(54) Title: LARGE DYNAMIC RANGE PROTEOMIC ANALYSIS METHODS AND COMPOSITIONS FOR PRACTICING THE SAME

(57) Abstract: Methods of determining whether a sample includes one or more analytes of interest are provided. Also provided are compositions, e.g., kits, systems and other compositions of matter, for practicing the subject methods. The subject methods and compositions find use in a variety of applications.

LARGE DYNAMIC RANGE PROTEOMIC ANALYSIS METHODS AND COMPOSITIONS FOR PRACTICING THE SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

Pursuant to 35 U.S.C. § 119 (e), this application claims priority to the filing
date of United States Provisional Patent Application Serial No. 60/732,262 filed
October 31, 2005; the disclosure of which is herein incorporated by reference.

INTRODUCTION

Proteomics involves the qualitative and quantitative measurement of gene
products and their activity by detecting and quantitating expression at the protein
level, rather than at the messenger RNA level. Proteomics also involves the study
of non-genome encoded events, including the post-translational modification of
proteins, interactions between proteins, and the location of proteins within a cell.
The structure, function, or level of activity of the proteins expressed by the cell are
also of interest.

Proteomics is of increasing interest for a number of reasons, including the
fact that measuring the mRNA abundances of a cell potentially provides only an
indirect and incomplete assessment of the protein content of the cell, as the level
of active protein that is produced in a cell is often determined by factors other than
the amount of mRNA produced, e.g. post-translational modifications, etc.

DETAILED DESCRIPTION

Methods of determining whether a sample includes one or more analytes,
such as proteinaceous analytes, of interest are provided. Aspects of the methods
include contacting the sample with a solid support that includes an affinity agent for
the analyte immobilized on a surface thereof and then detecting the presence of
resultant substrate surface bound analyte by assessing signal produced from a
large dynamic range signal producing system. Aspects of embodiments of the
large dynamic range signal producing system include the presence of a first
fluorescently labeled detection agent that specifically binds to the analyte; and a

second fluorescently labeled detection agent that specifically binds to the first
fluorescently labeled detection agent. In certain embodiments, the second
fluorescently labeled detection agent includes a greater number of fluorescent
moieties than the first fluorescently labeled detection agent. Also provided are kits,

5   systems and other compositions of matter for practicing the subject methods. The
subject methods and compositions find use in a variety of applications, including
but not limited to proteomic applications such as protein expression analysis, e.g.,
differential protein expression profiling.

10   Before the present invention is described in greater detail, it is to be
understood that this invention is not limited to particular embodiments described,
as such may vary. It is also to be understood that the terminology used herein is
for the purpose of describing particular embodiments only, and is not intended to
be limiting, since the scope of the present invention will be limited only by the

15   appended claims.
        Where a range of values is provided, it is understood that each intervening
value, to the tenth of the unit of the lower limit unless the context clearly dictates
otherwise, between the upper and lower limit of that range and any other stated or
intervening value in that stated range, is encompassed within the invention. The

20   upper and lower limits of these smaller ranges may independently be included in
the smaller ranges and are also encompassed within the invention, subject to any
specifically excluded limit in the stated range. Where the stated range includes
one or both of the limits, ranges excluding either or both of those included limits
are also included in the invention.

25   Unless defined otherwise, all technical and scientific terms used herein
have the same meaning as commonly understood by one of ordinary skill in the art
to which this invention belongs. Although any methods and materials similar or
equivalent to those described herein can also be used in the practice or testing of
the present invention, representative illustrative methods and materials are now

30   described.
        It is noted that, as used herein and in the appended claims, the singular
forms "a", "an", and "the" include plural referents unless the context clearly dictates

otherwise. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely," "only" and the like in connection with the recitation of claim elements, or use of a "negative" limitation.

5   As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present invention. Any recited method can be carried out in

10   the order of events recited or in any other order which is logically possible.

In further describing the subject invention, the methods are described first in greater detail, followed by a review of the kits and systems that find use in practicing the subject methods, as well as representative specific applications in

15   which the subject methods find use.

METHODS

General Features

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As summarized above, the subject invention provides highly sensitive methods of determining the presence, often in at least semi-quantitative if not quantitative terms, of one or more, including a plurality of, analytes in a sample. As the subject methods are highly sensitive, they are capable of detecting the

25   presence of an analyte in a sample where the analyte is present in a low concentration. In certain embodiments, the methods are capable of detecting an analyte in a sample where the analyte is present in a concentration as low as about 10 pg/mL or lower, where the concentration of the analyte may be as low as about 10 pg/mL, including as low as about 200 pg/mL or lower. As such, the lower

30   limit of detection of the subject methods, i.e., the lowest analyte concentration detectable by the subject methods is, in certain embodiments, at least about 10 pg/mL, such as at least about 100 pg/mL and including at least about 200 pg/mL. In representative embodiments, the methods are methods of detecting an analyte

in a sample where the analyte concentration ranges from about 50 pg/mL to about 300 ng/mL, such as from about 100 pg/mL to about 100 ng/mL and more including from about 200 pg/mL to about 30 ng/mL.

5   The subject methods are particularly suited for the detection of proteinaceous analytes, i.e., polypeptides, where the term polypeptide is used broadly to describe any molecule having two or more amino acid residues covalently bonded to each other by a peptide bond. As such, embodiments of the methods are suitable for use in detecting analytes that are small polypeptides as well as whole proteins and fragments thereof. In certain embodiments, the

10   analytes are proteins.

Aspects of the invention include a large dynamic range of sensitivity, such that the methods can be readily employed to determine a large dynamic range, i.e., wide concentration range, of analyte in a sample, such as from the lower limits reviewed above to large amounts (i.e., concentrations) of analyte, e.g.,

15   micrograms/ml, milligrams/ml, etc. This large dynamic range is, in certain embodiments, obtained without any sample dilution, such that large dynamic range can be obtained from a single sample without making one or more sample dilutions and testing each dilution individually.

Aspects of the invention include the use of a large dynamic range signal

20   producing system, as reviewed in greater detail below. Large dynamic range signal producing systems include first and second detection agents, where each detection agent is labeled with a distinguishable label, e.g., a fluorescent label. In certain embodiments, the number of labels in the second detection agent exceeds the number of labels in the first detection agent, e.g., by about 5 % or more, such

25   as by about 10% or more and including by about 25% or more.

In certain embodiments, the subject methods are methods of simultaneously detecting, e.g., quantitatively detecting, the presence of two or more, i.e., a plurality, of analytes in a sample, w, such as at least about 5 or more and including at least about 10 or more, where in representative embodiments at

30   least about 20 different analytes, such as at least about 50 and sometimes at least about 100 or more different analytes are assayed simultaneously, such as 200 or more, 300 or more, 400 or more, etc.

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Embodiments of the methods may be used with a variety of different types of samples and still provide the above-described sensitivity and large dynamic range. As such, the sample may be a highly complex sample, such as a crude cell extract, or an at least partially purified sample, as well as purified sample. Because

5   of the convenience, in representative embodiments the sample is a crude cell extract, where the only treatment employed may be a dilution step and/or removal of cellar debris step.

In further describing embodiments of the methods, aspects of the invention,

10   such as the types of solid supports, types of samples, large dynamic range signal producing system, etc., are now reviewed separately in greater detail.

Solid Support

In embodiments of the invention, a solid support (i.e., substrate) having at

15   least one surface immobilized affinity agent is employed. As such, embodiments of the invention may employ a solid support having a single type of surface immobilized affinity agent immobilized on a surface thereof. In yet other embodiments, the solid support includes a plurality of different affinity agents immobilized on a surface thereof. As such, embodiments of the methods include

20   the use of an array of a plurality of distinct affinity (i.e., binding) agents.

In certain embodiments, the affinity agent (i.e., analyte binding agent) is a molecule that has a high binding affinity for a target analyte. By high binding affinity is meant a binding affinity of at least about 10^{-4} M, such as at least about 10^{-6} M or higher, e.g., 10^{-9}M or higher. The affinity agent may be any of a variety of different

25   types of molecules, so long as it exhibits the requisite binding affinity for the target analyte when immobilized on the surface of a substrate.

In certain embodiments, the affinity agent is a small molecule or large molecule ligand. By small molecule ligand is meant a ligand ranging in size from about 50 to about 10,000 daltons, usually from about 50 to about 5,000 daltons

30   and more usually from about 100 to about 1000 daltons. By large molecule is meant a ligand ranging in size from about 10,000 daltons or greater in molecular weight.

The small molecule may be any molecule, as well as binding portion or fragment thereof, that is capable of binding with the requisite affinity to the target analyte. In certain embodiments, the small molecule is a small organic molecule that is capable of binding to the target analyte of interest. The small molecule may

5   include one or more functional groups necessary for structural interaction with the target analyte, e.g., groups necessary for hydrophobic, hydrophilic, electrostatic or even covalent interactions. Where the target analyte is a protein, the agent may include functional groups necessary for structural interaction with proteins, such as hydrogen bonding, hydrophobic-hydrophobic interactions, electrostatic

10   interactions, etc., and may include at least an amine, amide, sulfhydryl, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups.

Small molecule affinity agents may include cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more

15   of the above functional groups. Also of interest as small molecules are structures found among biomolecules, including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Such compounds may be screened to identify those of interest, where a variety of different screening protocols are known in the art.

20   The small molecule may be derived from a naturally occurring or synthetic compound that may be obtained from a wide variety of sources, including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including the preparation of randomized oligonucleotides and

25   oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known small molecules may be subjected to

30   directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc. to produce structural analogs.

As such, the small molecule may be obtained from a library of naturally occurring or synthetic molecules, including a library of compounds produced through combinatorial means, i.e., a compound diversity combinatorial library. When obtained from such libraries, the small molecule employed will have

5   demonstrated some desirable affinity for the protein target in a convenient binding affinity assay. Combinatorial libraries, as well as methods for the production and screening, are known in the art and described in: 5,741,713; 5,734,018; 5,731,423; 5,721,099; 5,708,153; 5,698,673; 5,688,997; 5,688,696; 5,684,711; 5,641,862; 5,639,603; 5,593,853; 5,574,656; 5,571,698; 5,565,324; 5,549,974; 5,545,568;

10   5,541,061; 5,525,735; 5,463,564; 5,440,016; 5,438,119; 5,223,409, the disclosures of which are herein incorporated by reference.

Also suitable for use as binding domains are polynucleic acid aptimers. Polynucleic acid aptamers may be RNA oligonucleotides which may act to selectively bind proteins, much in the same manner as a receptor or antibody

15   (Conrad et al., Methods Enzymol. (1996), 267(Combinatorial Chemistry), 336-367). In certain embodiments where the affinity ligand is a nucleic acid, e.g., an amptamer, the target analyte is not a nucleic acid. In certain embodiment, the affinity ligand is not a nucleic acid.

As pointed out, the affinity agent can also be a large molecule. Incertain

20   embodiments, large molecule affinity agents that are employed are antibodies, as well as binding fragments and mimetics thereof. Where antibodies are the affinity agent, they may be derived from polyclonal compositions, such that a heterogeneous population of antibodies differing by specificity are each tagged with the same tag nucleic acid, or monoclonal compositions, in which a

25   homogeneous population of identical antibodies that have the same specificity for the target protein are each tagged with the same tag nucleic acid. As such, the affinity agent may be either a monoclonal and polyclonal antibody. In yet other embodiments, the affinity agent is an antibody binding fragment or mimetic, where these fragments and mimetics have the requisite binding affinity for the target

30   protein. For example, antibody fragments, such as Fv, $F(ab)_2$ and Fab may be prepared by cleavage of the intact protein, e.g., by protease or chemical cleavage. Also of interest are recombinantly produced antibody fragments, such as single

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chain antibodies or scFvs, where such recombinantly produced antibody fragments retain the binding characteristics of the above antibodies. Such recombinantly produced antibody fragments generally include at least the VH and VL domains of the subject antibodies, so as to retain the binding characteristics of the subject

5   antibodies. These recombinantly produced antibody fragments or mimetics of the subject invention may be readily prepared using any convenient methodology, such as the methodology disclosed in U.S. Patent Nos. 5,851,829 and 5,965,371; the disclosures of which are herein incorporated by reference.

The above-described antibodies, fragments and mimetics thereof may be

10   obtained from commercial sources and/or prepared using any convenient technology, where methods of producing polyclonal antibodies, monoclonal antibodies, fragments and mimetics thereof, including recombinant derivatives thereof, are known to those of the skill in the art.

In certain embodiments, the solid support is one that displays a plurality of

15   distinct affinity agents immobilized on a surface, e.g., in an addressable manner, such as is found on an array. In representative embodiments, each of the plurality of different affinity agents immobilized on the solid support surface is one that includes at least an epitope binding domain of an antibody molecule. In these embodiments, the solid support employed in the methods are characterized by

20   having a plurality of probe spots, each made up of a distinct binding agent (i.e., a plurality of copies of distinct binding agent molecule) stably associated with the surface of a solid support.

Each probe composition of the subject arrays of certain of these embodiments is made up of multiple copies of a binding agent, where each binding

25   agent includes at least an epitope binding domain of antibody. By epitope binding domain is meant a region or portion of an antibody molecule that specifically binds to an antigen, more particularly a determinant or epitope of a given antigen. As such, in certain embodiments the binding agents are antibodies, as well as specific antigen binding fragments and mimetics thereof. Where antibodies are the binding

30   agent, they may be derived from polyclonal compositions, such that a heterogeneous population of antibodies differing by specificity are each immobilized on the substrate surface, or monoclonal compositions, in which a

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homogeneous population of identical antibodies that have the same specificity for the target analyte, e.g., protein, are each immobilized on the substrate surface. As such, the binding agent may be either a monoclonal or a polyclonal antibody in certain embodiments.

5   In yet other embodiments, the binding agent making up the subject probe compositions is an antibody binding fragment or mimetic, where these fragments and mimetics have the requisite binding affinity for the target analyte, e.g., protein. For example, antibody fragments, such as Fv, F(ab)$_2$ and Fab may be prepared by cleavage of the intact protein, e.g. by protease or chemical cleavage. Also of
10   interest are recombinantly produced antibody fragments, such as single chain antibodies or scFvs, where such recombinantly produced antibody fragments retain the binding characteristics of the above antibodies, i.e., they include the epitope binding domain (which means the whole domain or a least a functional portion thereof) of an antibody specific for the particular analyte. Such
15   recombinantly produced antibody fragments generally include at least the V$_H$ and V$_L$ domains of the subject antibodies, so as to retain the binding characteristics of the subject antibodies. These recombinantly produced antibody fragments or mimetics of the subject invention may be readily prepared using any convenient methodology, such as the methodology disclosed in U.S. Patent Nos. 5,851,829
20   and 5,965,371; the disclosures of which are herein incorporated by reference. The above-described antibodies, fragments and mimetics thereof may be obtained from commercial sources and/or prepared using any convenient technology, where methods of producing polyclonal antibodies, monoclonal antibodies, fragments and mimetics thereof, including recombinant derivatives thereof, are known to those of
25   the skill in the art. The binding agents have, in certain embodiments, a strong affinity for their analyte, where this affinity is at least about 10^{-6}, such as at least about 10^{-8} and includes ranges from about 10^{-8} to about 10^{-13}, such as from about 10^{-9} to about 10^{-12}, where the affinity is the affinity as measured following immobilization of the antibody onto the surface using the binding affinity assay
30   described in Pellequer, J. L., Van Regenmortel, M. H., J Endocrinol,, 139, (3) 495-501.

The probe spots made up of the binding agents as described above and present on the array may be any convenient shape, such as circular, elliptoid, oval or some other analogously curved shape. The total amount or mass of molecules present in each spot will be sufficient to provide for adequate binding and detection

5   of analytes during the assay in which the array is employed. In certain embodiments, the total mass of binding agents in each spot is at least about 10 pg, such as at least about 100 pg and including at least about 1 ng, where the total mass may be as high as 20 ng or higher, and in certain embodiments does not exceed about 10 ng and in certain embodiments does not exceed about 5 ng.

10   Where the probe spot has an overall circular dimension, the diameter of the spot may range from about 10 to about 5,000 μm, such as from about 20 to about 1,000 μm and including from about 50 to about 500 μm. The surface area of each spot may be at least about 100 μm^2, such as at least about 200 μm^2 and including at least about 400 μm^2, and may be as great as about 25 mm^2 or greater, and in

15   certain embodiments does not exceed about 5 mm^2, and in certain embodiments, does not exceed about 1 mm^2. The density of binding agents "probe" spots on the array, as well as the overall density of probe and non-probe spots (where the latter are described in greater detail below) may vary greatly. As used herein, the term spot refers to any spot on the array surface that is made up of binding agents,

20   whether control or probe binding agents, and as such includes both probe spots and non-probe spots. The density of the probe spots on the solid surface is, in certain embodiments, at least about 5/cm^2 such as at least about 10/cm^2 and may be as high as about 100/cm^2, about 200/cm^2, about 300/cm^2, about 500 /cm^2, about 1000/cm^2, about 5000/cm^2 or higher, and in certain embodiments does not

25   exceed about 1000/cm^2, and in these embodiments may not exceed about 500/cm^2 or about 400/cm^2, and in certain embodiments does not exceed about 300/cm^2. The spots may be arranged in a spatially defined and physically addressable manner, in any convenient pattern across or over the surface of the array, such as in rows and columns so as to form a grid, in a circular pattern, and

30   the like, where the pattern of spots may be present in the form of a grid across the surface of the solid support.

In the subject arrays, the spots of the pattern are stably associated with, i.e., immobilized on, the surface of a solid support, where the support may be a flexible or rigid support. By "stably associated" it is meant that the binding agents of the spots maintain their position relative to the solid support under incubation and
5   washing conditions, as described below. As such, the individual binding agent members that make up the spots can be non-covalently or covalently stably associated with the support surface based on technologies well known to those of skill in the art. Examples of non-covalent association include non-specific adsorption, binding based on electrostatic (e.g. ion, ion pair interactions),
10   hydrophobic interactions, hydrogen bonding interactions, specific binding through a specific binding pair member covalently attached to the support surface, and the like. Examples of covalent binding include covalent bonds formed between the spot binding agents and a functional group present on the surface of the rigid support, where the functional group may be naturally occurring or present as a
15   member of an introduced linking group. In certain embodiments, the binding agents making up the spots on the array surface are covalently bound to the support surface, e.g., through covalent linkages formed between moieties present on the binding agents, e.g., amines, and the substrate surface, etc, as may be present on a glass substrate, e.g., aminated glass. See e.g., the specific covalent
20   attachment protocol exemplified below.

As mentioned above, the array is present on either a flexible or rigid substrate. By flexible is meant that the support is capable of being bent, folded or similarly manipulated without breakage. Examples of solid materials which are flexible solid supports with respect to the present invention include membranes,
25   flexible plastic films, and the like. By rigid is meant that the support is solid and does not readily bend, i.e. the support is not flexible. As such, the rigid substrates of the subject arrays are sufficient to provide physical support and structure to the polymeric targets present thereon under the assay conditions in which the array is employed, particularly under high throughput handling conditions. Furthermore,
30   when the rigid supports of the subject invention are bent, they are prone to breakage.

The solid supports upon which the subject binding agent(s), e.g., in the form of patterns of spots, are presented in the subject substrates (e.g., arrays) may take a variety of configurations ranging from simple to complex, depending on the intended use of the array. Thus, the solid support could have an overall slide or

5   plate configuration, such as a rectangular or disc configuration. In certain embodiments, the solid support has a rectangular cross-sectional shape, having a length of from about 10 mm to 200 mm, such as from about 40 to 150 mm and including from about 75 to 125 mm and a width of from about 10 mm to 200 mm, such as from about 20 mm to 120 mm and including from about 25 to 80 mm, and

10   a thickness of from about 0.01 mm to 5.0 mm, such as from about 0.01 mm to 2 mm and including from about 0.01 to 1 mm. Thus, in one embodiment the support may have a micro-titre plate format, having dimensions of approximately 125x85 mm. In another representative embodiment, the support may be a standard microscope slide with dimensions of from about 25 x 75 mm.

15   The solid supports may be fabricated from a variety of materials. The materials from which the solid support is fabricated exhibit a low level of non-specific binding during binding events in certain embodiments. In certain embodiments, a material that is transparent to visible and/or UV light is employed. For flexible substrates, materials of interest include, but are not limited to: nylon,

20   both modified and unmodified, nitrocellulose, polypropylene, and the like. For rigid substrates, specific materials of interest include, but are not limited to: glass; plastics, e.g. polytetrafluoroethylene, polypropylene, polystyrene, polycarbonate, and blends thereof, and the like; metals, e.g. gold, platinum, and the like; etc. Also of interest are composite materials, such as glass or plastic coated with a

25   membrane, e.g., nylon or nitrocellulose, etc.

The solid supports of the arrays of these particular embodiments include at least one surface on which the pattern of spots is present, where the surface may be smooth or substantially planar, or have irregularities, such as depressions or elevations. The surface on which the pattern of spots is present may be modified

30   with one or more different layers of compounds that serve to modify the properties of the surface in a desirable manner. Such modification layers, when present, will generally range in thickness from a monomolecular thickness to about 1 mm,

usually from a monomolecular thickness to about 0.1 mm and more usually from a monomolecular thickness to about 0.001 mm. Modification layers of interest include: inorganic and organic layers such as metals, metal oxides, polymers, small organic molecules and the like. Polymeric layers of interest include layers of:

5   peptides, proteins, polynucleic acids or mimetics thereof, e.g. peptide nucleic acids and the like; polysaccharides, phospholipids, polyurethanes, polyesters, polycarbonates, polyureas, polyamides, polyethyleneamines, polyarylene sulfides, polysiloxanes, polyimides, polyacetates, polyacrylamides, and the like, where the polymers may be hetero- or homopolymeric, and may or may not have separate

10   functional moieties attached thereto, e.g. conjugated.

       In certain embodiments, e.g., where the binding agent is a whole antibody or analogous structure, an antibody universal binding layer is present on the substrate surface, e.g., covalently bound to the substrate surface, which layer acts as a linking group or tethering element between the antibody binding agent in the

15   substrate surface and serves to tether the antibody binding agent to the substrate surface. The basic principle is to utilize proteins and ligands with affinity towards antibodies (including but not limited to Protein A, Protein G, Protein L, Protein LA) which are covalently immobilized to a glass, plastic or any other type of surfaces. After the immobilization of the universal binding layer, the antibody binding agents

20   are deposited on the same locations and reversibly immobilized. The universal binding layer of affinity ligands thus forms a layer which protects the consequently bound antibodies from detrimental surface effects. An additional benefit is the directed mode of immobilization as compared to that of direct covalent attachment of the antibodies to activated surfaces. This results in 100 % availability of the

25   antigen binding sites on the antibodies for consequent detection of antigens. It also provides universal conditions for binding, since the formation of ligand/antibody complex is obtained under mild physiological conditions where as covalent immobilization of proteins is often performed under conditions that might be detrimental to their biological activity.

30   The total number of spots on the solid support surface will vary depending on the number of different probe spots (binding agent probe compositions) one wishes to display on the surface, as well as the number of non probe spots, e.g.,

control spots, orientation spots, calibrating spots and the like, as may be desired depending on the particular application in which the subject arrays are to be employed. In certain embodiments, the pattern present on the surface of the support will comprise at least about 10 distinct spots, such as at least about 20

5   spots, and including at least about 50 distinct spots, where the number of distinct spots may be as high as 10,000 or higher, but in certain embodiments does not exceed about 5,000 distinct spots, and in certain embodiments does not exceed about 3,000 distinct spots, e.g., does not exceed about 2,000 distinct spots. In certain embodiments, each distinct probe spot or probe composition is presented

10   in duplicate, i.e., so that there are two duplicate probe spots displayed on the array for a given target. The number of probe spots present in the array makes up a substantial proportion of the total number of spots on the array in certain embodiments, where in representative embodiments the number of probe spots is at least about 50 number %, such as at least about 80 number % and including at

15   least about 90 number % of the total number of spots on the array. As such, in certain embodiments the total number of spots on the array ranges from about 10 to about 20,000, such as from about 20 to about 10,000 and including from about 100 to 5,000.

In the arrays of certain embodiments (particularly those designed for use in

20   high throughput applications, such as high throughput analysis applications), a single pattern of probe spots may be present on the array or the array may include a plurality of different spot patterns, each pattern being as defined above. When a plurality of different spot patterns are present, the patterns may be identical to each other, such that the array comprises two or more identical spot patterns on its

25   surface, or the spot patterns may be different, e.g., in arrays that have two or more different sets of probes present on their surface, e.g., an array that has a pattern of spots corresponding to first population of target analytes and a second pattern of spots corresponding to a second population of analytes. Where a plurality of spot patterns are present on the array, the number of different spot patterns is at least

30   2, usually at least 6, more usually at least 24 or 96, where the number of different patterns will generally not exceed about 384.

In those embodiments where the array includes a plurality of spot patterns the array may include a plurality of reaction chambers, wherein each chamber has a bottom surface having associated therewith an pattern of spots and at least one wall, usually a plurality of walls surrounding the bottom surface. See e.g. U.S.

5   Patent No. 5,545,531, the disclosure of which is herein incorporated by reference. Of interest in certain embodiments are arrays in which the same pattern of spots in reproduced in 24 or 96 different reaction chambers across the surface of the array.

Within any given pattern of spots on the array, there may be a single spot that corresponds to (i.e., specifically binds to) a given analyte target or a number of

10   different spots that correspond to the same analyte, where when a plurality of different spots are present that correspond to the same analyte, the probe compositions of each spot that corresponds to the same analyte may be identical or different. In other words, a plurality of different analytes are represented in the pattern of spots, where each analyte may correspond to a single spot or a plurality

15   of spots, where the probe compositions among the plurality of spots corresponding to the same analyte may be the same or different. Where a plurality of spots (of the same or different composition) corresponding to the same analyte is present on the array, the number of spots in this plurality will be at least about 2 and may be as high as 10, but may not exceed about 5. As mentioned above, however, in

20   representative embodiments, any given analyte is represented by only a single type of probe spot, which may be present only once or multiple times on the array surface, e.g. in duplicate, triplicate etc.

The number of distinct or different probe spots present on the array, and therefore the number of different analytes represented on the array, is, in certain

25   embodiments, at least about 2, such as at least about 10 and including at least about 20, where in representative embodiments the number of different analytes represented on the array is at least about 50 and may be at least about 100. The number of different analytes represented on the array may be as high as 5,000 or higher, but in representative embodiments does not exceed about 3,000, such as

30   not more than about 2,500. An analyte is considered to be represented on an array if it is able to specifically bind to one or probe compositions on the array.

The arrays employed in aspects of the methods may be fabricated using any convenient protocol, where the protocol may vary depending on the nature of the substrate, the nature of any intervening surface layer, e.g., whether or not a universal binding layer is present, and the nature of the binding agents. Where the

5   solid support is a glass substrate or analogous material, typically the surface of the support is first activated to provide for functional groups suitable for use in the covalent bonding, either directly or through a linking group, of the binding agent. For example, glass surfaces may be aminated so as to display amine functional groups via silanization, according to well known surface chemistry protocols. In

10   many embodiments, the binding agent is then immobilized on the functionalized surface, e.g., through direct or indirect covalent bonding, e.g., by non-covalent binding to a covalently bound universal binding layer of molecules, as described above. Of interest in certain embodiments is the use of a surface activation agent, e.g., that provides a linking group capable of forming a covalent linkage between

15   aminated moieties, such as PIDTC and DVS, as exemplified in the experimental section below.

Following surface preparation, e.g., surface activation, a binding agent composition is immobilized on the substrate surface to produce a spot of the array. The binding agent composition is typically an aqueous composition. In

20   representative embodiments, the concentration ranges of the deposited binding agent composition is at least about 0.1 mg/mL, such as at least about 0.2 mg/mL, where the concentration may be as great as 1 mg/mL or greater. The purity of the binding agent composition is, in representative embodiments, at least about 90%, such as at least about 95% and including at least about 97% % pure.

25   The binding agent composition is deposited on the array surface using any convenient protocol. In certain embodiments, the binding agent composition is applied using a pin or analogous fluid deposition device. Also of interest are pipette devices, ink jet devices, etc., which are extensively described in the array preparation art. The particular device and protocol employed to spot the subject

30   binding agents is not critical, so long as it results in a functional probe spot, i.e., a probe spot that specifically binds to its target analyte.

Following deposition of the binding agent compositions to produce the
pattern of probe spots on the array, the surface is then contacted with a blocking
agent in order to block non-specific binding sites on the array surface. Any
convenient blocking agent may be employed, where representative blocking

5   agents include, but are not limited to, nonfat milk, BSA, gelatin, preimmune serum
and the like, where standard blocking protocols may be employed.

Following preparation and blocking, as described above, the array may be
stored for a period of time prior to use, as desired. The array may be stored in any
convenient format, including both dry and wet formats, so long as the activity of the

10   array, i.e., the binding ability of the probe spots on the array for their specific
analytes, is not adversely affected. By not adversely affected is meant that the
sensitivity of the array does not change with respect to a given analyte as
compared to the array immediately following blocking by a value that exceeds
about 10 fold, and usually does not change by a value that exceeds about 5 fold.

15   In many embodiments, the period of time for which the array is stored prior to use
in the subject methods, described in greater detail below, is at least about 2 days,
usually at least about 6 months and more usually at least about 9 months and may
be as long as about 1 year or longer, where the array is typically not stored for a
period that exceeds about 6 months prior to use.

20

Sample

The sample that is contacted with the solid support surface may vary
greatly, depending upon the nature of the assay to be performed. In certain
embodiments, the sample is an aqueous fluid sample. The amount of fluid sample

25   also varies with respect to the nature of the device, the nature of the sample, etc.
In representative embodiments, the amount of sample that is contacted with the
solid support surface ranges from about 100 µl to about 10 ml, such as from about
1 mL to about 8 ml and including from about 4 to about 6 ml.

In certain embodiments, the fluid sample is a naturally occurring sample,

30   where the sample may or may not be modified prior to contact with the solid
support. In certain embodiments, the fluid sample is obtained from a physiological
source, where the physiological source may be eukaryotic, with physiological

sources of interest including sources derived from single celled organisms such as yeast and multicellular organisms, including plants and animals, such as mammals, where the physiological sources from multicellular organisms may be derived from particular organs or tissues of the multicellular organism, or from

5   isolated cells or cellular compartments, e.g., nucleus, cytoplasm, etc., derived therefrom.

In obtaining the fluid sample, the initial physiological source (e.g., tissue, collection of cells, etc.) may be subjected to a number of different processing steps, where such processing steps might include tissue homogenization, protein

10   extraction and the like, where such processing steps are known to the those of skill in the art. Of particular interest in certain embodiments is the use of cellular extracts as the sample.

In certain embodiments, the initial fluid sample derived from a particular source, e.g., a cell extract, may be subjected to a fractionation protocol that

15   reduces the complexity of the protein composition of the sample. By reduce the complexity is meant that the total mass of all of the proteins in the sample is reduced by at least about 10 fold, such as by at least about 100 fold and including by at least about 1000 fold.

In certain embodiments, the fractionation protocol employed is one that

20   reduces the amount of highly abundant proteins in the sample. In this embodiment, a pool of covalently attached antibodies, e.g., one or more columns of antibodies, is employed for enrichment of antigen analytes of interest from an initial sample, e.g., whole cell extracts. After reversible adsorption of the antigens of interest on the multi-antibody column, the non adsorbed material is washed away with

25   washing buffer and the specifically retarded antigens are eluted and collected for further labeling and incubation with the array containing binding agent spots for the antigen/analytes of interest, e.g., the same antibodies that were used for initial enrichment. In this manner, the initial sample is fractionated so as to reduce the complexity and enrich the sample for the analytes of interest.

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Incubation

Following sample preparation, the analyte containing fluid sample is contacted with the solid support surface and contact is maintained under sufficient conditions and for a period of time sufficient for binding of analyte to specific binding agent on the solid support surface to occur. In certain embodiments, the array and analyte containing sample are incubated together for at least about 10 min., such as at least about 20 min., and including at least about 30 min., where the incubation time may be as long as about 480 min. or longer, but in certain embodiments does not exceed about 60 min. During incubation, the solid support surface and sample are maintained at a temperature that may range in certain embodiments from about 20 °C to about 28 °C, such as from about 22 °C to about 26 °C. In certain embodiments, the array and sample are subjected to mixing or agitation during the incubation step.

in certain embodiments, a feature of the incubation step is that present in the analyte containing fluid medium contacted with the substrate surface during incubation is a metal ion chelating polysaccharide, i.e., a polysaccharide that chelates metal ions, wherein the polysaccharide may be a charged molecule. The polysaccharide may be a naturally occurring or synthetic molecule, and may be a homopolymeric or heteropolymeric compound. The molecular weight of the polysaccharide may vary greatly, but in certain embodiments ranges from about 10,000 to 2,500,000 daltons, such as from about 10,000 to 500,000 daltons, where in certain embodiments a population of polysaccharide molecules of different weights are present in the analyte containing fluid medium during incubation. The total amount of metal chelating polysaccharide present in the analyte containing fluid medium may range from about 0.01% to about 2%, such as from about 0.2% to about 1%. In certain embodiments, the metal chelating polysaccharide is a heteropolymeric compound that includes polygalactouronate sequences. Of particular interest in certain embodiments are pectins, like fruit pectins, e.g., citrus pectin (e.g., lemon, orange), apple pectin, tomato pectin, and the like.

During incubation, the pH of the liquid medium may be maintained at a value ranging from about 6.5 to about 8.5, such as from about 7.0 to about 8.0. Also present may be one or more buffers, e.g., Tris, sodium citrate and the like;

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salts, e.g., NaCl, sodium sulfate, and the like; surfactants/surfactants, e.g., Pluronics, Tweens, glycerol, ethylene glycol, etc.

5 While the contact of the solid support surface and analyte containing fluid medium may be accomplished using any convenient protocol, in certain embodiments, the initial sample is first pre-incubated with an incubation buffer that includes the metal ion chelating polysaccharide to produce a preincubated analyte containing sample, which preincubated sample is then contacted with the substrate for the incubation period. In these embodiments, the incubation buffer employed at least includes the metal ion chelating polysaccharide as described above. In

10 addition, the incubation buffer typically includes a number of additional components, including buffering agents, salts, surfactants, etc.

In certain embodiments, the substrate and sample may be contacted and incubated in the presence of a known amount of a competitor that competes with said analyte for binding to said affinity agent immobilized on said substrate

15 surface. For example, where the assay format is a competitive format, a known amount of an analyte competitor, e.g., analyte or binding fragment thereof, may be included in the sample.

Washing

20 Following incubation, non-support bound components of the analyte containing medium contacted with the support surface during incubation are separated or removed from the surface in certain embodiments. This separation step may be accomplished using one or more washing steps, as desired, in which the substrate surface is contacted and separated from, including flushed with, one

25 or more different fluid compositions.

In certain embodiments, the support surface is subjected to a sequential washing protocol, in which the support surface is washed with a plurality of distinct washing solutions. The number of different washings employed in these embodiments varies, but ranges in certain embodiments from about 3 to 10, such

30 as from about 5 to 9 and including from about 6 to 8, where in certain embodiments, 7 distinct washings are employed. In these embodiments, the series of different washing mediums employed provides a modulation or change in the

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nature of the washing medium and components therein, e.g., in order to subject
the array surface to a sequential or step-wise change or modulation of conditions,
e.g., amount/type of detergent, salt concentration, buffering agent, additives, etc.
In these embodiments, the different washing conditions to which the support is

5   subjected during the sequential wash protocol are ones that provide for a decrease
in background and cross-reactivity during detection, and therefore an increase in
signal to noise ratio and/or selectivity, so as to provide the sensitive results
discussed above. In certain embodiments, the washing conditions are ones that
provide for an increase in signal to noise ratio and/or selectivity of at least about 2-

10   fold, such as at least about 5-fold and including at least about 10 fold and
compared to a control assay in which only a single wash step with a wash fluid that
is the same as the incubation fluid is performed. In certain embodiments, the
sequential wash protocol is characterized by initially employing a high salt wash,
e.g., to remove electrostatically bound molecules, followed by sequential use of

15   wash fluids of decreasing detergent composition, and/or a change of buffers, e.g.,
from Tris to sodium citrate. Of interest in certain embodiments is the use of a
fluorescence quenching decreasing agent, e.g., polyethyleneimine and other
agents that provide for a decrease in fluorescence quenching and therefore an
increase in signal to noise ratio.

20

Large Dynamic Range Signal Producing System

        As indicated above, aspects of the subject methods include the use of a
large dynamic range signal producing system. A feature of the large dynamic
range signal producing systems employed in embodiments of the invention is the

25   presence of: (i) a first fluorescently labeled detection agent that specifically binds
to a surface immobilized member, e.g., target analyte; and (ii) a second
fluorescently labeled detection agent that specifically binds to the first fluorescently
labeled detection agent. The first and second detection agents may specifically
bind directly or indirectly to their targets (e.g., target analyte or first detection

30   agent, respectively), such that they may bind directly to their targets or via one or
more binding mediation members, e.g., non-covalent specific binding pair
members, such as ligands, *e.g.* biotin, fluorescein, digoxigenin, other haptens,

polyvalent cations, chelator groups and the like. In certain embodiments, the first detection agent binds directly to the target analyte, e.g., that is bound to the surface immobilized binding agent. In representative embodiments, the second fluorescently labeled detection agent directly binds to the first fluorescently labeled

5   detection agent. In certain embodiments, the second fluorescently labeled detection agent indirectly binds to the first fluorescently labeled detection agent.

In certain embodiments, the first fluorescently labeled detection agent is capable of indirectly binding to a plurality of second fluorescently labeled detection agents. Aspects of these embodiments include the use of a detection agent that

10   can be bound by a signal amplification agent, e.g., a biotinylated antibody as reviewed below, such that a plurality of second detection agents can be indirectly bound to the first detection agent.

In certain embodiments, the first fluorescently labeled detection agent includes: an affinity agent, such as antibody or binding fragment thereof, that

15   specifically binds to the target analyte; and a first fluorescent label. As such, the first detection agent is labeled with a first fluorescent label. By labeled is meant that the detection agent is joined to, either covalently bonded to or stably but non-covalently bound to, a first fluorescent label. Examples of fluorescent moieties or labels of interest include, but are not limited to: coumarin and its derivatives, e.g. 7-

20   amino-4-methylcoumarin, aminocoumarin, bodipy dyes, such as Bodipy FL, cascade blue, fluorescein and its derivatives, e.g. fluorescein isothiocyanate, Oregon green, rhodamine dyes, e.g. texas red, tetramethylrhodamine, eosins and erythrosins, cyanine dyes, e.g. Cy3 and Cy5, macrocyclic chelates of lanthanide ions, e.g. quantum dye™, fluorescent energy transfer dyes, such as thiazole

25   orange-ethidium heterodimer, TOTAB, etc.

In certain embodiments, the first fluorescent label is a proteinaceous label, such as a phycobiliprotein or complex thereof, e.g., a phycobilisome. Various phycobiliproteins can be used as the first fluorescent moiety. These phycobiliproteins may be allophycocyanin, allophycocyanin B, C-phycocyanin, R-

30   phycocyanin, C-phycoerythrin, B-phycoerythrin, B-phycoerythrin I and II, and the like. The phycobiliproteins may be obtained from various species of cyanobacteria (blue-green algae), red algae and cryptomonads. Phycobiliproteins and complexes

thereof, including both naturally occurring and synthetic, are described in greater detail in U.S. Patent Nos. 5,055,556; 4,859,582; 4,542,104; 4,520,110, as well as U.S. Patent Application Nos. 20010055783 and 20030134325; the disclosures of which are herein incorporated by reference.

5   In representative embodiments, the second fluorescently labeled detection agent of the large dynamic range signal producing system includes: an affinity agent that specifically binds to the first fluorescently labeled detection agent, either directly or indirectly; and a second fluorescent label. In certain embodiments, the second detection agent is made up of a single molecule that includes the above

10   recited components, while in other embodiments the second detection agent is made up of two or more distinct molecules, wherein the different elements are present on different molecules. Aspects of this second embodiment include embodiments where the affinity agent is present on a first molecule that specifically binds to the first detection agent, and then the fluorescent label is present on a

15   second molecule that specifically binds to the affinity agent comprising molecule, e.g., via a high affinity specific binding member pair interaction, such as a biotin/avidin or streptavidin interaction. As such, in certain embodiments, the second detection agent includes a first molecule that is a biotinylated affinity agent for the first detection agent, and a second molecule that includes avidin or

20   streptavidin conjugated to a second fluorescent moeity.

The second fluorescent moiety may include a single fluorescent entity or a plurality of distinct entities that are associated (either covalently or non-covalently) with each other to produce a fluorescent complex, e.g., in the form of a naturally occurring or synthetic phycobilisome. As with the first detection agent, the

25   fluorescent moiety of the second detection agent may any of a variety of different fluorescent entities. In representative embodiments, the second fluorescent moiety is made up of one or more fluorescent proteinaceous molecules, such as phycobiliproteins.

In one representative embodiment, the second fluorescent detection agent

30   is a conjugate of avidin or streptavidin and a stabilized, e.g., crosslinked, complex of two or more, e.g., three or more, different or distinct phycobiliproteins, e.g., APCT, B-PE and R-PC, such as is marketed by Martek Biosciences under the

tradename PBXL-3. See e.g., Technical Bulletin 2, Martek Biosciences, June
2002. In yet other embodiments, the second fluorescent moiety is made up of non-
covalently associated or bonded different phycobiliproteins, e.g., such as APC in
combination with one or both of B-PE and R-PC. In such embodiments, ratio of

5   different phycobiliproteins may vary, but in certain embodiments may be 1 APC
and/or R-PC for every 5 to 10 B-PE.

In certain embodiments, the first and second fluorescent labels, when
immobilized on the solid support surface, e.g., via specific binding to the support
immobilized affinity agent capture analyte (or mimetic thereof or agent itself,

10   depending on the particular assay format, e.g., direct or competitive), are present
in a fluorescent energy transfer (FET), such as a fluorescent resonance energy
transfer (FRET) relationship. As such, the first and second fluorescent moieties are
chosen to provide this relationship, e.g., by having the first fluorescent moiety of
the first detection agent serve as a donor and the second fluorescent moiety of the

15   second detection agent serve as an acceptor. Two fluorescent labels are viewed
as being a FRET pair for purposes of the present invention if, when positioned
sufficiently close to each other (typically less than about 100Å, and usually less
than about 80Å), they participate in fluorescence resonance energy transfer, such
that excitation of one of the labels gives rise to emission from the other of the two

20   labels. A variety of FRET pairs of fluorescent labels are known to those of skill in
the art and may be employed. The energy donors of the pairs (e.g., the first
fluorescent moieties) will generally be compounds which absorb in the range of
about 300 to about 800 nm, such as in the range of about 450 to about 700 nm,
and are capable of transferring energy to an acceptor fluorophore, which absorbs

25   light of a wavelength 15 nm, more usually 20 nm or higher, than the absorption
wavelength of the donor. The acceptor will emit in the range of about 400 to about
900 nm in certain embodiments.

In certain embodiments, the FRET relationship that is present is not
complete, but instead only partial, such that not all of the energy emitted from the

30   donor is taken up by the acceptor. As such, embodiments are characterized in that
less than 90% of the energy is transmitted, such as less than 80%, including less
than 70% or less, so that an emission signal is still obtainable from the first

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fluorescent moiety. In certain embodiments, there will be at least 10% efficiency of transfer at a distance of from about 1-10 nm between chromophoric groups, such as at least about 25 % efficiency of transfer and including at least 50% efficiency of transfer at from about 3-10 nm, such as from about 4-8 nm.

5   In certain embodiments, the analytes of interest present in the sample are contacted with at least some of, if not all of, the members of the large dynamic range signal producing system prior to contact with the support surface. In certain embodiments, the analytes in the sample are not contacted with the signal producing system prior to incubation. Any convenient protocol is acceptable so

10   long as the signal producing system is contacted with the sample contacted support at some point prior to detection, described below. As such, the surface bound analytes may be labeled following incubation and an initial wash step.

Detection

15   Following washing, the support surface is read or scanned for the presence of binding complexes, e.g., between analytes in the assayed sample and binding agents on the substrate surface. In other words, analyte/binding agent complexes on the surface of the array are detected in representative embodiments.

        Any convenient protocol may be employed for detecting the binding agents

20   on the support surface. The detection method may be qualitative or quantitative depending on the particular application in which the subject method is being performed, where the particular detection protocol employed may or may not use a detectable label. Representative detection protocols that may be employed include those described in WO 00/04389 and WO 00/04382; the disclosures of the priority

25   applications of which are herein incorporated by reference. In certain embodiments, a fluorescence scanner device, i.e., fluorimeter, is employed. In certain embodiments, the methods include assessing or evaluating signal at two or more different wavelengths, e.g., a first wavelength corresponding to an emission maximum of the first fluorescent moiety and a second wavelength corresponding

30   to an emission maximum of the second fluorescent moiety. In certain embodiments, the signal that is assessed is one that is excited by a narrow wavelength band of excitation light.

Analyte Determination

Following detection of signal from the signal producing system, e.g., which is proportional to the amount of surface bound binding complexes on the support

5   surface, the presence of any surface bound binding complexes is then related to the presence of the one or more analytes in the sample. In representative embodiments, the signal intensity value obtained from the signal producing system is quantitatively related to the presence of the corresponding analyte in the sample, so as to provide a quantitative determination of the analyte amount in the

10   sample. This relating step is readily accomplished in that the position on the surface at which a particular surface bound complex is located indicates the identity of the analyte or protein, since the binding agent for the protein is attached to a known specific location on the surface. Thus, this relating step merely comprises determining the location on the surface on which a binding complex is

15   present, comparing that location to a reference that provides information regarding the correlation of each location to a particular analyte and thereby deriving the identity of the analyte in the sample. In sum, the location of the surface bound binding complexes is used to determine the identity of the one or more analytes of interest in the sample.

20   By way of further illustration, the following representative protein assay is summarized. Where one is interested in assaying a sample for the presence of 100 different proteins, an array displaying a collection of 100 different antibody binding agents is prepared, where each different antibody binding agents in the collection specifically binds to a different protein member of the 100 different

25   proteins being assayed. The array is then contacted with the sample being assayed under conditions sufficient for binding complexes to be produced between the probe binding agent spots and their corresponding target proteins in the sample. Any resultant binding complexes on the surface of the array are then detected and the location of the detected binding complexes is used to determine

30   which of the 100 proteins of interest is present in the sample.

In certain embodiments, two or more physiological sources, e.g., cell extracts, are assayed according to the above protocols in order to generate

analyte profiles for the two or more sources that may be compared. In such
embodiments, analyte containing sample may be separately contacted to identical
arrays or together to the same array under binding conditions, depending on
whether a means for distinguishing the patterns generated by the different
5   populations of analytes is employed, e.g. distinguishable labels.

In certain embodiments, the results are internally validating, e.g., where
overlapping signals are obtained from the disparate components of the large
dynamic signal producing system.

10   KITS

Also provided by the subject invention are kits for use in practicing the
subject methods, as described above. The subject kits typically include a solid
support having a binding agent immobilized on a surface thereof, such as an array
of binding agents, as described above, e.g., an array of binding agents where each
15   agent includes an epitope binding domain of an antibody. In addition, the kits of
representative embodiments include a large dynamic range signal producing
system of first and second detection agents, as described above. In addition, the
subject kits may further include an incubation buffer, as described above, or at
least components for making such a buffer, i.e., an incubation buffer that includes
20   a metal ion chelating polysaccharide. The subject kits may also include an
extraction/labeling buffer as described above, as well as one or more wash buffers.
In addition, the subject kits may include a fractionation means, e.g., where
fractionated steps are employed, such as the binding agent/antibody columns
described above. Furthermore, the kits may include one or more positive or
25   negative controls, e.g., pre-labeled antigen known to have a corresponding control
spot on the array, etc.

The various components of the kit may be present in separate containers or
certain compatible components may be precombined into a single container, as
desired.

30   In addition to the above components, the subject kits mayfurther include
instructions for practicing the subject methods. These instructions may be present
in the subject kits in a variety of forms, one or more of which may be present in the
kit. One form in which these instructions may be present is as printed information

on a suitable medium or substrate, e.g., a piece or pieces of paper on which the information is printed, in the packaging of the kit, in a package insert, etc. Yet another means would be a computer readable medium, e.g., diskette, CD, etc., on which the information has been recorded. Yet another means that may be present

5   is a website address which may be used via the internet to access the information at a removed site. Any convenient means may be present in the kits.

SYSTEMS

       Also provided are systems for use practicing the subject methods. The

10   subject systems include at least the solid supports and first and second detection agents as described above, and a detector device for detecting surface bound complexes following incubation. The subject systems may also include additional components necessary for practicing a given embodiment of the subject methods, e.g., incubation buffer, extraction/labeling buffer, washing fluid, labeling reagents,

15   etc., as described above.

UTILITY

       The subject methods find use in a variety of different applications, where certain applications of interest include analyte detection, drug development, toxicity

20   testing, clinical diagnostics, differential protein expression analysis, etc.

       One application of particular interest in which the subject invention finds use is proteomics, in which the subject methods are used to characterize the proteome or some fraction of the proteome of a physiological sample, e.g. a cell, population of cells, population of proteins secreted by a cell or population of cells, etc. By

25   proteome is meant the total collection or population of intracellular proteins of a cell or population of cells and the proteins secreted by the cell or population of cells. In using the subject methods in proteomics applications, the subject methods are employed to measure the presence, and usually quantity, of the proteins which have been expressed in the cell of interest, i.e., are present in the assayed

30   physiological sample derived from the cell of interest. In certain applications, the subject methods are employed to characterize and then compare the proteomes of two or more distinct cell types, e.g., a diseased and normal cell. Proteomics

applications in which the subject invention finds use are further described in WO 00/04382, WO 00/04389 and WO 00/04390, and the priority U.S. Patent applications on which these international applications are based, the disclosures of which priority applications are herein incorporated by reference.

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Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those 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

10   from the spirit or scope of the appended claims.

Accordingly, the preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody

15   the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions.

20   Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same

25   function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of present invention is embodied by the appended claims.

WHAT IS CLAIMED IS:

1.   A method of determining the presence of an analyte in a sample, said method comprising:

5   (a)   contacting said sample with a solid support comprising an affinity agent for said analyte immobilized on a surface thereof; and

          (b)   detecting the presence of resultant surface bound analyte by assessing signal produced from a signal producing system that includes:

                    (i)   a first fluorescently labeled detection agent that specifically
10   binds to said analyte; and

                    (ii)   a second fluorescently labeled detection agent that
                            specifically binds to said first fluorescently labeled detection
                            agent;

          wherein said second fluorescently labeled detection agent comprises a
15   greater number of fluorescent moieties than said first fluorescently labeled detection agent;

          to determine whether said analyte is present in said sample.

2.   The method according to Claim 1, wherein said second fluorescently
20   labeled detection agent directly binds to said first fluorescently labeled detection agent.

3.   The method according to Claim 1, wherein said second fluorescently labeled detection agent indirectly binds to said first fluorescently labeled detection
25   agent.

4.   The method according to Claim 3, wherein said first fluorescently labeled detection agent is capable of indirectly binding to a plurality of said second fluorescently labeled detection agents.

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5.   The method according to Claim 1, wherein said affinity agent is an antibody or binding fragment thereof.

6.   The method according to Claim 1, wherein said first fluorescently labeled detection agent comprises:

        an antibody or binding fragment thereof that specifically binds to said

5   analyte; and

        a first fluorescent label.

7.   The method according to Claim 6, wherein said first fluorescent label is a proteinaceous fluorescent label.

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8.   The method according to Claim 7, wherein said proteinaceous fluorescent label is a phycobilliprotein.

9.   The method according to Claim 1, wherein said second fluorescently

15   labeled detection agent comprises;

        an affinity agent that specifically binds to said first fluorescently labeled detection agent; and

        a second fluorescent label.

20   10.   The method according to Claim 9, wherein said affinity agent and second fluorescent label are present on the same molecule.

11.   The method according to Claim 10, wherein said affinity agent is present on a first molecule and said second fluorescent label is present on a second

25   molecule.

12.   The method according to Claim 11, wherein said first molecule is an antibody.

30   13.   The method according to Claim 12, wherein said first molecule comprises biotin.

14.   The method according to Claim 13, wherein said second molecule comprises avidin or streptavidin.

15.   The method according to Claim 9, wherein said second fluorescent label comprises a plurality of fluorescent moieties.

16.   The method according to Claim 15, wherein said plurality of fluorescent moieties are covalently bonded to each other.

17.   The method according to Claim 15, wherein said plurality of fluorescent moieties are non-covalently bonded to each other.

18.   The method according to Claim 9, wherein said fluorescent label is a phycobilliprotein.

19.   The method according to Claim 1, wherein said method comprises washing said solid support following contact with said sample.

20.   The method according to Claim 1, wherein said method comprises evaluating signal at two different light wavelengths.

21.   The method according to Claim 1, wherein said solid support comprises a plurality of distinct affinity agents each specific for a different analyte immobilized in an addressable manner on said surface.

22.   The method according to Claim 21, wherein each of said affinity agents at least comprises a specific epitope binding domain of an antibody.

23.   The method according to Claim 1, wherein said sample is combined with at least one of said first and second detection agents prior to contacting said sample with said support surface.

24.   The method according to Claim 1, wherein said sample is combined with both of said first and second detection agents prior to contacting said sample with said substrate.

5   25.   The method according to Claim 1, wherein said sample is contacted with said substrate, said substrate is washed and then said washed substrate is contacted with said first and second detection agents.

26.   The method according to Claim 1, wherein said first and second
10   fluorescently labeled detection agents include distinct fluorescent labels that are capable of an incomplete FET relationship.

27.   The method according to Claim 1, wherein said sample comprises a known amount of a competitor that competes with said analyte for binding to said affinity
15   agent immobilized on said substrate surface.

28.   A kit comprising:
    (a)   a solid support comprising an affinity agent immobilized on a surface thereof; and
20   (b)   a signal producing system that includes:
        (i)   a first fluorescently labeled detection agent that specifically binds to said analyte; and
        (ii)   a second fluorescently labeled detection agent that specifically binds to said first fluorescently labeled detection
25   agent;
wherein said second fluorescently labeled detection agent comprises a greater number of fluorescent moieties than said first fluorescently labeled detection agent.

30   29.   The kit according to Claim 28, wherein said second fluorescently labeled detection agent directly binds to said first fluorescently labeled detection agent.

30.   The kit according to Claim 28, wherein said second fluorescently labeled detection agent indirectly binds to said first fluorescently labeled detection agent.

31.   The kit according to Claim 30, wherein said first fluorescently labeled
5   detection agent is capable of indirectly binding to a plurality of second fluorescently labeled detection agents.

32.   The kit according to Claim 28, wherein said affinity agent is an antibody or binding fragment thereof.
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33.   The kit according to Claim 28, wherein said first fluorescently labeled detection agent comprises:

        an antibody or binding fragment thereof that specifically binds to said analyte; and
15   a first fluorescent label.

34.   The kit according to Claim 33, wherein said first fluorescent label is a proteinaceous fluorescent label.

20   35.   The kit according to Claim 34, wherein said proteinaceous fluorescent label is a phycobilliprotein.

36.   The kit according to Claim 28, wherein said second fluorescently labeled detection agent comprises;
25   an affinity agent that specifically binds to said first fluorescently labeled detection agent; and
        a second fluorescent label.

37.   The kit according to Claim 36, wherein said affinity agent and second
30   fluorescent label are present on the same molecule.

38.   The kit according to Claim 36, wherein said affinity agent is present on a first molecule and said second fluorescent label is present on a second molecule.

39.   The kit according to Claim 38, wherein said first molecule comprises biotin.

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40.   The kit according to Claim 39, wherein said second molecule comprises avidin or streptavidin.

41.   The kit according to Claim 36, wherein said second fluorescent label

10   comprises a plurality of fluorescent moieties.

42.   The kit according to Claim 41, wherein said plurality of fluorescent moieties are covalently bonded to each other.

15   43.   The kit according to Claim 41, wherein said plurality of fluorescent moieties are non-covalently bonded to each other.

44.   The kit according to Claim 36, wherein said fluorescent label is a phycobilliprotein.

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45.   The kit according to Claim 28, wherein said kit further comprises a wash buffer.

46.   The kit according to Claim 28, wherein said solid support comprises a

25   plurality of distinct affinity agents each specific for a different analyte immobilized in an addressable manner on said surface.

47.   The kit according to Claim 46, wherein each of said affinity agents at least comprises a specific epitope binding domain of an antibody.

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48. The kit according to Claim 47, wherein said first and second fluorescently labeled detection agents include distinct fluorescent labels that are capable of an incomplete FET relationship.

5 49. The kit according to Claim 28, wherein said kit further comprises a known amount of a competitor that competes with said analyte for binding to said affinity agent immobilized on said substrate surface.