% piperdine/DMF (2 x 20 min), then washed with DMF (8 times) and equally divided into 18 separate reaction vessels. In each separate vessel, a single Fmoc-amino acid was coupled to the resin (BOP/NMM, 5-10 told excess) for 4-7 hours. The individual resins were washed and combined using the "split/mix" library technique (Furka *et al., Int. J. Peptide Protein Res.*, 37, 487-493 (1991); Lam *et al., Nature*, 354, 82-84 (1991); International Patent Application Publication Number WO 92/00091 (1992); U.S. Patent No. 5,010,175; U.S. Patent No.5,133,866; and U.S. Patent No. 5,498,538). The cycle of deprotection and coupling was repeated until the amino acid sequence was completed (six cycles for a hexamer library). The final Fmoc was removed from peptide resins using 20% piperidine/DMF in separate reaction vessels during the last coupling cycle. Side-chain protecting groups were removed with TFA treatment (TFA:H_2O:Phenol, 90:5:5) for 2 hours. Resins were washed extensively and dried under a vacuum. Peptide densities achieved were typically in the range of 0.06-0.12 mmol/g of resin. The amino acids may be either L or D-stereoisomers or racemates.

Sequencing and peptide composition of peptide ligand-resin bead complexes were confirmed, and the degree of substitution of the resin was calculated by quantitative amino acid analysis at Commonwealth Biotechnologies, Inc., Richmond, Virginia. Sequencing was performed at Protein Technologies Laboratories, Texas A&M University, by Edman degradation using a Hewlett Packard G1005A.

Devices for the preparation of combinatorial libraries are commercially available (see, e.g., 357 MPS, 390 MPS, Advanced Chem Tech, Louisville, Kentucky, Symphony, Rainin, Woburn, Massachusetts, 433A, Applied Biosystems, Foster City, California,

9050 Plus, Millipore, Bedford, Massachusetts). In addition, numerous combinatorial libraries are themselves commercially available (see, e.g., ComGenex, Princeton, New Jersey, Tripos, Inc., St. Louis, Missouri, 3D Pharmaceuticals, Exton, Pennsylvania, Martek Biosciences, Columbia, Marlyand, etc.).

In some peptide library embodiments, the peptides are expressed on the surface of recombinant bacteriophage to produce large, easily screened libraries. Using the "phage method" (Scott and Smith, *Science* 249:386-390, 1990; Cwirla, *et al, Proc. Natl. Acad. Sci.*, 87:6378-6382, 1990; Devlin *et al., Science*, 49:404-406; 1990), very large libraries can be constructed (10^6-10^8 chemical entities). A second approach uses primarily chemical methods, of which the Geysen method (Geysen *et al., Molecular Immunology* 23:709-715, 1986; Geysen *et al. J. Immunologic Method* 102:259-274, 1987; and the method of Fodor *et a*l. (*Science* 251:767-773, 1991) are examples. Furka *et al.* (*14th International Congress of Biochemistry*, Volume #5, Abstract FR:013, 1988; Furka, *Int. J. Peptide Protein Res.* 37:487-493, 1991), Houghton (U.S. Patent Number 4,631,211, issued December 1986) and Rutter *et al.* (U.S. Patent Number 5,010,175, issued April 23, 1991) describe methods to produce a mixture of peptides that can be tested as agonists or antagonists.

Other chemistries for generating chemical diversity libraries can also be used. Such chemistries include, but are not limited to: peptides (e.g., International Patent Application Publication Number WO 91/19735), encoded peptides (e.g., International Patent Application Publication Number WO 93/20242), random bio-oligomers (e.g., International Application Patent Publication Number WO 92/00091), benzodiazepines (e.g., U.S. Patent Number 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs *et al., Proc. Nat. Acad. Sci.* USA 90:6909-6913 (1993)), vinylogous polypeptides (Hagihara *et al., J. Amer. Chem. Soc.* 114:6568 (1992)), nonpeptidal peptidomimetics with glucose scaffolding (Hirschmann *et al., J. Amer. Chem. Soc.* 114:9217-9218 (1992)), analogous organic syntheses of small compound libraries (Chen *et al., J. Amer. Chem. Soc.* 116:2661 (1994)), oligocarbamates (Cho *et al., Science* 261:1303 (1993)), and/or peptidyl phosphonates (Campbell *et al., J. Org. Chem.* 59:658 (1994)), nucleic acid libraries (see Ausubel, Berger and Sambrook, all *supra*), peptide nucleic acid libraries (see, e.g., U.S. Patent Number 5,539,083), antibody libraries

(see, e.g., Vaughn *et al., Nature Biotechnology*, 14(3):309-314 (1996) and International Patent Application Serial Number PCT/US96/10287), carbohydrate libraries (see, e.g., Liang *et al., Science*, 274:1520-1522 (1996) and U.S. Patent Number 5,593,853), small organic molecule libraries (see, e.g., benzodiazepines, Baum C&EN, January 18, page 33 (1993); isoprenoids, U.S. Patent Number 5,569,588; thiazolidinones and metathiazanones, U.S. Patent Number 5,549,974; pyrrolidines, U.S. Patent Numbers 5,525,735 and 5,519,134; morpholino compounds, U.S. Patent Numbers 5,506,337; benzodiazepines, 5,288,514, and the like).

Linker Moieties

Binding moieties of the present invention optionally include linker moieties that allow targeted and/or reversible coupling of the binding moiety to a solid support. Exemplary linker moieties include epitope and his-tags, which are attached to the biomolecule to be captured to form a fusion protein. In these instances, a cleavable linker sequence, such as those specific for Factor Xa or enterokinase (Invitrogen, San Diego, California) may be optionally included between the biomolecule and the capture moiety to facilitate isolation and/or separation of the components of the fusion molecule. Protein domains specifically recognized by designer ligands may also be used as linker moieties (See, e.g., Deisenhofer, J., *Biochemistry 20* (1981) 2361-2370). Many other equivalent linker moieties are known in the art; see, e.g., Hochuli, *Chemische Industrie*, 12:69-70 (1989); Hochuli, *Genetic Engineering, Principle and Methods*, 12:87-98 (1990), Plenum Press, New York; and Crowe, *et al.* (1992) *OIAexpress: The High Level Expression & Protein Purification System*, QIAGEN, Inc. Chatsworth, California.; which are incorporated herein by reference. Antigenic determinants and other characteristic properties of the biomolecule to be adsorbed may also serve as capture moiety tags. Exemplary linker moieties include poly-histidine (poly-his) or poly-histidine-glycine (poly-his-gly) tags; the flu HA tag polypeptide and its antibody 12CA5 [Field *et al., Mol. Cell. Biol.*, 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan *et al., Molecular and Cellular Biology*, 5:3610-3616 (1985)); and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody (Paborsky *et al., Protein Engineering*, 3(6):547-553 (1990)). Other tag polypeptides include the Flag-peptide (Hopp *et al., BioTechnology*, 6:1204-1210 (1988)); the KT3

epitope peptide (Martin *et al., Science*, 255:192-194 (1992)); a α-tubulin epitope peptide (Skinner *et al., J. Biol. Chem.*, 266: 15163-15166 (1991)); and the T7 gene 10 protein peptide tag (Lutz-Freyermuth *et al., Proc. Natl. Acad. Sci.* USA, 87: 6393-6397 (1990)).

C. Capturing Analytes From A Test Sample Using Binding Moieties

Analytes present in a test sample are captured by contacting the test sample with the binding moieties under conditions that allow each binding moiety to couple with its corresponding analyte. As inferred above, binding moieties may be contacted with the test sample directly, or the binding moieties may be first attached to a solid support, such as a dipstick or insoluble polymeric bead, membrane or powder.

In the case in which the binding moieties are part of a bead library, the ratio of bead volume to sample volume for a complex, concentrated sample such as serum can be between, for example, 1:150 and 1:1. The smaller the ratio of beads to sample, the greater the ability to increase the relative concentration of low abundance or rare analyte species. At a constant bead:sample volume of 1:10, volumes of beads used with serum can be at least between 0.0005 ml and 15 ml of beads (including 0.020 ml). In the event that a dilute protein solution is used, such as 0.01 mg/ml protein concentration, and the capacity of a library is about 10 mg/ml total protein, a ratio of bead volume to sample volume of 1:100 and 1:1000 or more will results in the protein being concentrated on the beads.

In one embodiment, the binding moiety is coupled to a solid support prior to contacting the test sample. In this alternative embodiment, the solid support is simply contacted with the test sample for a time sufficient to allow the binding moiety to bind to the analyte, then the solid support is withdrawn from the test sample with the analyte bound to it via formation of a complex between the analyte and the binding moiety.

In one embodiment, the binding moieties include a linker moiety. In this embodiment the binding moieties are contacted directly to the test sample in a manner that allows analytes present in the test sample to bind to the binding moieties. After sufficient time has elapsed, a solid support that includes a complementary capture moiety to the capture moiety of the binding moiety is contacted to the test sample. This allows

the binding moiety to couple with the solid support through the capture moiety, while retaining the bound analyte.

Contacting the binding moiety with the test sample may be accomplished by admixing the two, contacting the test sample with the binding moiety, flowing the test sample over a solid support having binding moieties attached thereto, and other methods that would be obvious to those of ordinary skill in the art. The binding moieties and the analytes are kept in contact for a time sufficient to allow the binding moieties to reach binding equilibrium with the sample. Under typical laboratory conditions this is at least 10 minutes.

Solid Supports

Acceptable supports for use in the present invention can vary widely. A support can be porous or nonporous. It can be continuous or non-continuous, flexible or nonflexible. A support can be made of a variety of materials including ceramic, glassy, metallic, organic polymeric materials, or combinations thereof.

Preferred supports include organic polymeric supports, such as particulate or beaded supports, woven and nonwoven webs (such as fibrous webs), microporous fibers, microporous membranes, hollow fibers or tubes. Polyacrylamide and mineral supports such as silicates and carbonates (e.g., hydroxyl apatite) can also be used. Woven and nonwoven webs may have either regular or irregular physical configurations of surfaces. Particularly preferred embodiments include solid supports in the form of spherical or irregularly-shaped beads or particles.

Porous materials are useful because they provide large surface areas. The porous support can be synthetic or natural, organic or inorganic. Suitable solids have a porous structure with pores of a diameter of at least about 1.0 nanometer (nm) and a pore volume of at least about 0.1 cubic centimeter/gram (cm^3/g). Preferably, the pore diameter is at least about 30 nm because larger pores will be less restrictive to diffusion. Preferably, the pore volume is at least about 0.5 cm^3/g for greater potential capacity due to greater surface area surrounding the pores. Preferred porous supports include particulate or beaded supports such as agarose, hydrophilic polyacrylates, polystyrene, mineral oxides and Sepharose, including spherical and irregular-shaped beads and particles. Porous materials have an additional advantage in that the porosity may be controlled

adventitiously to restrict the binding of large aggregates or cells such as red blood cells from blood. This allows preferential retention of the smaller, permeable proteins such those found in plasma.

For significant advantage, the supports for binding moieties are preferably hydrophilic. Preferably, the hydrophilic polymers swell in water to allow for greater infiltration of analytes. Examples of such supports include natural polysaccharides such as cellulose, modified celluloses, agarose, cross-linked dextrans, amino-modified cross-linked dextrans, guar gums, modified guar gums, xanthan gums, locust bean gums and hydrogels. Other examples include cross-linked synthetic hydrophilic polymers such as polyacrylamide, polyacrylates, polyvinyl alcohol (PVA) and modified polyethylene glycols.

Attachment of the binding moieties to the solid support may be accomplished through a variety of mechanisms. The solid support can be derivatized with a fully prepared binding moiety by attaching a previously prepared binding moiety to the solid support. Alternatively, the binding moiety may be formed on the solid support by attaching a precursor molecule to the solid support and subsequently adding additional precursor molecules to the growing chain bound to the solid support by the first precursor molecule. This mechanism of building the adsorbent on the solid support is particularly useful when the binding moiety is a polymer, particularly a biopolymer such as a polypeptide, polynucleotide or polysaccharide molecule. A biopolymer adsorbent can be provided by successively adding monomeric components (e.g., amino acids, nucleotides or simple sugars) to a first monomeric component attached to the solid support using methods known in the art. See, e.g., U.S. Patent Number 5,445,934 (Fodor *et al.*).

In certain embodiments, for example combinatorial libraries, each solid support, e.g., each bead can have only one binding moiety attached to it (within the limits of combinatorial chemistry).

However, in another embodiment, each solid support can have a plurality of different binding moieties attached. For example, a combinatorial library of peptides can be manufactured using the split-and-pool process. These peptides can be cleaved from the beads to which they attached, mixed, and then attached to a new set of beads, without any sorting of the peptides by beads. In this way, each bead will have many different

binding moieties attached. Accordingly, this invention provides combinatorial libraries of binding moieties in which a plurality of different members of the combinatorial library are attached to the same solid support. As few as one and as many as 10, 100, 1000, 10,000, 1,000,000, 1,000,000,000 or more different binding moieties may be coupled to a single solid support. In certain embodiments the solid support is in the form of beads, with a single, different, binding moiety type bound to each bead. For example in a peptide binding moiety library, peptides representing one possible permutation of amino acids would be bound to one bead, peptides representing another possible permutation to another bead, and so on.

Binding moieties may be coupled to a solid support using reversible or non-reversible interactions. For example, non-reversible interactions may be made using a support that includes at least one reactive functional group, such as a hydroxyl, carboxyl, sulfhydryl, or amino group that chemically binds to the binding moiety, optionally through a spacer group. Suitable functional groups include N-hydroxysuccinimide esters, sulfonyl esters, iodoacetyl groups, aldehydes, epoxy, imidazolyl carbamates, and cyanogen bromide and other halogen-activated supports. Such functional groups can be provided to a support by a variety of known techniques. For example, a glass surface can be derivatized with aminopropyl triethoxysilane in a known manner. In some embodiments, binding moieties are coupled to a solid support during synthesis, as is known to those of skill in the art (e.g., solid phase peptide and nucleic acid synthesis).

Alternatively, reversible interactions between a solid support and a binding moiety may be made using linker moieties associated with the solid support and/or the binding moiety. A variety of linker moieties suitable for use with the present invention are known, some of which are discussed above. Use of linker moieties for coupling diverse agents is well known to one of ordinary skill in the art, who can apply this common knowledge to form solid support/binding moiety couplings suitable for use in the present invention with no more than routine experimentation.

Microparticulate Solid Supports

A preferred embodiment of the present invention utilizes small, beaded, microparticulate solid supports that are less than 1000 μm, preferably less than 100, 10, 1 or 0.1μm in diameter. Such supports are typically formed by mechanical milling or

otherwise reducing larger beads to a powder consistency. Microparticulate solid supports are desirable because they possess increased surface area to volume ratio compared to a larger, non-porous bead. Microparticulate solid supports also decrease the volume of support necessary to contain a combinatorial library of the invention, thereby allowing more complex libraries to be used. Using existing equipment however, it is difficult to synthesize combinatorial libraries on very small (< 10 μm) beads due to the limitations in frit sizes of the filter systems used. To overcome this problem the combinatorial library may be synthesized in bulk on a bead that may then be fragmented by mechanically grinding, crushing, or sonicating it to form a powder or collection of micro-particles.

Using these techniques, microparticulate solid supports coupled to different binding moieties may be produced. These in turn may be extensively mixed to form a more uniform composition relative to mixing larger or various sizes of different beads.

The microparticulate solid support may be covalently attached to an activated surface to make a "dipstick" or chip through an epoxy group, N-hydroxysuccinimide, dimethyl 3,3'-dithiopropionimidate, or glutaraldehyde so as to form a chemical bond with the ligands of the combinatorial library or with the base matrix of the polymer on which the ligands were synthesized. This may be achieved through cross-linking to the N-terminal amino group of a peptide library.

Non-reacted cross-linking groups on the surface may be reacted with a small chemical such a mercapto-ethanol or ethanolamine to prevent further reactivity. In addition, surfaces may be further treated to prevent non-specific adhesion of protein.

Target molecules bound to binding moieties coupled to microparticulate solid supports may be washed in one or a variety of ways, e.g. with buffer at different salt concentrations and pH, and the bound proteins eluted in solutions of low pH, low or high ionic strength, strong chaotropes, acetonitrile/formic acid, etc.

Eluted target molecules may be analyzed for protein composition according to molecular weight by several methods, including, but not limited to, mass spectrometry, SDS-PAGE, capillary electrophoresis, or by pI through isoelectric focusing.

Alternatively, target molecules may be eluted through electrophoresis. In this embodiment the microparticulate solid supports containing bound target molecules may be soaked with an appropriate solution such as Laemmli buffer and the proteins resolved

by SDS-PAGE analysis. An alternative buffer may contain urea and the proteins may be separated by electrophoresis into an isoelectric focusing gel. Alternatively, the target molecules may be eluted following digestion with proteases such as trypsin and the resulting peptides removed by washing.

Alternatively, the microparticulate solid supports may be compounded with a bulking agent and compacted into tablet form. In this format it may be added directly to a sample solution or instead, first suspended in buffer.

Microparticulate solid supports may be placed into solution such as agarose or acrylamide and cross-linked into a gel itself or cross-linked to each other through a polymerization reaction with a cross-linker on a fiber to form a monolithic material.

Alternatively, microparticulate solid supports may be immobilized onto a thin film of adhesive.

Another approach is the entrapment of microparticulate solid supports in a porous matrix. Such matrixes could include nonwoven fibers or webs with the particles possibly being incorporated during the melt-blowing stage.

Microparticles can be incorporated into a single sheet or stack of membranes as desired to achieve the appropriate desired binding capacity, in which case the microparticulate solid supports are entrapped between the layers by calendering or hydroentanglement.

The membrane composition can be selected from natural or synthetic sources including polyester and polypropylene fibers and meshes. Of course, one of skill in the art will be aware that many of the techniques described in this section are generally applicable to other embodiments of the present invention.

Removing Non-bound Analytes

A feature of the present invention is that treatment of analytes according to the methods described herein preferably concentrates and partially purifies bound analyte in addition to reducing the variance between analyte concentrations. Implementation of this feature to the fullest includes optionally washing any non-bound analytes from the analyte bound to the binding moieties on the solid support.

Washing away non-bound analytes is preferably performed by contacting the analyte bound to the binding moiety with a mild wash solution. The mild wash solution

is designed to remove contaminants including aggregates and cells and non-bound analytes frequently found in the test sample originally containing the analyte. Typically a wash solution will be at a physiologic pH and ionic strength and the wash will be conducted under ambient conditions of temperature and pressure.

Formulation of wash solutions suitable for use in the present invention can be performed by one of skill in the art without undue experimentation. Methods for removing contaminants, including low stringency washing methods, are published, for example in Scopes, *Protein Purification: Principles and Practice* (1982); Ausubel, *et al.* (1987 and periodic supplements); *Current Protocols in Molecular Biology*; Deutscher (1990) "Guide to Protein Purification" *in Methods in Enzymology* vol. 182, and other volumes in this series.

D. Isolating Captured Analytes From Binding Moieties

Bound analyte may be eluted from the binding moieties and isolated using a variety of methods, preferably by using an aqueous elution buffer that disrupts the interaction between the binding moiety and the analyte. Any suitable elution buffer may be used for this purpose, including denaturing agents such as chaotropes and organic solvents. Exemplary elution buffers include aqueous salt solutions of very low or high ionic strength, low or high pH, detergent solutions, and organic solvents. Solutions and suspensions of agents that competitively bind to binding moieties of the invention may also be used in elution buffers, provided that such competitive binding agents do not interfere with subsequent collection or analysis of the analytes of interest. The elution buffer(s) chosen are highly application-specific and may be readily identified by one of ordinary skill in the art through materials commonly available in the public domain or through routine experimentation (see, e.g., *Scopes, Protein Purification: Principles and Practice* (1982); and Deutscher (1990) *"Guide to Protein Purification" in Methods in Enzymology* vol. 182, and other volumes in this series).

A typical experimental sequence includes washing with physiological concentrations of sodium chloride and elution with high concentrations (1M and above) of sodium chloride (to collect proteins adsorbed by a dominant ion exchange interaction), followed by ethylene glycol (eluent for protein interacting mainly by hydrophobic associations), followed by lowering the pH to 2.5 (to denature proteins) to alter the

overall charge of the protein and prevent ionic interactions and finally by a chaotrope such as 6M guanidine-HCl.

Examples of suitable elution buffers include those that modify surface charge of an analyte and/or binding moiety, such as pH buffer solutions. pH buffer solutions used to disrupt surface charge through modification of acidity preferably are strong buffers, sufficient to maintain the pH of a solution in the acidic range, i.e., at a pH less than 7, preferably less than 6.8, 6.5, 6.0, 5.5, 5.0, 4.0 or 3.0; or in the basic range at a pH greater than 7, preferably greater than 7.5, 8.0, 8.3, 8.5, 9.0, 9.3, 10.0 or 11.0. In certain embodiments, the elution buffer can comprise 9 M urea at pH 3, 9 M urea at pH 11 or a mixture of 6.66% MeCN/13.33% IPA/79.2% H_2O/0.8% TFA. The selection of one method versus another depends on the analytical method used for the eluted sample.

Alternatively, solutions of high salt concentration having sufficient ionic strength to mask charge characteristics of the analyte and/or binding moiety may be used. Salts having multi-valent ions are particularly preferred in this regard, e.g., sulphates and phosphates with alkali earth or transition metal counterions, although salts dissociating to one or more monovalent ions are also suitable for use in the present invention, provided that the ionic strength of the resulting solution is at least 0.1, preferably 0.25, 0.3, 0.35, 0.4, 0.5, 0.75, 1.0 mol l^{-1} or higher. By way of example, many protein analyte/binding moiety interactions are sensitive to alterations of the ionic strength of their environment; therefore, analyte may be isolated from the binding moiety by contacting the bound analyte with a salt solution, preferably an inorganic salt solution such as sodium chloride. This may be accomplished using a variety of methods including bathing, soaking, or dipping a solid support to which the analyte is bound into the elution buffer, or by rinsing, spraying, or washing the elution buffer over the solid support. Such treatments will release the analyte from the binding moiety coupled to the solid support. The analyte may then be recovered from the elution buffer.

Chaotropic agents, such as guanidine and urea, disrupt the structure of the water envelope surrounding the binding moiety and the bound analyte, causing dissociation of complexes between the analyte and binding moiety. Chaotropic salt solutions suitable for use as elution buffers of the present invention are application-specific and can be formulated by one of skill in the art through routine experimentation. For example, a

suitable chaotropic elution buffer may contain urea or guanidine ranging in concentration from 0.1 to 9 M.

Detergent-based elution buffers modify the selectivity of the affinity molecule with respect to surface tension and molecular complex structure. Suitable detergents for use as elution buffers include both ionic and nonionic detergents. Non-ionic detergents disrupt hydrophobic interactions between molecules by modifying the dielectric constant of a solution, whereas ionic detergents generally coat receptive molecules in a manner that imparts a uniform charge, causing the coated molecule to repel like-coated molecules. For example, the ionic detergent sodium dodecyl sulphate (SDS) coats proteins in a manner that imparts a uniform negative charge. Examples of non-ionic detergents include Triton X-100, TWEEN, NP-40 and Octyl-glycoside. Examples of zwitterionic detergents include CHAPS.

Another class of detergent-like compounds that disrupt hydrophobic interactions through modification of a solution's dielectric constant includes ethylene glycol, propylene glycol and organic solvents such as ethanol, propanol, acetonitrile, and glycerol.

A preferred elution buffer of the present invention includes a matrix material suitable for use in a mass spectrometer. A matrix material may be included in the elution buffer. Some embodiments of the invention may optionally include eluting analyte(s) from binding moieties directly to mass spectrometer probes, such as protein or biochips.

Other elution buffers suitable for the present invention include combinations of buffer components mentioned above. Elution buffers formulated from two or more of the foregoing elution buffer components are capable of modifying the selectivity of molecular interaction between subunits of a complex based on multiple elution characteristics.

Analytes isolated using the present invention may have a range of concentrations of analytes or concentration variance between analytes that is less than the range of concentration of analytes or concentration variance originally present in the test sample. For example, after manipulation using the methods of the present invention, isolated analytes which have a range of concentrations of analytes or concentration variance from other isolated analytes that is decreased by at least a factor of two, more preferably a

factor of 10, 20, 25, 50, 100, 1000 or more, from the concentration variance between the same analytes present in the test sample prior to subjecting the test sample to any of the methods described herein. Preferably, the method of the invention is performed with a minimal amount of elution buffer, to ensure that the concentration of isolated analyte in the elution buffer is maximized. More preferably, the concentration of at least one isolated analyte will be higher in the elution buffer than previously in the test sample.

After isolating the captured analytes, the analytes may be further processed by concentration or fractionation based on some chemical or physical property such as molecular weight, isoelectric point or affinity to a chemical or biochemical ligand. Fractionation methods for nucleic acids, proteins, lipids and polysaccharides are well-known in the art and are discussed in, for example, Scopes, *Protein Purification: Principles and Practice* (1982); Sambrook *et al.*, *Molecular Cloning--A Laboratory Manual* (2nd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, New York, (Sambrook) (1989); and *Current Protocols in Molecular Biology*, F. M. Ausubel *et al.*, eds., *Current Protocols*, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1994 Supplement) (Ausubel).

E. Detecting Isolated Analytes

After analytes have been eluted and isolated free of binding moieties, the analyte may be detected, quantified or otherwise characterized using any technique available to those of ordinary skill in the art. A feature of applying the analysis techniques of the present invention to complex test samples is the reduction of the range of analyte concentrations for isolated analytes relative to the large range in analyte concentration found in the original test sample. This reduction in analyte concentration range allows a much larger percentage of analytes found in the original test sample to be detected and characterized without recalibrating the detection device than would be available for analyte detection using the original test sample itself. The actual reduction in analyte concentration range achieved is dependent on a variety of factors including the nature of the original test sample and the nature and diversity of the binding moieties used. Generally, the reduction in analyte concentration variance using the techniques described herein is sufficient to allow at least 25%, more preferably at least 30%, 40%, 50%, 60%, 70%, 75% or 80% of the analytes isolated, to be detected without instrument re-

calibration. Ideally, the present invention allows at least 90%, 95%, 98% or more of the analytes isolated to be detected without instrument re-calibration.

Detecting analytes isolated using the techniques described herein may be accomplished using any suitable method known to one of ordinary skill in the art. For example, colorimetric assays using dyes are widely available. Alternatively, detection may be accomplished spectroscopically. Spectroscopic detectors rely on a change in refractive index; ultraviolet and/or visible light absorption, or fluorescence after excitation with a suitable wavelength to detect reaction components. Exemplary detection methods include fluorimetry, absorbance, reflectance, and transmittance spectroscopy. Changes in birefringence, refractive index, or diffraction may also be used to monitor complex formation or reaction progression. Particularly useful techniques for detecting molecular interactions include surface plasmon resonance, resonant mirror techniques, grating-coupled waveguide techniques, and multi-polar resonance spectroscopy. These techniques and others are well known and can readily be applied to the present invention by one skilled in the art, without undue experimentation. Many of these methods and others may be found for example, in "Spectrochemical Analysis" Ingle, J. D. and Crouch, S. R., Prentice Hall Publ. (1988) and "Analytical Chemistry" Vol. 72, No. 17.

A preferred method of detection is by mass spectrometry. Mass spectrometry techniques include, but are not limited to ionization (I) techniques such as matrix assisted laser desorption (MALDI), continuous or pulsed electrospray (ESI) and related methods (e.g., IONSPRAY or THERMOSPRAY), or massive cluster impact (MCI); these ion sources can be matched with detection formats including linear or non-linear reflection time-of-flight (TOF), single or multiple quadropole, single or multiple magnetic sector, Fourier Transform ion cyclotron resonance (FTICR), ion trap, and combinations thereof (e.g., ion-trap/time-of-flight). For ionization, numerous matrix/wavelength combinations (MALDI) or solvent combinations (ESI) can be employed. Subattomole levels of analyte have been detected, for example, using ESI (Valaskovic, G. A. *et al.*, (1996) *Science* 273:1199-1202) or MALDI (Li, L. *et al.*, (1996) *J. Am. Chem. Soc.* 118:1662-1663) mass spectrometry. ES mass spectrometry has been introduced by Fenn *et al.* (*J. Phys. Chem.* 88, 4451-59 (1984); International Patent Application Publication Number WO 90/14148)

and current applications are summarized in recent review articles (R. D. Smith *et al.*, *Anal. Chem.* 62, 882-89 (1990) and B. Ardrey, *Electrospray Mass Spectrometry, Spectroscopy Europe*, 4, 10-18 (1992)). MALDI-TOF mass spectrometry has been introduced by Hillenkamp *et al.* ("Matrix Assisted UV-Laser Desorption/Ionization: A New Approach to Mass Spectrometry of Large Biomolecules," *Biological Mass Spectrometry* (Burlingame and McCloskey, editors), Elsevier Science Publishers, Amsterdam, pp. 49-60, 1990). With ESI, the determination of molecular weights in femtomole amounts of sample is very accurate due to the presence of multiple ion peaks that may be used for the mass calculation. An alternative analytical method of the present invention utilizes Surfaces Enhanced for Laser Desorption/Ionization (SELDI), as discussed for example in U.S. Patent Number 6,020,208. Mass spectroscopy is a particularly preferred method of detection in those embodiments of the invention where elution of analytes directly onto a mass spectrometer probe or biochip occurs, or where the elution buffer contains a matrix material or is combined with a matrix material after elution of analytes from the binding moieties.

Another method of detection widely used is electrophoresis separation based on one or more physical properties of the analyte(s) of interest. A particularly preferred embodiment for analysis of polypeptide and protein analytes is two-dimensional electrophoresis. A preferred application separates the analyte by isoelectric point in the first dimension, and by size in the second dimension. Methods for electrophoretic analysis of analytes vary widely with the analyte being studied, but techniques for identifying a particular electrophoretic method suitable for a given analyte are well known to those of skill in the art.

II. IDENTIFICATION OF BIOMARKERS

Another embodiment of the present invention is the use of the beaded binding moiety libraries described above in the identification of biomarkers for the diagnosis of diseases, infection or pollution. Biomarkers may be identified in any of the samples noted above, but preferably are identified from samples such as blood, urine, cerebrospinal fluid and the like, taken from living beings, most preferably human beings. There are several ways in which biomarkers may be identified.

A "biomarker" is virtually any biological compound, such as a protein and a fragment thereof, a peptide, a polypeptide, a proteoglycan, a glycoprotein, a lipoprotein, a carbohydrate, a lipid, a nucleic acid, an organic on inorganic chemical, a natural polymer, and a small molecule, that is present in the biological sample and that may be isolated from, or measured in, the biosample. Furthermore, a biomarker can be the entire intact molecule, or it can be a portion thereof that may be partially functional or recognized, for example, by an antibody or other specific binding protein. A biomarker can be an epitope-specific antibody. A biomarker is considered to be informative if a measurable aspect of the biomarker is associated with a given phenotype, such as a particular disease state in a living being, or level of pollution in a body of water. Such a measurable aspect may include, for example, the presence, absence, or concentration of the biomarker in the biological sample from the individual and/or its presence as part of a profile of biomarkers. Such a measurable aspect of a biomarker is defined herein as a "feature." A feature may also be a ratio of two or more measurable aspects of biomarkers, which biomarkers may or may not be of known identity, for example. A "biomarker profile" comprises at least two such features, where the features can correspond to the same or different classes of biomarkers such as, for example, a nucleic acid and a carbohydrate. A biomarker profile may also comprise at least 3, 4, 5, 10, 20, 30 or more features. In one embodiment, a biomarker profile comprises hundreds, or even thousands, of features. In another embodiment, the biomarker profile comprises at least one measurable aspect of at least one internal standard.

A "phenotype" is an observable physical or biochemical characteristic of an organism, as determined by both genetic makeup and environmental influences. Alternatively, in the context of the present invention, a phenotype may also be associated with non-living aspects of nature, for example the phenotype of a body of water includes those aspects of the body of water that are detectable, either physically or chemically. For example, the phenotype of a lake includes the water temperature, acidity, mineral content, oxygen content, whether it is capable of sustaining life and if so, what types of life.

A "phenotypic change" is a detectable change in a parameter associated with a given phenotype. For instance, a phenotypic change may include an increase or decrease

of a biomarker in a bodily fluid, where the change is associated with a disease state. A phenotypic change may further include a change in a detectable aspect of a given state of a patient that is not a change in a measurable aspect of a biomarker. For example, a change in phenotype may include a detectable change in body temperature, respiration rate, pulse, blood pressure, or other physiological parameter. Such changes can be determined via clinical observation and measurement using conventional techniques that are well-known to the skilled artisan. As used herein, "conventional techniques" are those techniques that classify an individual based on phenotypic changes without obtaining a biomarker profile according to the present invention.

Using the claimed invention to identify diagnostic biomarkers in a species or tissue requires the availability of at least two biosamples. The biosamples provided may be from a control group and a test group, a control group and a test individual, taken from the same individual at different times or any other permutation that is readily apparent to one of skill in the art.

Each biosample obtained is treated with a beaded binding moiety library as described herein. In this way, more putative biomarkers are available for analysis, as described in the examples section herein below. This occurs because the binding moiety library narrows the variance in the concentration range of analytes present in the sample, thereby allowing both low abundance and high abundance analytes to be detected.

After treatment with a binding moiety library of the present invention, analytes for each of the biosamples that are bound by the binding moieties are eluted and pooled separately. The pooled samples are then analyzed to determine if any of the common analytes in the samples display differential expression (enhanced expression in one biosample vs. the other), or is expressed in one biosample but not the other. Analytes displaying such differential expression are considered putative biomarkers for the phenotypic change or difference observed between the sources of the respective biosamples. Further statistical and analytic testing may then be performed to correlate the biomarker with the phenotypic change with a desired degree of certainty.

Preferred methods of analytical analysis for use in identifying biomarkers are the same as those described above for identifying analytes binding the binding moieties of the invention generally.

III. KITS

The present invention also includes kits containing components that allow one of ordinary skill in the art to perform the techniques described herein. The most basic of kits for this purpose provide a plurality of binding moieties, each binding moiety in an amount selected to capture a pre-determined amount of a different analyte. In some kit embodiments of the invention the binding moieties are supplied coupled to a solid support, preferably insoluble beads. In other embodiments the solid support and binding moieties are supplied separately. When supplied separately, the binding moieties and/or solid supports include a capture moiety that allows the operator of the invention to couple binding moiety to solid support during the course of practicing the invention described herein. Kits providing separate binding moieties and solid supports may optionally provide additional reagents necessary to perform the reaction coupling the binding moieties to the solid supports.

Kits of the present invention also include a plurality of containers containing components for sample preparation and analyte isolation. Exemplary components of this nature include one or more wash solutions sufficient for removing non-bound material from a binding moiety specifically bound to an analyte, and at least one elution solution sufficient to release analyte specifically bound by a binding moiety.

Kit embodiments may optionally include instructions for using the library of binding moieties in the methods of this invention.

Although the forgoing invention has been described in some detail by way of illustration and example for clarity and understanding, it will be readily apparent to one ordinary skill in the art in light of the teachings of this invention that certain variations, changes, modifications and substitution of equivalents may be made thereto without necessarily departing from the spirit and scope of this invention. As a result, the embodiments described herein are subject to various modifications, changes and the like, with the scope of this invention being determined solely by reference to the claims appended hereto. Those of skill in the art will readily recognize a variety of non-critical parameters that could be changed, altered or modified to yield essentially similar results.

While each of the elements of the present invention is described herein as containing multiple embodiments, it should be understood that, unless indicated

otherwise, each of the embodiments of a given element of the present invention is capable of being used with each of the embodiments of the other elements of the present invention and each such use is intended to form a distinct embodiment of the present invention.

As can be appreciated from the disclosure above, the present invention has a wide variety of applications. The invention is further illustrated by the following examples, which are only illustrative and are not intended to limit the definition and scope of the invention in any way.

EXAMPLES

Example 1. Incubation of Library with Unfractionated, Undiluted Human Pooled Plasma

To aid analysis of complex samples, this method is useful to decrease the concentration differential. Human plasma is one of the most complex and difficult to analyze materials: proteins are present in concentration range greater than 10^{10} (Anderson and Anderson); decreasing this range will aid in the analysis of trace proteins. Under the conditions of this method, incubation of plasma with the ligand library will increase the number of proteins that can be detected and subsequently analyzed as compared with analysis of the unprocessed starting material.

A. Sample Preparation

Frozen, pooled, human platelet-poor plasma (PPP) was thawed at 37°C and filtered through 0.8 and 0.45 μm filters. Four replicates of approximately 1 ml of a library of hexamer peptide ligands on Toyopearl AF Amino 650 M resin (65 μm average diameter, ~2x 10^6 beads/ml; Tosoh Biosciences, Montgomeryville, Pennsylvania) with EACA-Ala spacer were each incubated with 9 ml of plasma for 1 hour at room temperature, rotating. The resin was drained and washed with 1 ml citrate buffer (20 mM citrate, 140 mM NaCl, pH 7.0). This wash solution was retained, as well as samples of the loading plasma and initial flow through. Bead libraries were subsequently washed with 20 column volumes of citrate.

100 µl of resin from samples 2 and 4 were incubated with an equal volume (100 µl) of 2 x LDS buffer + DTT (Invitrogen, Carlsbad, California) for 10 minutes at 90° C and centrifuged. The supernatant was collected and saved for analysis.

200 µl of resin from replicates 1 - 4 were incubated with 400 µl 6M GuHCl or 400 µl 6M urea for 1 hour in a batch format. The resin was allowed to drain and the flow through was collected for analysis. The GuHCl and urea concentrations in the eluates were reduced to 1M Urea on G-25 columns as follows: the G-25 columns were equilibrated twice with 200 µl 1M urea for 5 minutes, then centrifuged at 2000 rpm for 3 minutes. 20 µl of the urea and GuHCl samples were added to the tubes and centrifuged again under the same conditions. The flow through was collected.

B. LDS-PAGE Analysis

Initial PPP, flow through and wash were diluted 1:25 with citrate buffer, then 1:2 with 2x sample buffer. 14 µl of the treated GuHCl and urea supernatants were heated in 5 µl 4x LDS buffer + 2 µl DTT reducing agent for 10 minutes at 90°C. Two wells were loaded with approximately 10 µl of beads from samples 2 and 4.23 µl of each of the remaining samples were run on a 4-12% Bis-Tris gel (NuPage, Invitrogen) in MOPS buffer at 200 V. The gels were stained with Simply Blue (Invitrogen) according to the manufacturer's instructions. The results are shown in Figures 1 and 4.

Several bands that are not visible in the original plasma are present in the treated samples, while the very intense albumin band present in the original plasma (~64 kD) is substantially reduced. These results demonstrate that the method described does decrease the concentration range of proteins as detected by this method, thereby increasing the number of proteins that can be detected and analyzed compared with analysis of the starting material.

Example 2. Reduction of Concentration Variance After Removal of IgG.

In many proteomic applications, one of the first steps of sample preparation is removal of albumin and IgGs, as these high abundance proteins mask the detection of lower abundance species. Removal of these proteins, however, also often removes trace species associated with them, and also involves loss of sample. It would be advantageous to have a method of sample preparation that does not require IgG depletion before

analysis. This example demonstrates that removal of IgGs is not required to visualize protein species that are not detected in intact plasma. The pattern of proteins detected in LDS-PAGE is compared in plasma that has and has not been depleted of IgGs.

A. Sample Preparation

Frozen, pooled, human platelet-poor plasma (PPP) was thawed at 37°C and filtered through 0.8 and 0.45 μm filters. IgG was removed from the plasma as follows: 5 ml Protein G Sepharose Fast-flow resin (Amersham, T&S) was packed in a Bio-Rad column, 10 ml of filtered PPP was added at a 10 cm/h flow rate (controlled by a peristaltic pump) and the flow through was collected.

Approximately 1 ml library of hexamer peptide ligands on Toyopearl 650 M amino resin (65 μm average diameter, ~ 2x 10^6 beads/ml; Tosoh Biosciences, Montgomeryville, Pennsylvania) with EACA-Ala spacer was incubated with 9 ml of flow through (above) for 1 hour/room temp/rotating. Clots that formed during incubation were removed by pipette. The resin was drained and washed with 1 ml citrate buffer (20 mM citrate, 140 mM NaCl, pH 7.0), followed by 10 ml T-citrate (citrate buffer+0.05% Tween-20) and 10 ml citrate buffer. The flow through and first 1 ml of wash were collected for analysis. The resin was divided into 3 approximately equal, 200 μl aliquots.

One resin aliquot was heated with an equal volume (200 μl) 2x LDS buffer + DTT (Invitrogen, Carlsbad, California) for 10 minutes at 90°C and centrifuged. The supernatant was collected and saved for analysis. The remaining resin aliquots were incubated with 500 μl 6M GuHCl or 500 μl 6M urea for 1 hour in batch format. The resin was allowed to drain and the flow through was collected for analysis.

The GuHCl and urea concentrations in the eluates were reduced from 6M to 1M concentration and half the original volume (2x concentrated) by buffer exchange over G-25 columns.

B. LDS-PAGE Analysis

Initial PPP, IgG-depleted PPP, flow through and wash, as well as samples of GuHCl and urea supernatants were heated in LDS buffer+DTT reducing agent for 10 minutes at 90°C. The final dilutions of the LDS buffer, GuHCl, and Urea eluates were 0.25x , 1x, and 1x, respectively. The PPP, IgG-depleted plasma, flow through, and wash

were diluted 50x. The Protein G LDS and Glycine eluates were incubated with 2x LDS buffer + DTT. 23 µl of each samples were run on a 4-12% Bis-Tris gel, in MOPS buffer, at 200 V. Samples of plasma prepared earlier according to the methods above from which the IgG was not removed were run as well. The gels were stained with Simply Blue followed by SilverQuest according to the manufacturer's instructions. The data is presented in Figure 2.

Although in the starting, flow through and wash samples there is a clear decrease in proteins at MW 50 and 25 KDa (sizes of the reduced immunoglobulin heavy and light chains), there are no significant differences in the LDS-PAGE eluates from plasma both with and without IgG as visualized on the gels. The signals from the urea and GuHCl samples are indistinct due to sample handling issues. These data indicate that there is no obvious effect of removing IgGs. There may be other reasons that it is preferable to remove and retain the IgGs, perhaps for independent analysis; however, removal is not required to analyze trace proteins by this method.

Example 3. Reduction of Range of Concentrations of Proteins in Human Serum

Previous examples have demonstrated the usefulness of the described method with undiluted and unfractionated human plasma. n clinical diagnostics the starting sample frequently is serum, not plasma. The following example demonstrates the feasibility of using the described method to prepare serum for analysis. .

A. Serum Preparation

Five 7 ml tubes of human blood were allowed to clot at 4°C overnight. The clotted blood was centrifuged at 4,000 rpm for 5 minutes in a Sorvall centrifuge RT7, serum collected, and filtered through 0.8 and 0.45 µm filters.

B. Sample Preparation:

1. Toyopearl-Based Library Incubation

Approximately 1 ml Toyopearl AF Amino 650 M library (65 µm average diameter, ~2x 10^6 beads/ml; Tosoh Biosciences, Montgomeryville, Pennsylvania) was incubated with 9 ml serum for 1 hour/RT/rotating. 200 µl of resin were heated with 200

µl 2x (LDS buffer + DTT reducing agent) for 10 minutes at 90°C. The supernatant was collected and saved at -20°C for analysis. 200 µl of resin were incubated with 400 µl 6M GuHCl, for 1 hour in batch format. The flow through was collected for analysis, the GuHCl exchanged to 1M urea by buffer exchange through a G25 column, and kept at room temperature. The initial and non-bound serum fractions were diluted 1:25 with citrate, then 1:2 with 2x LDS/DTT. Samples were heated for 10 minutes at 90°C and then frozen at -20°C. 200 µl serum and 200 µl of each non-bound fraction were analyzed.

C. LDS-PAGE Analysis

14 µl of exchanged 6MGuHCl samples were heated with 5 µl 4x LDS buffer and 2 µl 10 x DTT for 10 minutes, at 90°C. The frozen LDS samples were re-heated for 10 min, at 90°C. 20 µl of each sample was loaded per well into two 4-12% Bis Tris gels. The gels were run with MOPS running buffer at 200 V until the dye front reached the bottom of gels. Gels were stained with Simply Blue protein stain according to the manufacturer's instructions and destained with H_2O. The gels are presented in Figure 3.

There is a substantial increase in the number of bands visible in serum following incubation with Toyopearl library (compare lane 2 with lanes 4 and 5). The pattern of bands is very similar to the pattern obtained with incubation of library with plasma (compare lane 4, FIG. 3, with lane 7, FIG. 1). These results indicate that preparation of serum samples with the method of this invention increases the number of bands that can be analyzed by LDS-PAGE, and decreases the concentration of the most abundant proteins in the eluates as compared with the starting serum.

Example 4. Increasing numbers of ligands bind to increased concentrations of a target analyte.

For the invention to be useful to detect biomarkers, the difference in the concentration of a biomarker analyte in one sample compared with a second, comparable sample, must be maintained. This is accomplished when increasing numbers of ligands in a library bind to the analyte when it is present at higher concentrations, as shown in the example, below.

Methods:

180 µl aliquot of well mixed hexamer library, synthesized on Toyopearl AF Amino 650 M resin, was measured out for 6 dilutions. The beads were suspended in an equal volume of citrate buffer. The resin slurry was then suspended in 1.2 ml citrate buffer, separated into 220 µl aliquots, and mixed with buffer spiked with CRP (Human plasma CRP, cat#236603, lot#D00002163 (Calbiochem), C=6.1 mg/ml ≈ 265 µM, diluted by citrate buffer to 1 and 10 µM) prepared as described below:

	[rCRP] desired		volume of	stock conc	Citrate buffer
a.	0	-			220 ul
b.	10 nM	(230 ng/ml)	2.2 ul	1 uM	220 ul
c.	25 nM	(575 ng/ml)	5.5 ul	1 uM	220 ul
d.	50 nM	(1150 ng/ml)	1.1 ul	10 uM	220 ul
e.	100 nM	(2300 ng/ml)	2.2 uL	10 uM	220 ul
f.	250 nM	(5750 ng/ml)	5.5 ul	10 uM	220 ul

Samples were incubated at room temperature for 1 hr, then resin was pelleted by centrifugation at 6000 rpm for 2 min. Library was washed in 440 µl of citrate buffer and centrifuged Micro Spin column as before. Resin was diluted in 220 µl of citrate buffer, carefully mixed and transferred into 0.5 ml eppendorf tubes. Six aliquots of 14.66 µl (= 1 ul of swollen beads) was added to 100 µl of 0.5% LMP agarose, carefully mixed with 900 µl of 0.5% LMP agarose and applied in sections to a 1% agarose gel base. Bound protein was transferred for 3 hrs in 1M NaCl, the membrane replaced and a second transfer was performed for 3 hours in 6m guanidine HCl. Approximately 34,000 beads were present in 6 areas on the gel.

The membranes were rinsed with H_2O and blocked with Casein blocker overnight. CRP on the membranes was detected using a CRP-8 monoclonal antibody (Cat#C-1688, Lot# 094K4803), C=9.1 mg/ml (Sigma) diluted 1:9100 by TBS + 0.05% Tween 20 (TBS/T) and incubated with membranes for 1.5 h at RT on shaker. The membrane was washed in TBS/T buffer (3 times X150 ml for 5 min). The membranes then were incubated with 60 ml of secondary IgG, conjugated with phosphatase (goat anti mouse IgG, human serum adsorbed (Cat#075-1802, Lot# XE084, Kirkegaard & Perry laboratories), diluted 1:12500 diluted in TBS/T for 50 min at RT. The membranes were washed and CRP detected with CDP-Star (TROPIX) substrate and exposed to film (Kodak).

Results:

As demonstrated in Figure 7, an increase in the number and intensity of spots, associated with beads that bind CRP, can be observed when the library is incubated with increasing concentrations of CRP. These data indicate that, when an increasing concentration of an analyte is incubated with a given amount of library, or when two samples with different concentrations of the same analyte are incubated with the same amount of library, the analyte will bind to the ligands in the library in a dose-dependant manner. This is because the library comprises a collection of ligands with a vast range of affinities to any particular target. The ligands with the highest affinities are able to bind to the analyte at low concentrations; as the concentration increases, ligands with lower affinities to the target then are able to bind to the analyte. This is demonstrated in Figure 7 by the increasing number of spots, representing an increasing number of ligands that bind to the analyte that can be observed on the films with increasing analyte (CRP) concentration. Thus, the number of ligands that are able to bind to CRP is proportional to the concentration, and that ligands that originally did not bind detectable amounts of low concentrations of CRP are able to bind the target when present at higher, but non-saturating concentrations. Thus, the amount of a target bound to and eluted from library is proportional to the concentration of the target in the original starting material.

Example 5. Use of combinatorial library for improved troponin detection and analysis by ELISA.

Troponin I (Biodesign, Cat #A86862H Lot # 3C07903) was spiked into human plasma or human citrated blood at different concentrations (0-100ng/ml). Toyopearl AF Amino 650 M library was swollen in DMF, washed with, and stored in 20% MeOH. The library was washed with PBS and equilibrated with citrate buffer directly before the experiments were performed. Blood samples (1 ml) were added to the column containing resins (100 µl of bed volume) and allowed to flow through by gravity. The resin was washed three times with 1 ml of PBS to remove non-bound proteins. To remove extra PBS, columns were centrifuged for 4000g for 1 min in an Eppendorf tube. Bound proteins were eluted with 250 µl of 0.05M HCl, pH 1.9, and immediately neutralized with 85 µl 0.5M NaH_2PO_4 pH 7.5. Fifty µl of 1% BSA with 0.05% Tween 20 was added

to stabilize the proteins. Evaluation of troponin in plasma and library-treated blood samples was performed by Troponin I ELISA, Bio-Quant kit (#BQ 015C) according to the manufacturer's manual. The result is shown in Figure 8. These data demonstrate that resin concentrates troponin from blood samples (▼) spiked with troponin, improving the detection compared with spiked, untreated whole blood (■). The library maintains the concentration differential between samples, with more troponin detected from samples with higher initial concentrations than from samples with lower initial concentrations.

Although the foregoing invention has been described in some detail by way of illustration and example for clarity and understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit and scope of the appended claims.

All publications and patent applications cited in this specification (with the exception of the priority applications) are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

What is claimed is:

1. A method comprising the steps of:

(a) providing a first sample comprising a plurality of different analyte species present in the first sample in a first range of concentrations;

(b) contacting the first sample with an amount of a library comprising at least 100 different binding moieties;

(c) capturing amounts of the different analyte species from the first sample with the different binding moieties and removing unbound analyte species; and

(d) isolating the captured analyte species from the binding moieties to produce a second sample comprising a plurality of different analyte species present in the second sample in a second range of concentrations; wherein the amount of the library is selected to capture amounts of the different analyte species so that the second range of concentrations is less than the first range of concentrations.

2. The method of claim 1, wherein the first sample comprises at least 100, at least 1,000, at least 10,000, at least 100,000, at least 1,000,000 or at least 10,000,000 different analyte species.

3. The method of claim 1, wherein the library comprises at least 1,000, at least 10,000, at least 100,000, at least 1,000,000 or at least 10,000,000 different binding moieties.

4. The method of claim 1, wherein the binding moieties comprise bio-organic polymers.

5. The method of claim 1, wherein the binding moieties are bound to a solid support or supports.

6. The method of claim 1, wherein the library is a non-selective library.

7. The method of claim 1, wherein the different binding moieties are comprised in a complete or incomplete combinatorial library.

8. The method of claim 1, wherein the second sample has a diversity of analyte species that is substantially the same as the first sample.

9. The method of claim 1, wherein the sample is selected from the group consisting of amniotic fluid, blood, cerebrospinal fluid, intraarticular fluid, intraocular fluid, lymphatic fluid, milk, perspiration plasma, saliva, semen, seminal plasma, serum, sputum, synovial fluid, tears, umbilical cord fluid, urine, biopsy homogenate, cell culture fluid, cell extracts, cell homogenate, conditioned medium, fermentation broth, tissue homogenate and derivatives of these.

10. The method of claim 1, further comprising detecting analyte species in the second sample.

11. The method of claim 1, wherein removing unbound analytes comprises washing the captured analytes with a wash buffer.

12. The method of claim 1, wherein the analytes are selected from the group consisting of polypeptides, nucleic acids, complex carbohydrates, complex lipids, synthetic inorganic compounds and synthetic organic compounds.

13. The method of claim 1, further comprising fractionating the analytes in the second sample based on a physical or chemical property.

14. The method of claim 1, further comprising identifying at least one of the isolated analytes.

15. The method of claim 1, further comprising contacting a biospecific binding moiety with the second sample and determining whether the biospecific binding moiety has captured an analyte species from the second sample.

16. The method of claim 4, wherein the bio-organic polymers are selected from the group consisting of peptides, oligonucleotides and oligosaccharides.

17. The method of claim 1, wherein the binding moieties are selected from antibodies and aptamers.

18. The method of claim 5, wherein the solid support or supports is a collection of beads or particles.

19. The method of claim 5, wherein the solid support or supports is selected from the group consisting of fibers, monoliths, membranes and plastic strips.

20. The method of claim 6, wherein the non-selective library is selected from the group consisting of a germ line antibody library, a phage display library of recombinant binding proteins, a dye library or a non-combinatorial library in which the binding specificity of the members is not pre-selected, a combinatorial library and portions thereof.

21. The method of claim 7, wherein the combinatorial library is a hexapeptide library.

22. The method of claim 10, wherein the analytes are detected using a method selected from the group consisting of calorimetric, spectrophotometric, magnetic resonance, mass spectroscopic, electrophoretic, chromatographic, enzymatic, and sequence analysis.

23. The method of claim 13, wherein fractionating comprises segregating the analytes using a technique selected from the group consisting of chromatography, electrophoresis, capillary electrophoresis, filtration and precipitation.

24. The method of claim 18, wherein each bead or particle is attached to a substantially different binding moiety.

25. The method of claim 18, wherein a plurality of different binding moieties are attached to the same bead or particle.

26. The method of claim 18, wherein the beads or particles have a diameter of less than 1 μm.

27. The method of claim 18, wherein the beads or particles are formed milling microparticulate beads using a method selected from the group consisting of crushing, grinding, and sonicating.

28. The method of claim 27, wherein the microparticulate beads are a polymeric matrix formed from a natural or synthetic polymer.

29. The method of claim 27, wherein the particles are coupled to a second solid support to form an array or dipstick.

30. A kit for detecting a plurality of analytes in a sample, comprising:
(i) a container comprising a library of at least 100 different binding moieties; and
(ii) instructions for using the library to perform the method of claim 1.

31. The kit of claim 30, wherein the binding moieties are coupled to the solid support or supports.

32. The kit of claim 30, wherein the library comprises a hexapeptide combinatorial library or a portion thereof wherein the hexapeptides are attached to particles.

33. The kit of claim 30, further comprising a binding buffer for capturing analytes with the binding moieties.

34. The kit of claim 30, further comprising an elution buffer for eluting captured analytes from the binding moieties.

35. A library comprising at least 100 different binding moieties, wherein a plurality of different binding moieties are attached to the same solid support or supports.

36. The library of claim 35, wherein the binding moieties comprise a combinatorial hexapeptide library or portion thereof.

37. A method for identifying a diagnostic biomarker, the method comprising the steps of:

(a) providing a first set of biosamples from a first set of organisms having a first phenotype;

(b) providing a second set of biosamples from a second set of organisms having a second phenotype;

(c) performing the method of claim 1 on each of the biosamples, thereby creating a third and fourth set of biosamples, respectively;

(d) detecting analyte species in each of the third and fourth set of biosamples;

(e) identifying at least one analyte species that is differentially present in the third and fourth set of biosamples, whereby the at least one analyte species is a biomarker for distinguishing the first phenotype from the second phenotype.

38. The method of claim 37, wherein step (e) comprises identifying a biomarker profile that provides better predictive power than any one of the biomarkers in the profile, alone.

39. A method for reducing the relative amounts of analytes in a sample, the method comprising the steps of:

(a) providing a first sample comprising a first plurality of different analytes having a first variance in amounts;

(b) contacting the first sample with a plurality of different binding moieties, each binding moiety present in a determined amount;

(c) capturing a portion of the first different analytes from the first sample with the different binding moieties and removing uncaptured analytes; and

(d) isolating the captured analytes from the binding moieties to produce a second sample comprising a second plurality of different analytes having a second variance in amounts; wherein the determined amount of each of the plurality of different binding moieties is selected to capture amounts of the different analytes whereby the second variance in amounts is less than the first variance in amounts.

40. A method for detecting biomarkers comprising:

(a) providing a first set of biosamples from a first set of organisms having a first phenotype;

(b) providing a second set of biosamples from a second set of organisms having a second phenotype;

(c) performing the method of claim 1 on each of the biosamples, thereby creating a third and fourth set of biosamples, respectively, wherein the difference in the concentration of the biomarker analyte between the first and second biosamples is maintained between the third and fourth biosamples;

(d) detecting analyte species in each of the third and fourth set of biosamples; wherein an analyte species differentially present in the third and fourth set of biosamples is a biomarker.

41. The method of claim 40, wherein the analytes are selected from the group consisting of polypeptides, nucleic acids, complex carbohydrates, complex lipids, synthetic inorganic compounds and synthetic organic compounds.

42. The method of claim 40, wherein at least one biomarker is identified.

43. The method of claim 42, wherein the biomarker is troponin.

44. The method of claim 42, wherein the biomarker is CRP.

45. The method of claim 40, wherein the biosample is selected from the group consisting of amniotic fluid, blood, cerebrospinal fluid, intraarticular fluid, intraocular fluid, lymphatic fluid, milk, perspiration plasma, saliva, semen, seminal plasma, serum, sputum, synovial fluid, tears, umbilical cord fluid, urine, biopsy homogenate, cell culture fluid, cell extracts, cell homogenate, conditioned medium, fermentation broth, tissue homogenate and derivatives of these.

FIG. 1

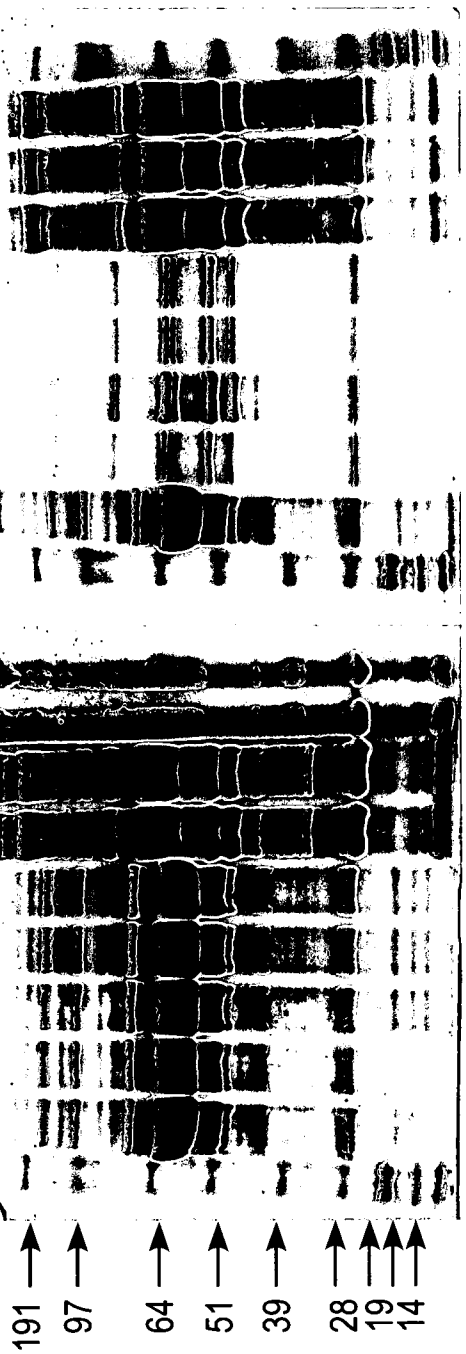
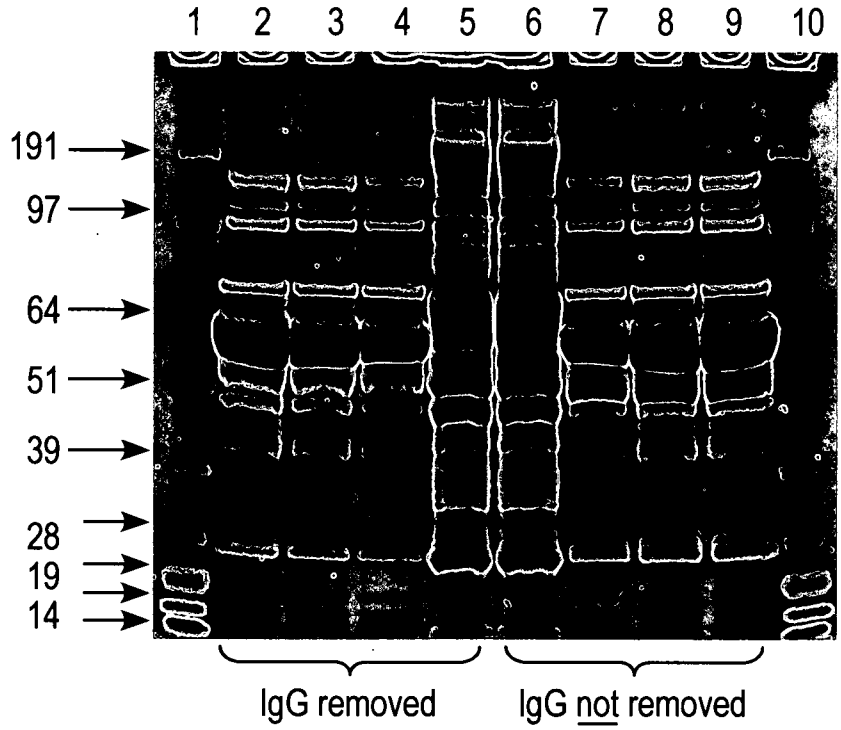

1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10
191
97
64
51
39
28
19
14
1. MW marker
2. PPP (diluted 1:50)
3. Flow through 1 (diluted 1:50)
4. Flow through 2 (diluted 1:50)
5. Flow through 3 (diluted 1:50)
6. Flow through 4 (diluted 1:50)
7. LDS eluate 2 (4x diluted)
8. LDS eluate 4 (4x diluted)
9. Directly loaded beads 2
10. Directly loaded beads 4
1. MW marker
2. PPP (diluted 1:50)
3. 6M urea eluate 1 (to 1 M urea)
4. 6M urea eluate 2 (to 1 M urea)
5. 6M urea eluate 3 (to 1 M urea)
6. 6M urea eluate 4 (to 1 M urea)
7. 6M GuHCl eluate 2 (to 1 M urea)
8. 6M GuHCl eluate 3 (to 1 M urea)
9. 6M GuHCl eluate 4 (to 1 M urea)
10. MW

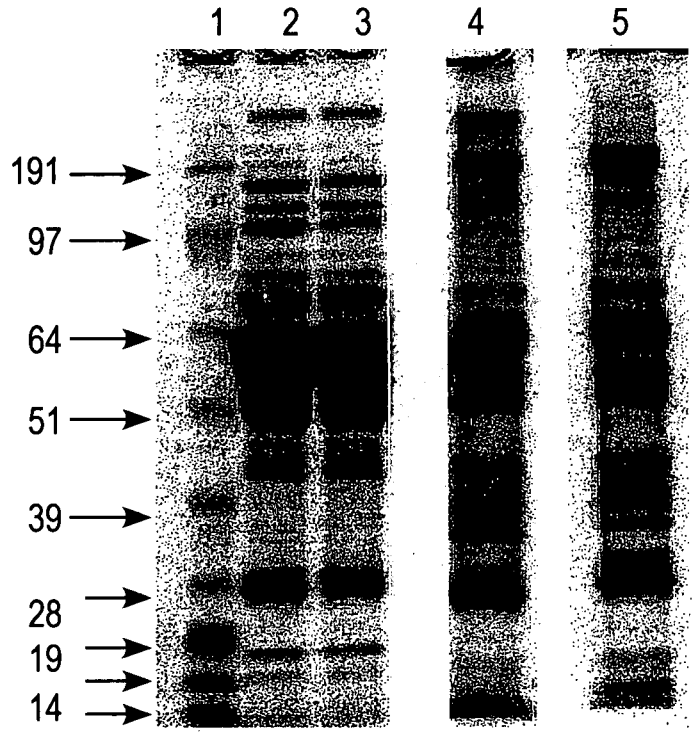
FIG. 2

PPP (IgG removed) vs.
unmodified PPP
on 65 μm hexamer library



1. MW marker
2. PPP, IgG removed (diluted 1:50)
3. Flow through (diluted 1:50)
4. Wash (diluted 1:50)
5. LDS buffer eluate (diluted 1:4)
6. LDS buffer eluate (diluted 1:4)
7. Wash (diluted 1:50)
8. Flow through (diluted 1:50)
9. PPP, unmodified (diluted 1:50)
10. MW marker

FIG. 3



1. MW marker
2. Serum (diluted 1:50)
3. Flow through (dil 1:50)
4. LDS eluate
5. 6M GuHCl eluate

FIG. 4

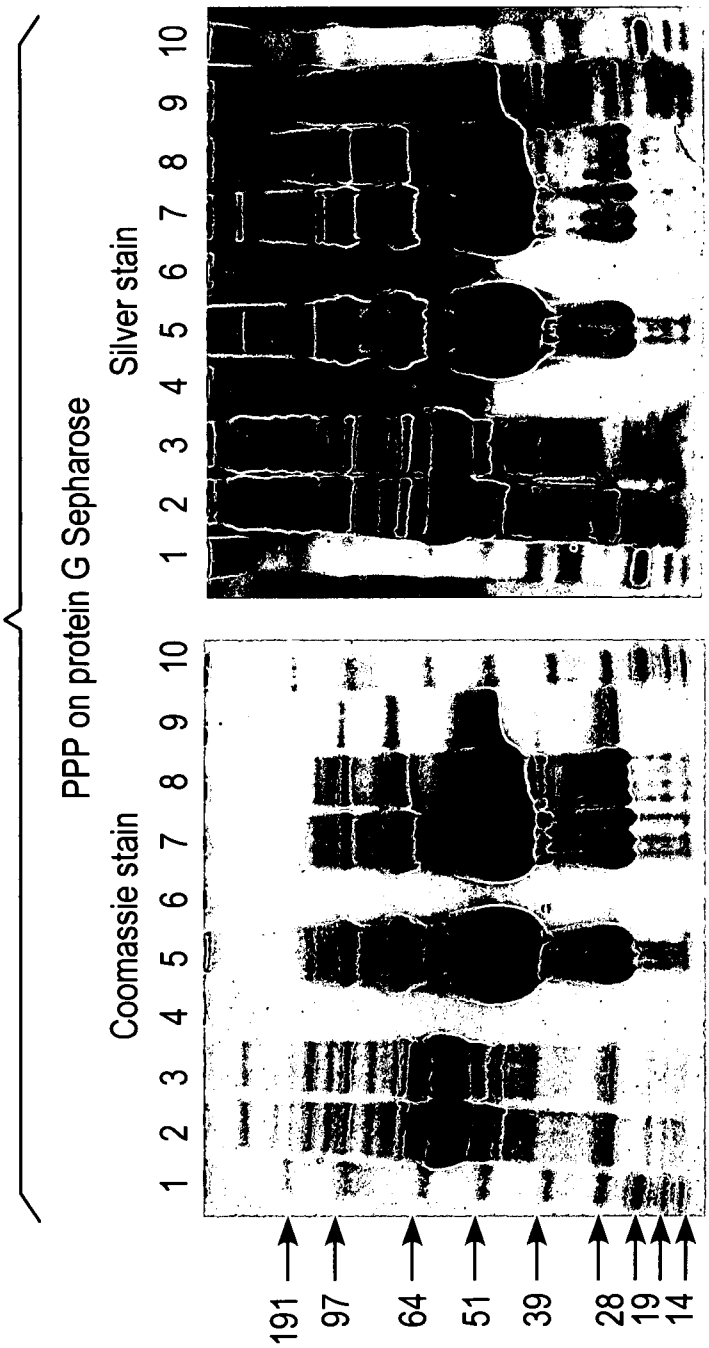
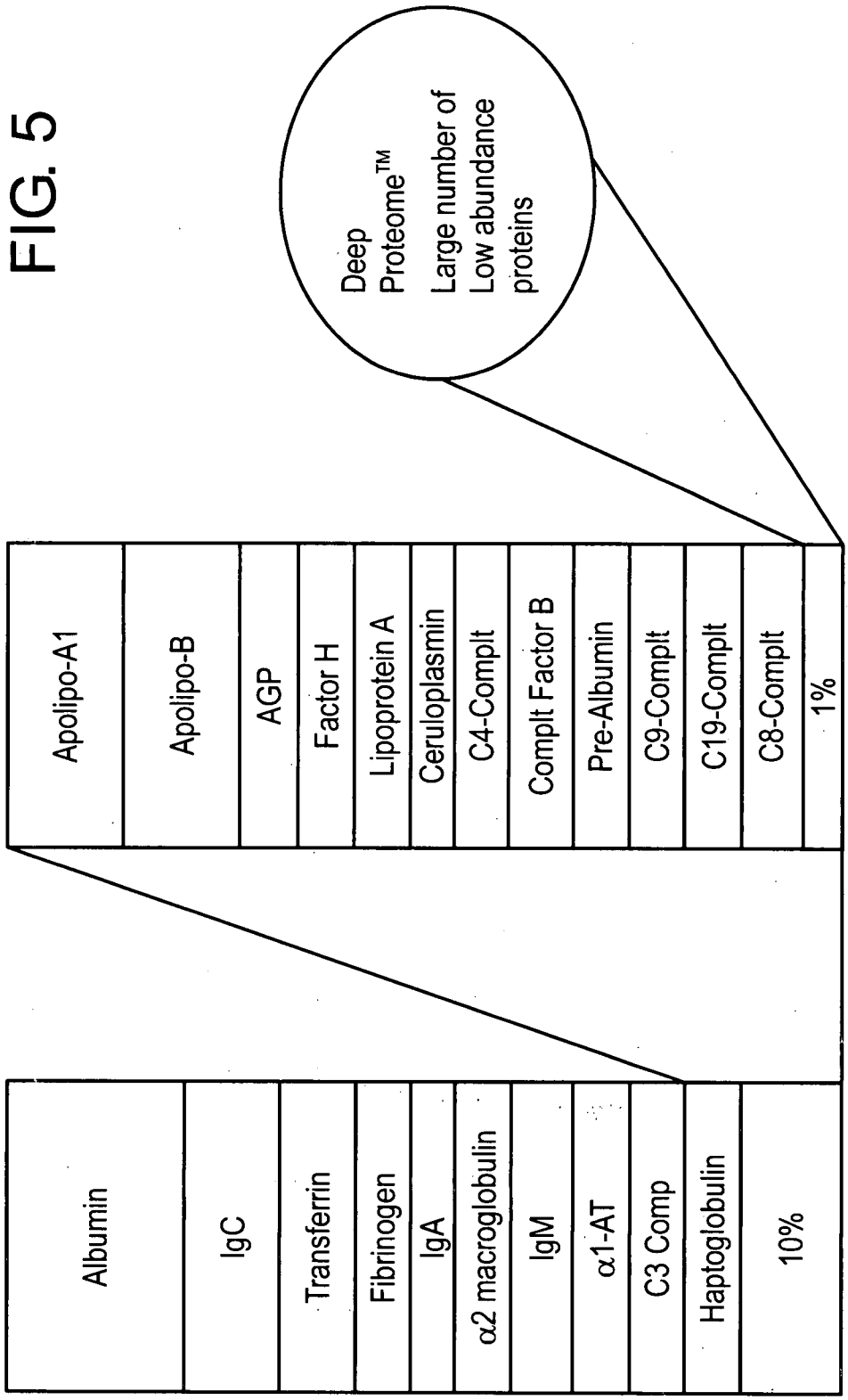

PPP on protein G Sepharose
Coomassie stain
Silver stain
1 2 3 4 5 6 7 8 9 10
191
97
64
51
39
28
19
14
1. MW marker
2. PPP (diluted 1:50)
3. PPP, IgG removed (diluted 1:50)
4. Empty
5. LDS buffer eluate of ProteinG Sepharose
6. Empty
7. Fraction I eluted
8. Fraction II eluted
9. Fraction III eluted
10. MW marker

FIG. 5


Albumin
IgC
Transferrin
Fibrinogen
IgA
α2 macroglobulin
IgM
α1-AT
C3 Comp
Haptoglobulin
10%
Apolipo-A1
Apolipo-B
AGP
Factor H
Lipoprotein A
Ceruloplasmin
C4-Complt
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Pre-Albumin
C9-Complt
C19-Complt
C8-Complt
1%
Deep Proteome™
Large number of Low abundance proteins

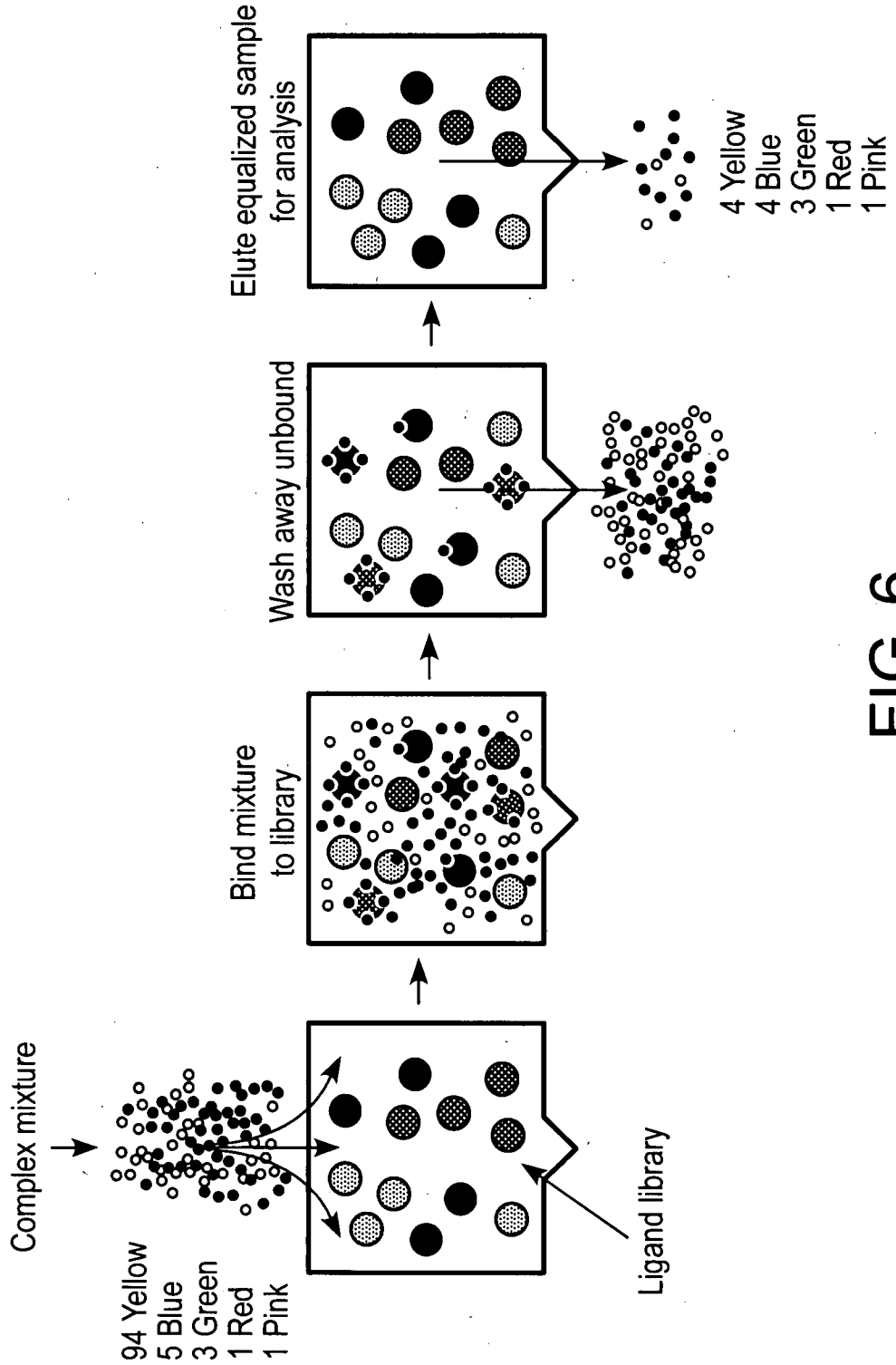

Complex mixture
94 Yellow
5 Blue
3 Green
1 Red
1 Pink
Ligand library
Bind mixture to library
Wash away unbound
Elute equalized sample for analysis
4 Yellow
4 Blue
3 Green
1 Red
1 Pink

FIG. 6

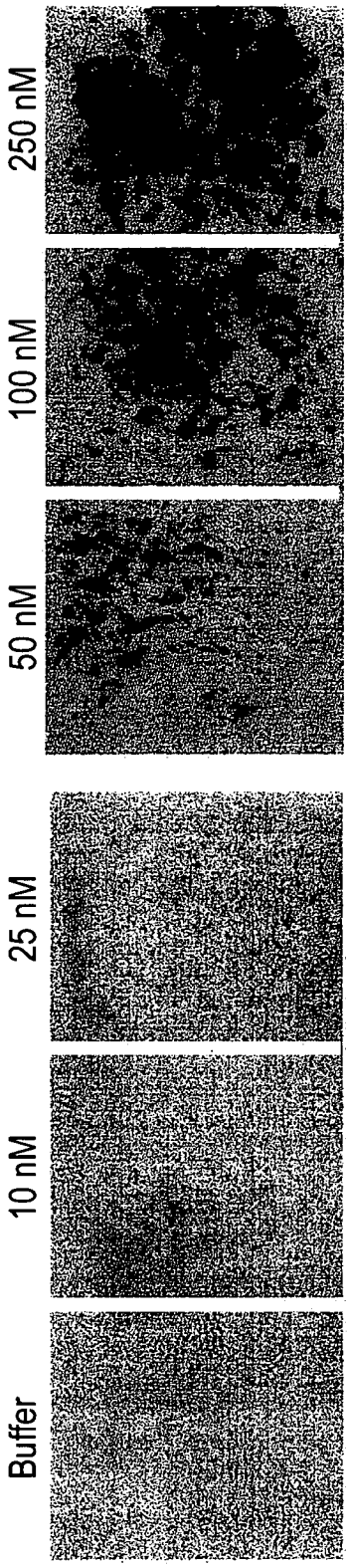


FIG. 7

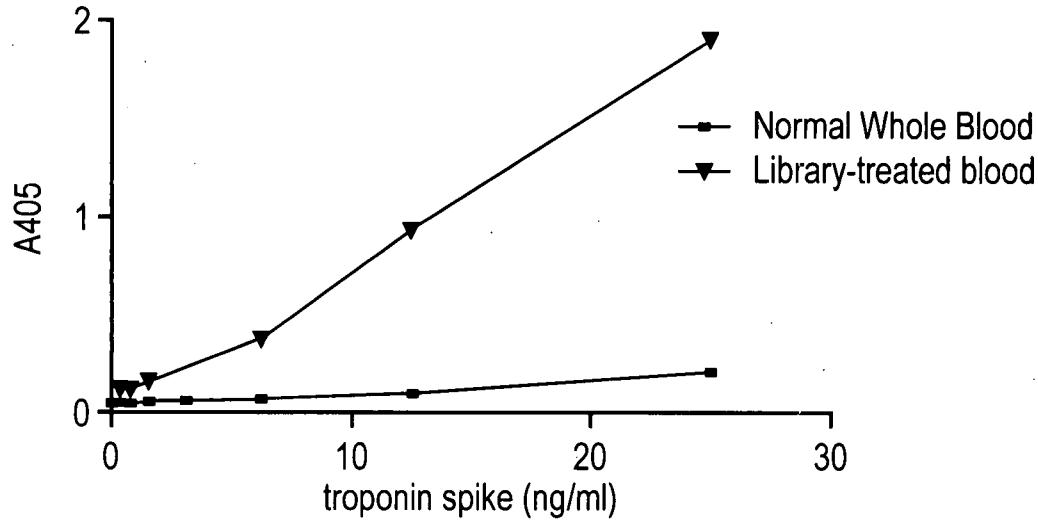

2
1
0
A405
0
10
20
30
troponin spike (ng/ml)
Normal Whole Blood
Library-treated blood

FIG. 8