In addition, the methods of the invention utilize a substrate to which the complementary chemical binding moieties corresponding to the first feature on each target analyte are attached. In the certain methods described herein, a separate substrate bearing the complementary chemical binding moiety that binds the first feature of a target analyte is provided for each target analyte. The invention also provides methods that further allow the use of additional substrate configurations. In one such configuration, the complementary chemical binding moieties corresponding to the first feature of each of the target analytes are bound to the same substrate. In another such configuration, the complementary chemical binding moieties corresponding to the first feature of each of the target analytes are bound to the same substrate in a manner such that the complementary chemical binding moieties corresponding to one target analyte are localized to a different spatial region of the substrate than are the complementary chemical binding moieties corresponding to the other target analytes. These alternative configurations are beneficial in some uses and implementations of the invention.

The substrate used in the methods of the invention serve three primary purposes. One such purpose is to provide a surface upon which the target analyte can be selectively immobilized while materials in the medium that are not target analytes are not so immobilized. This is accomplished by attaching complementary chemical binding moieties that correspond to the first feature of a target analyte to the surface of the substrate and allowing the target analyte to bind to the complementary chemical binding moiety and thus become immobilized on the surface of the substrate. A second such purpose is to facilitate the separation of the target analyte immobilized on the surface of the substrate from the medium. In certain embodiments of the invention, the substrate can be made of a magnetic material that can, along with any material bound to its surface, be separated from the medium by the application of a magnetic field. Numerous other means of separating a substrate from a medium are known and can be likewise employed. Two of many possible illustrative examples are substrates made of materials that are either more or less dense than the medium and which can, therefore, be separated from the medium by sedimentation or floatation, respectively; and substrates that are sufficiently large that they can be separated from the medium by filtration. A substrate can be suspendable in the medium as described herein. Alternatively, the methods of the invention can benefit from the use of a macroscopic substrate such as, but not limited to a

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microscope slide where spatially separated regions containing the different complementary chemical binding moieties can be formed. The third such purpose of the substrate is that, by virtue of its ability to bind target analytes, it causes the local concentration of the target analytes at the surface of the substrate to be substantially higher than the concentration of the target analytes in the medium. Taken together, these three purposes significantly improve the sensitivity and specificity of the analysis and provide a broad range of options from which the optimum implementation of the invention for any specific use or purpose can be selected.

The methods described herein can utilize a detection probe that comprises a gold nanoparticle approximately 10 to 50 nM in diameter to which are attached a complementary chemical binding moiety that selectively binds to the second feature of a target analyte and a plurality of oligonucleotide pairs. The identity of the target analyte to which any particular detection probe responds can be encoded in the base sequence of the oligonucleotide that is attached to the gold nanoparticle. The oligonucleotide encoding the identity of a particular target analyte has variably been referred to as the "barcode" or the "biobarcode" for that target analyte.

In certain embodiments, the methods of the invention may alternatively utilize detections probes such as described as follows in order to derive benefits such as simple procedures and fast results while retaining sensitivity of methods utilizing gold nanoparticles as described herein. In particular, the surface area of a spherical particle scales as the square of its diameter. The surface area of a one micron diameter sphere is, for example, approximately 1100 times greater than that of a 30nM diameter particle. This means that a one micron diameter spherical particle can accommodate approximately 1100 times as many oligonucleotides as can a 30nM particle, and, as the measurable signal in these inventions is a function of this number of oligonucleotides, a detection probe constructed using a one micron particle can be expected to yield approximately 1100 times the measurable signal per binding event than is available from a 30nM particle. This increased "gain" or "amplification" resulting from the use of a larger particle permits considerable simplifications of the analysis procedure without sacrificing sensitivity. However, the rate of diffusion of the particle and, thus, the frequency with which they move into contact with the target analyte to be detected and the time required to achieve binding to any particular fraction of the target analyte that is

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present scales inversely with its diameter. The time available for these binding events, therefore, imposes a practical limit upon the largest size of particle that may be employed while still meeting the time constraints that are imposed by any particular use or implementation of the invention. The optimum particle size for any particular use or implementation is, therefore, determined by the balance between the foregoing factors and is typically in the range of between 0.5 and 5.0 microns.

One type of detection probe can be structured identically to the previously described probes based upon 30nM gold particles except that a 1 micron (1000nM) polystyrene microparticle is employed. A second type of detection probe has particular utility when the presence of only a small number of target analytes is to be simultaneously determined. These detection probes of the second type encode the identity of the target analyte in the characteristics of a "detectable entity" bound to an oligonucleotide that is, in turn, bound to the probe particle rather than in the sequence of bases comprising the oligonucleotide. In this context, a "detectable entity" may, for example, be a fluorophore with the identity of the target analyte being encoded in the emission wavelength of a fluorophore. Other suitable detectable entities may include, but are not limited to radioisotopes, electrochemically active species, Raman active species, chemiluminescent species, and the like. Detection probes of this type are particularly suitable for detection by flow cytometric methods and for the in-situ detection of target analytes. A third type of detection probe further allows the oligonucleotides bearing detectable entities to be chemically cleaved from the microparticle to facilitate detection in solution. These improved detection probes provide additional options for optimizing the implementation of the invention for any specific use or purpose.

The present invention relates to a method that utilizes oligonucleotides as biochemical barcodes for detecting multiple analytes in a sample. The approach takes advantage of recognition elements (e.g., proteins or nucleic acids) functionalized either directly or indirectly with nanoparticles and the previous observation that hybridization events that result in the aggregation of gold nanoparticles can significantly alter their physical properties (e.g. optical, electrical, mechanical).[8-12] The general idea is that each recognition element can be associated with a different oligonucleotide sequence (a DNA barcode) with discrete and tailorable hybridization and melting properties and a physical signature associated with the nanoparticles that changes upon melting to decode a series

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of analytes in a multi-analyte assay. Therefore, one can use the melting temperature of a DNA-linked aggregate and a physical property associated with the nanoparticles that changes upon melting to decode a series of analytes in a multi-analyte assay. The barcodes herein are different from the ones based on physical diagnostic markers such as nanorods,[23] flourophore-labeled beads,[24] and quantum dots,[25] in that the decoding information is in the form of chemical information stored in a predesigned oligonucleotide sequence.

The present invention provides several broadly applicable strategies for using nanoparticle probes (preferably gold nanoparticle probes), heavily functionalized with oligonucleotides, to detect a variety of biomolecules, for example, single or multiple polyvalent proteins, in one sample. In particular known methods for detection of multiple proteins in one sample is complicated and often requires time consuming, expensive assay protocols. In this regard, others have recently used fluorophore-labeled peptidonucleic acids and DNA microarrays to recognize multiple protein targets in one solution. [15-17] However, this method relies on the binding of the proteins labeled with oligonucleotides to a microarray surface. The final step of the method described herein is based solely on the surface chemistry of ordinary DNA. Therefore, it can incorporate many of the high sensitivity aspects of state-of-the-art nanoparticle DNA detection methods, [9,11] but allows one to detect a variety of biomolecules, such as proteins, rather than DNA without having the proteins present during the detection event. For surface assays, proteins are typically more difficult to work with than short oligonucleotides because they tend to exhibit greater nonspecific binding to solid supports, which often leads to higher background signals. Finally, for the homogeneous assay, the unusually sharp melting profiles associated with these nanoparticle structures will allow one to design more biobarcodes than what would be possible with probes that exhibit normal and broad DNA melting behavior.

The present invention contemplates the use of any suitable particle having oligonucleotides attached thereto that are suitable for use in detection assays. In practicing this invention, however, microparticles and nanoparticles are preferred. The size, shape and chemical composition of the particles will contribute to the properties of the resulting probe including the DNA barcode. These properties include optical properties, optoelectronic properties, electrochemical properties, electronic properties,

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stability in various solutions, pore and channel size variation, ability to separate bioactive molecules while acting as a filter, etc. The use of mixtures of particles having different sizes, shapes and/or chemical compositions, as well as the use of nanoparticles having uniform sizes, shapes and chemical composition, are contemplated. Examples of suitable particles include, without limitation, nano- and microsized core particles, aggregate particles, isotropic (such as spherical particles) and anisotropic particles (such as non-spherical rods, tetrahedral, prisms) and core-shell particles such as the ones described in U.S. Patent application no. 10/034,451, filed December 28, 2002 and International application no. PCT/US01/50825, filed December 28, 2002, which are incorporated by reference in their entirety. In practicing the invention, the detection probes are preferably generated prior to conducting the actual assay. Alternatively, the detection probes may be generated in situ while conducting the assay.

Thus, in one embodiment of the invention, nanoparticle-conjugate probes are provided. Nanoparticles useful in the practice of the invention include metal (*e.g.*, gold, silver, copper and platinum), semiconductor (*e.g.*, CdSe, CdS, and CdS or CdSe coated with ZnS) and magnetic (*e.g.*, ferromagnetite) colloidal materials. Other nanoparticles useful in the practice of the invention include ZnS, ZnO, TiO_2, AgI, AgBr, HgI_2, PbS, PbSe, ZnTe, CdTe, In_2S_3, In_2Se_3, Cd_3P_2, Cd_3As_2, InAs, and GaAs. The size of the nanoparticles is preferably from about 5 nm to about 150 nm (mean diameter), more preferably from about 30 to about 100 nm, most preferably from about 40 to about 80 nm. The size of microparticles of the invention is preferably from about 1 μm to about 150 μm (mean diameter), more preferably from about 1 μm to about 50 μm, most preferably about 1 μm. The size of the microparticles or nanoparticles used in a method of the invention can be varied as required by their particular use or application. The variation of size can be advantageously used to optimize certain physical characteristics of the nanoparticles, for example, optical properties or amount surface area that can be derivatized. The nanoparticles may also be rods, prisms, or tetrahedra.

Methods of making metal, semiconductor and magnetic nanoparticles are well known in the art. See, *e.g.*, Schmid, G. (ed.) *Clusters and Colloids* (VCH, Weinheim, 1994); Hayat, M.A. (ed.) *Colloidal Gold: Principles, Methods, and Applications* (Academic Press, San Diego, 1991); Massart, R., *IEEE Taransactions On Magnetics*, 17, 1247 (1981); Ahmadi, T.S. et al., *Science*, 272, 1924 (1996); Henglein, A. et al., *J. Phys.*

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Chem., 99, 14129 (1995); Curtis, A.C., et al., *Angew. Chem. Int. Ed. Engl.*, 27, 1530 (1988).

Methods of making ZnS, ZnO, TiO_2, AgI, AgBr, HgI_2, PbS, PbSe, ZnTe, CdTe, In_2S_3, In_2Se_3, Cd_3P_2, Cd_3As_2, InAs, and GaAs nanoparticles are also known in the art. See, *e.g.*, Weller, *Angew. Chem. Int. Ed. Engl.*, 32, 41 (1993); Henglein, *Top. Curr. Chem.*, 143, 113 (1988); Henglein, *Chem. Rev.*, 89, 1861 (1989); Brus, *Appl. Phys. A.*, 53, 465 (1991); Bahncmann, in Photochemical Conversion and Storage of Solar Energy (eds. Pelizetti and Schiavello 1991), page 251; Wang and Herron, *J. Phys. Chem.*, 95, 525 (1991); Olshavsky et al., *J. Am. Chem. Soc.*, 112, 9438 (1990); Ushida et al., *J. Phys. Chem.*, 95, 5382 (1992).

Suitable nanoparticles are also commercially available from, *e.g.*, Ted Pella, Inc. (gold), Amersham Corporation (gold) and Nanoprobes, Inc. (gold).

Presently preferred for use in detecting nucleic acids are gold nanoparticles. Gold colloidal particles have high extinction coefficients for the bands that give rise to their beautiful colors. These intense colors change with particle size, concentration, interparticle distance, and extent of aggregation and shape (geometry) of the aggregates, making these materials particularly attractive for colorimetric assays. For instance, hybridization of oligonucleotides attached to gold nanoparticles with oligonucleotides and nucleic acids results in an immediate color change visible to the naked eye (see, *e.g.*, the Examples).

The nanoparticles, the oligonucleotides or both are functionalized in order to attach the oligonucleotides to the nanoparticles. Such methods are known in the art. For instance, oligonucleotides functionalized with alkanethiols at their 3'-termini or 5'-termini readily attach to gold nanoparticles. See Whitesides, *Proceedings of the Robert A. Welch Foundation 39th Conference On Chemical Research Nanophase Chemistry*, Houston, TX, pages 109-121 (1995). See also, Mucic et al. *Chem. Commun.* 555-557 (1996) (describes a method of attaching 3' thiol DNA to flat gold surfaces; this method can be used to attach oligonucleotides to nanoparticles). The alkanethiol method can also be used to attach oligonucleotides to other metal, semiconductor and magnetic colloids and to the other nanoparticles listed above. Other functional groups for attaching oligonucleotides to solid surfaces include phosphorothioate groups (see, *e.g.*, U.S. Patent No. 5,472,881 for the binding of oligonucleotide-phosphorothioates to gold surfaces),

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substituted alkylsiloxanes (see, *e.g.* Burwell, *Chemical Technology*, 4, 370-377 (1974) and Matteucci and Caruthers, *J. Am. Chem. Soc.*, 103, 3185-3191 (1981) for binding of oligonucleotides to silica and glass surfaces, and Grabar et al., *Anal. Chem.*, 67, 735-743 for binding of aminoalkylsiloxanes and for similar binding of mercaptoaklylsiloxanes). Oligonucleotides terminated with a 5' thionucleoside or a 3' thionucleoside may also be used for attaching oligonucleotides to solid surfaces. The following references describe other methods which may be employed to attached oligonucleotides to nanoparticles: Nuzzo et al., *J. Am. Chem. Soc.*, 109, 2358 (1987) (disulfides on gold); Allara and Nuzzo, *Langmuir*, 1, 45 (1985) (carboxylic acids on aluminum); Allara and Tompkins, *J. Colloid Interface Sci.*, 49, 410-421 (1974) (carboxylic acids on copper); Iler, The Chemistry Of Silica, Chapter 6, (Wiley 1979) (carboxylic acids on silica); Timmons and Zisman, *J. Phys. Chem.*, 69, 984-990 (1965) (carboxylic acids on platinum); Soriaga and Hubbard, *J. Am. Chem. Soc.*, 104, 3937 (1982) (aromatic ring compounds on platinum); Hubbard, *Acc. Chem. Res.*, 13, 177 (1980) (sulfolanes, sulfoxides and other functionalized solvents on platinum); Hickman et al., *J. Am. Chem. Soc.*, 111, 7271 (1989) (isonitriles on platinum); Maoz and Sagiv, *Langmuir*, 3, 1045 (1987) (silanes on silica); Maoz and Sagiv, *Langmuir*, 3, 1034 (1987) (silanes on silica); Wasserman et al., *Langmuir*, 5, 1074 (1989) (silanes on silica); Eltekova and Eltekov, *Langmuir*, 3, 951 (1987) (aromatic carboxylic acids, aldehydes, alcohols and methoxy groups on titanium dioxide and silica); Lec et al., *J. Phys. Chem.*, 92, 2597 (1988) (rigid phosphates on metals).

In one aspect of this embodiment of the invention, nanoparticles conjugated with dendrimers labeled with at least two types of oligonucleotides are provided. Dendritic molecules are structures comprised of multiple branching unit monomers, and are used in various applications. See, e.g., Barth et al., Bioconjugate Chemistry 5:58-66 (1994); Gitsov & Frechet, Macromolecules 26:6536-6546 (1993); Hawker & Frechet, J. Amer. Chem. Soc. 112:7638-7647 (1990a); Hawker & Frechet, Macromolecules 23:4726-4729 (1990b); Hawker et al., J. Chem. Soc. Perkin Trans. 1:1287-1297 (1993); Lochmann et al. J. Amer. Chem. Soc. 115:7043-7044 (1993); Miller et al., J. Amer. Chem. Soc. 114:1018-1025 (1992); Mousy et al., Macromolecules 25:2401-2406 (1992); Naylor et al., J. Amer. Chem. Soc. 111:2339-2341 (1989); Spindler & Frechet, Macromolecules Macromolecules 26:4809-4813 (1993); Turner et al., Macromolecules 26:4617-4623 (1993); Wiener et al., Magnetic Resonance Med. 31(1):1-8 (1994); Service, 267:458-459

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(1995); Tomalia, Sci. Amer. 62-66 (1995); and U.S. Pat. Nos. 4,558,120; 4,507,466; 4,568,737; 4,587,329; 4,857,599; 5,527,524; 5,338,532 to Tomalia, and U.S. Patent 6,274,723 to Nilsen, all of which are incorporated herein, in their entirety. Dendritic molecules provide important advantages over other types of supermolecular architectures, such as contacting a maximum volume a minimum of structural units, ability to more easily control size, weight, and growth properties, and the multiple termini can be derivatized to yield highly labeled molecules with defined spacing between the labels, or provide sites of attachment for other molecules, or mixtures thereof. See generally U.S. Patent 6,274,723 and the above cited references for methods of synthesis.

Nucleic acid dendrimers that are useful in the methods of the invention are any of those known in the art that can be functionalized with nucleic acids or generated from nucleic acids/oligonucleotides. Such dendrimers can be synthesized according to disclosures such as Hudson et al., "Nucleic Acid Dendrimers: Novel Biopolymer Structures," Am. Chem. Soc. 115:2119-2124 (1993); U.S. Patent 6,274,723; and U.S. Pat. No. 5,561,043 to Cantor.

In another aspect of this embodiment the invention, a universal nanoparticle-oligonucleotide conjugate is provided. The universal provide may be used in an assay for any target nucleic acid that comprises at least two portions. This "universal probe" comprises (i) oligonucleotides of a single "capture" sequence attached to it that are complementary to at least a portion of a reporter oligonucleotide (e.g., barcode DNA), and to a portion of a target recognition oligonucleotide (one embodiment of the universal probe is described in Figures 16A and 16B). The target recognition oligonucleotides comprise a sequence having at least two portions; the first portion comprises complementary sequence to the capture sequence attached to the nanoparticle, and the second portion comprises complementary sequence to the first portion of the particular target nucleic acid sequence. Various types of target recognition oligonucleotides can be used to great advantage with the universal probe, such that a library of target recognition oligonucleotides can be switched or interchanged in order to select for particular target nucleic acid sequences in a particular test solution. A second type of oligonucleotide, which comprises sequence complementary to the second portion of the target nucleic acid, is attached to a support surface, such as a magnetic particle or glass slide.

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Contacting the universal probe with a solution comprising the reporter oligonucleotide (barcode DNA) and the target recognition oligonucleotide under conditions that allow for hybridization create a universal probe that is "activated" for contacting with a solution that may contain the target nucleic acid (Fig. 16A, top reaction scheme). The test solution can be contacted under conditions that allow for hybridization, in sequence or in combination with either or both of the "activated" universal probe or the second type of oligonucleotide, which is attached to a support. Once adequate time is allowed for complex formation, the uncomplexed test solution components are removed from the complex, and the reporter oligonucleotides are detected. One embodiment of this assay is depicted in Fig. 16B.

These universal probes can be manipulated for increased advantage, which depend on the particular assay to be conducted. The probes can be "tuned" to various single target nucleic acid sequences, by simply substituting or interchanging the target recognition oligonucleotides such that the second portion comprises complementary sequence to the target nucleic acid of interest. Similarly, if multiple target nucleic acid sequences are to be assayed in a single test solution, the reporter oligonucleotides can comprise a sequence that is specific for each target nucleic acid. Thus, detection of the reporter oligonucleotide of known and specific sequence, would indicate the presence of the particular target nucleic acid in the test solution.

In other aspect of this embodiment of the invention, the oligonucleotides are bound to nanoparticles using sulfur-based functional groups. U.S. patent application nos. 09/760,500 and 09/820,279 and international application nos. PCT/US01/01190 and PCT/US01/10071 describe oligonucleotides functionalized with a cyclic disulfide which are useful in practicing this invention. The cyclic disulfides preferably have 5 or 6 atoms in their rings, including the two sulfur atoms. Suitable cyclic disulfides are available commercially or may be synthesized by known procedures. The reduced form of the cyclic disulfides can also be used.

Preferably, the linker further comprises a hydrocarbon moiety attached to the cyclic disulfide. Suitable hydrocarbons are available commercially, and are attached to the cyclic disulfides. Preferably the hydrocarbon moiety is a steroid residue. Oligonucleotide-nanoparticle conjugates prepared using linkers comprising a steroid residue attached to a cyclic disulfide have unexpectedly been found to be remarkably

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stable to thiols (e.g., dithiothreitol used in polymerase chain reaction (PCR) solutions) as compared to conjugates prepared using alkanethiols or acyclic disulfides as the linker. Indeed, the oligonucleotide-nanoparticle conjugates of the invention have been found to be 300 times more stable. This unexpected stability is likely due to the fact that each oligonucleotide is anchored to a nanoparticle through two sulfur atoms, rather than a single sulfur atom. In particular, it is thought that two adjacent sulfur atoms of a cyclic disulfide would have a chelation effect which would be advantageous in stabilizing the oligonucleotide-nanoparticle conjugates. The large hydrophobic steroid residues of the linkers also appear to contribute to the stability of the conjugates by screening the nanoparticles from the approach of water-soluble molecules to the surfaces of the nanoparticles.

In view of the foregoing, the two sulfur atoms of the cyclic disulfide should preferably be close enough together so that both of the sulfur atoms can attach simultaneously to the nanoparticle. Most preferably, the two sulfur atoms are adjacent each other. Also, the hydrocarbon moiety should be large so as to present a large hydrophobic surface screening the surfaces of the nanoparticles.

The oligonucleotide-cyclic nanoparticle conjugates that employ cyclic disulfide linkers may be used as probes in diagnostic assays for detecting target analytes in a sample as described in U.S. patent application nos. 09/760,500 and 09/820,279 and international application nos. PCT/US01/01190 and PCT/US01/10071. These conjugates have been found to improve the sensitivity of diagnostic assays in which they are used. In particular, assays employing oligonucleotide-nanoparticle conjugates prepared using linkers comprising a steroid residue attached to a cyclic disulfide have been found to be about 10 times more sensitive than assays employing conjugates prepared using alkanethiols or acyclic disulfides as the linker.

Each nanoparticle will have a plurality of oligonucleotides attached to it. As a result, each nanoparticle-oligonucleotide conjugate can bind to a plurality of oligonucleotides or nucleic acids having the complementary sequence.

Oligonucleotides of defined sequences are used for a variety of purposes in the practice of the invention. Methods of making oligonucleotides of a predetermined sequence are well known. See, *e.g.*, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (2nd ed. 1989) and F. Eckstein (ed.) *Oligonucleotides and Analogues*, 1st Ed.

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(Oxford University Press, New York, 1991). Solid-phase synthesis methods are preferred for both oligoribonucleotides and oligodeoxyribonucleotides (the well-known methods of synthesizing DNA are also useful for synthesizing RNA). Oligoribonucleotides and oligodeoxyribonucleotides can also be prepared enzymatically. For oligonucleotides having bound thereto a specific binding complement to a target analyte, any suitable method for attaching the specific binding complement such as proteins to the oligonucleotide may be used.

Any suitable method for attaching oligonucleotides onto the particle, nanoparticle, or nanosphere surface may be used. A particularly preferred method for attaching oligonucleotides onto a surface is based on an aging process described in U.S. application nos. 09/344,667, filed June 25, 1999; 09/603,830, filed June 26, 2000; 09/760,500, filed January 12, 2001; 09/820,279, filed March 28, 2001; 09/927,777, filed August 10, 2001; and in International application nos. PCT/US97/12783, filed July 21, 1997; PCT/US00/17507, filed June 26, 2000; PCT/US01/01190, filed January 12, 2001; PCT/US01/10071, filed March 28, 2001, the disclosures which are incorporated by reference in their entirety. The aging process provides nanoparticle-oligonucleotide conjugates with unexpected enhanced stability and selectivity

The method comprises providing oligonucleotides preferably having covalently bound thereto a moiety comprising a functional group which can bind to the nanoparticles. The moieties and functional groups are those that allow for binding (*i.e.*, by chemisorption or covalent bonding) of the oligonucleotides to nanoparticles. For instance, oligonucleotides having an alkanethiol, an alkanedisulfide or a cyclic disulfide covalently bound to their 5' or 3' ends can be used to bind the oligonucleotides to a variety of nanoparticles, including gold nanoparticles.

The oligonucleotides are contacted with the nanoparticles in water for a time sufficient to allow at least some of the oligonucleotides to bind to the nanoparticles by means of the functional groups. Such times can be determined empirically. For instance, it has been found that a time of about 12-24 hours gives good results. Other suitable conditions for binding of the oligonucleotides can also be determined empirically. For instance, a concentration of about 10-20 nM nanoparticles and incubation at room temperature gives good results.

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Next, at least one salt is added to the water to form a salt solution. The salt can be any suitable water-soluble salt. For instance, the salt may be sodium chloride, magnesium chloride, potassium chloride, ammonium chloride, sodium acetate, ammonium acetate, a combination of two or more of these salts, or one of these salts in phosphate buffer. Preferably, the salt is added as a concentrated solution, but it could be added as a solid. The salt can be added to the water all at one time or the salt is added gradually over time. By "gradually over time" is meant that the salt is added in at least two portions at intervals spaced apart by a period of time. Suitable time intervals can be determined empirically.

The ionic strength of the salt solution must be sufficient to overcome at least partially the electrostatic repulsion of the oligonucleotides from each other and, either the electrostatic attraction of the negatively-charged oligonucleotides for positively-charged nanoparticles, or the electrostatic repulsion of the negatively-charged oligonucleotides from negatively-charged nanoparticles. Gradually reducing the electrostatic attraction and repulsion by adding the salt gradually over time has been found to give the highest surface density of oligonucleotides on the nanoparticles. Suitable ionic strengths can be determined empirically for each salt or combination of salts. A final concentration of sodium chloride of from about 0.1 M to about 1.0 M in phosphate buffer, preferably with the concentration of sodium chloride being increased gradually over time, has been found to give good results.

After adding the salt, the oligonucleotides and nanoparticles are incubated in the salt solution for an additional period of time sufficient to allow sufficient additional oligonucleotides to bind to the nanoparticles to produce the stable nanoparticle-oligonucleotide conjugates. As will be described in detail below, an increased surface density of the oligonucleotides on the nanoparticles has been found to stabilize the conjugates. The time of this incubation can be determined empirically. A total incubation time of about 24-48, preferably 40 hours, has been found to give good results (this is the total time of incubation; as noted above, the salt concentration can be increased gradually over this total time). This second period of incubation in the salt solution is referred to herein as the "aging" step. Other suitable conditions for this "aging" step can also be determined empirically. For instance, incubation at room temperature and pH 7.0 gives good results.

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The conjugates produced by use of the "aging" step have been found to be considerably more stable than those produced without the "aging" step. As noted above, this increased stability is due to the increased density of the oligonucleotides on the surfaces of the nanoparticles which is achieved by the "aging" step. The surface density achieved by the "aging" step will depend on the size and type of nanoparticles and on the length, sequence and concentration of the oligonucleotides. A surface density adequate to make the nanoparticles stable and the conditions necessary to obtain it for a desired combination of nanoparticles and oligonucleotides can be determined empirically. Generally, a surface density of at least 10 picomoles/cm^2 will be adequate to provide stable nanoparticle-oligonucleotide conjugates. Preferably, the surface density is at least 15 picomoles/cm^2. Since the ability of the oligonucleotides of the conjugates to hybridize with nucleic acid and oligonucleotide targets can be diminished if the surface density is too great, the surface density is preferably no greater than about 35-40 picomoles/cm^2.

As used herein, "stable" means that, for a period of at least six months after the conjugates are made, a majority of the oligonucleotides remain attached to the nanoparticles and the oligonucleotides are able to hybridize with nucleic acid and oligonucleotide targets under standard conditions encountered in methods of detecting nucleic acid and methods of nanofabrication.

It has been found that the hybridization efficiency of nanoparticle-oligonucleotide conjugates can be increased dramatically by the use of recognition oligonucleotides which comprise a recognition portion and a spacer portion. "Recognition oligonucleotides" are oligonucleotides which comprise a sequence complementary to at least a portion of the sequence of a nucleic acid or oligonucleotide target. In this embodiment, the recognition oligonucleotides comprise a recognition portion and a spacer portion, and it is the recognition portion which hybridizes to the nucleic acid or oligonucleotide target. The spacer portion of the recognition oligonucleotide is designed so that it can bind to the nanoparticles. For instance, the spacer portion could have a moiety covalently bound to it, the moiety comprising a functional group which can bind to the nanoparticles. These are the same moieties and functional groups as described above. As a result of the binding of the spacer portion of the recognition oligonucleotide to the nanoparticles, the recognition portion is spaced away from the surface of the nanoparticles and is more accessible for hybridization with its target. The length and

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sequence of the spacer portion providing good spacing of the recognition portion away from the nanoparticles can be determined empirically. It has been found that a spacer portion comprising at least about 10 nucleotides, preferably 10-30 nucleotides, gives good results. The spacer portion may have any sequence which does not interfere with the ability of the recognition oligonucleotides to become bound to the nanoparticles or to a nucleic acid or oligonucleotide target. For instance, the spacer portions should not have sequences complementary to each other, to that of the recognition olignucleotides, or to that of the nucleic acid or oligonucleotide target of the recognition oligonucleotides. Preferably, the bases of the nucleotides of the spacer portion are all adenines, all thymines, all cytidines, or all guanines, unless this would cause one of the problems just mentioned. More preferably, the bases are all adenines or all thymines. Most preferably the bases are all thymines.

It has further been found that the use of diluent oligonucleotides in addition to recognition oligonucleotides provides a means of tailoring the conjugates to give a desired level of hybridization. The diluent and recognition oligonucleotides have been found to attach to the nanoparticles in about the same proportion as their ratio in the solution contacted with the nanoparticles to prepare the conjugates. Thus, the ratio of the diluent to recognition oligonucleotides bound to the nanoparticles can be controlled so that the conjugates will participate in a desired number of hybridization events. The diluent oligonucleotides may have any sequence which does not interfere with the ability of the recognition oligonucleotides to be bound to the nanoparticles or to bind to a nucleic acid or oligonucleotide target. For instance, the diluent oligonulceotides should not have a sequence complementary to that of the recognition olignucleotides or to that of the nucleic acid or oligonucleotide target of the recognition oligonucleotides. The diluent oligonucleotides are also preferably of a length shorter than that of the recognition oligonucleotides so that the recognition oligonucleotides can bind to their nucleic acid or oligonucleotide targets. If the recognition oligonucleotides comprise spacer portions, the diluent oligonulceotides are, most preferably, about the same length as the spacer portions. In this manner, the diluent oligonucleotides do not interefere with the ability of the recognition portions of the recognition oligonucleotides to hybridize with nucleic acid or oligonucleotide targets. Even more preferably, the diluent oligonucleotides have the same sequence as the sequence of the spacer portions of the recognition oligonucleotides.

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In another embodiment of the invention, particle complex probes are provided. Each type of particle complex probe contains a predetermined reporter oligonucleotide or barcode for a particular target analyte. In the presence of target analyte, aggregates are produced as a result of the binding interactions between the particle complex and the target analyte. These aggregates can be isolated and analyzed by any suitable means, e.g., thermal denaturation, to detect the presence of one or more different types of reporter oligonucleotides. In practicing this invention, nanoparticle complex probes are preferred.

Thus, in one aspect of the invention, the particle complex probe comprises a particle having oligonucleotides bound thereto, one or more DNA barcodes, and an oligonucleotide having bound thereto a specific binding complement to a specific target analyte, wherein (i) the DNA barcode has a sequence having at least two portions; (ii) at least some of the oligonucleotides attached to the particle have a sequence that is complementary to a first portion of a DNA barcode; (iii) the oligonucleotide having bound thereto a specific binding complement have a sequence that is complementary to a second portion of a DNA barcode; and (iv) the DNA barcode in each type of particle complex probe has a sequence that is different and that serves as an identifier for a particular target analyte.

In another aspect of this embodiment, the particle complex probe comprises a particle having at least two types of oligonucleotides bound thereto, one or more DNA barcodes, and an oligonucleotide having bound thereto a specific binding complement to a target analyte, wherein a first type of oligonucleotides bound to the probe having a sequence that is complementary to at least a portion of the DNA barcode, the second type of oligonucleotide bound to the probe having a sequence that is complementary to at least a portion of the sequence of the oligonucleotide having a specific binding complement.

In another aspect of this embodiment the particle complex probe comprising a particle having oligonucleotides bound thereto, one or more DNA barcodes, and a specific binding complement to a target analyte, wherein at least a portion of the oligonucleotides bound to the particle have a sequence that is complementary to at least a portion of the sequence of the DNA barcode and where the DNA barcode serves as an identifier for a specific target analyte.

In yet another embodiment of the invention, a particle complex probe is provided. Thus in one embodiment of the invention, a particle complex probe is provided which

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comprises a particle having oligonucleotides bound thereto, a DNA barcode, and an oligonucleotide having bound thereto a specific binding complement to a specific target analyte, wherein (i) the DNA barcode has a sequence having at least two portions; (ii) at least some of the oligonucleotides attached to the particle have a sequence that is complementary to a first portion of a DNA barcode; (iii) the oligonucleotide having bound thereto a specific binding complement have a sequence that is complementary to a second portion of a DNA barcode; and (iv) the DNA barcode in each type of particle complex probe has a sequence that is different and that serves as an identifier for a particular target analyte.

In another embodiment of the invention, a particle complex probe is provided which comprises a particle having at least two types of oligonucleotides bound thereto, a DNA barcode, and an oligonucleotide having bound thereto a specific binding complement to a target analyte, wherein a first type of oligonucleotides bound to the probe having a sequence that is complementary to at least a portion of the DNA barcode, the second type of oligonucleotide bound to the probe having a sequence that is complementary to at least a portion of the sequence of the oligonucleotide having a specific binding complement.

In yet another embodiment of the invention, a particle complex probe is provided which comprises a particle having oligonucleotides bound thereto, a DNA barcode, and a specific binding complement to a target analyte, wherein at least a portion of the oligonucleotides bound to the particle have a sequence that is complementary to at least a portion of the sequence of the DNA barcode and where the DNA barcode serves as an identifier for a specific target analyte.

In yet another embodiment of the invention, a detection probe is provided which comprises a nanoparticle; a member of a specific binding pair bound to the nanoparticle; at least one type of oligonucleotide bound to the nanoparticle; and at least one type of DNA barcode each having a predetermined sequence, wherein each type of DNA barcode is hybridized to at least a portion of the at least one type of oligonucleotide.

Preferably the particles comprise nanoparticles as described above such as metal, semiconductor, insulator, or magnetic nanoparticles. Preferably the particles are gold nanoparticles. The the specific binding complement or binding pair member and the target analyte are members of a specific binding pair which comprises nucleic acid,

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oligonucleotide, peptide nucleic acid, polypeptide, antibody, antigen, carbohydrate, protein, peptide, amino acid, hormone, steroid, vitamin, drug, virus, polysaccharides, lipids, lipopolysaccharides, glycoproteins, lipoproteins, nucleoproteins, oligonucleotides, antibodies, immunoglobulins, albumin, hemoglobin, coagulation factors, peptide and protein hormones, non-peptide hormones, interleukins, interferons, cytokines, peptides comprising a tumor-specific epitope, cells, cell-surface molecules, microorganisms, fragments, portions, components or products of microorganisms, small organic molecules, nucleic acids and oligonucleotides, metabolites of or antibodies to any of the above substances.

In another embodiment of the invention, methods are provided for detecting for the presence or absence of one or more target analytes, the target analyte having at least two binding sites, in a sample. In one aspect of this embodiment of the invention, a method is provided which comprises the steps of:

providing a substrate;

providing one or more types of particle probes, each type of probe comprising a particle having one or more specific binding complements to a specific target analyte and one or more DNA barcodes bound thereto, wherein the specific binding complement of each type of particle probe is specific for a particular target analyte, and the DNA barcode for each type of particle probe serves as a marker for the particular target analyte;

immobilizing the target analytes onto the substrate;

contacting the immobilized target analytes with one or more types of particle probes under conditions effective to allow for binding between the target analyte and the specific binding complement to the analyte and form a complex in the presence of the target analyte;

washing the substrate to remove unbound particle probes; and

optionally amplifying the DNA barcode; and

detecting for the presence or absence of the amplified DNA barcode wherein the presence or absence of the marker is indicative of the presence or absence of a specific target analyte in the sample.

In one aspect of this embodiment of the invention, the target analyte is a protein or hapten and its specific binding complement is an antibody comprising a monoclonal or polyclonal antibody.

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In another aspect of the invention, any suitable substrate may be used. The substrate may be arrayed with one or more types of capture probes for the target analytes.

In this aspect of the invention, the barcode may be isolated. Analyte detection occurs indirectly by ascertaining for the presence of reporter oligonucleotide or biobarcode by any suitable means such as a DNA chip.

DNA barcode can optionally be amplified by any suitable means including PCR amplification, and then be detected by any suitable DNA detection system using any suitable detection probes. The particle is preferably labeled with a sufficient amount of DNA barcodes to provide sufficient signal amplification and eliminate the need for DNA barcode amplification. In practicing this invention, amplification by the PCR method is preferred. PCR amplification (herein also referred to as *B*PCR) of the DNA barcode allows one to detect a protein target at attomolar level. The assay as illustrated in Figure 6, utilizes a new type of nanoparticle heavily functionalized with hybridized oligonucleotides (biobarcodes)[36] and polyclonal detection antibodies to recognize a target analyte, the prostate specific antigen (PSA) (Example 5). In addition, polyamine microparticles (1μm diameter) with magnetic iron oxide cores are funtionalized with PSA monoclonal antibodies (Figure 6 and Example 5). The gold nanoparticles and the polyamine microparticles sandwich the PSA target, generating a complex with a large ratio of barcode DNA to protein target (for 13 nm particles, each particle can support up to 200 strands of DNA; this represents the upper limit for this size particle). Application of a magnetic field draws the magnetic particles to the wall of the reaction vessel in a matter of seconds, allowing one to separate both reacted and unreacted microparticles but only reacted nanoparticles from the reaction mixture. Washing the aggregate structures in Nanopure water (18 MOhms) dehybridizes barcode DNA from nanoparticle-immobilized complements. Using the magnetic separator, the aggregate can be easily removed from the assay solution, leaving the barcode DNA, which can be amplified using PCR, and subsequently and quickly identified by standard DNA detection methodologies (scanometric[11], gel electrophoresis, or fluorophore-labeling approaches). PSA was chosen as the initial target for these studies because of its importance in the early detection of prostate cancer, one of the most common cancers and second leading cause of cancer death in American men [47, 48]. Importantly, identification of disease relapse following the surgical treatment of prostate cancer using PSA as a marker present at low levels (10s of

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copies), could be extremely beneficial and enable the delivery of curative adjuvant therapies [46, 49].

Examples 5-6 demonstrate that *B*PCR is an extremely powerful method for detecting protein analytes, namely PSA, at low attomolar concentrations in the presence of background proteins using either gel electrophoresis or scanometric microarray detection. The work demonstrates several advantages over current protein detection methods. First, the target binding protein of the assay is homogeneous. Therefore, one can add a large quantity of magnetic particles to the reaction vessel to facilitate the binding kinetics between the detection antibody and target analyte. This leads to an assay that is faster than heterogeneous systems and also allows one to increase sensitivity because the capturing step is more efficient. Second, the use of the nanoparticle biobarcodes provides a high ratio of PCR-amplifiable DNA to labeling antibody serving to substantially increase assay sensitivity. For example, the *B*PCR assay reported herein was able to detect PSA at 3 aM concentration while a PCR-based immunoassay has been reported to have a detection limit of 3 fM for the same target analyte [43]. Third, this assay obviates the need for complicated conjugation chemistry for attaching DNA to the labeling antibodies. Barcode DNA is bound to the nanoparticle probe through hybridization at the start of the labeling reaction and liberated for PCR amplification using a simple wash step. Ad the labeling antibody and DNA are present on the same particle, there is no need for the addition of further antibodies or DNA-protein conjugates prior to the PCR amplification of barcode DNA. In addition, the barcode DNA is removed from the detection assay, and PCR is carried out on samples of barcode DNA that is free from PSA, most of the biological sample, the microparticles, and nanoparticles. This substantially reduces background signal. Finally, this protein detection scheme has the potential for massive multiplexing and the simultaneous detection of many analytes in one solution. Although the PSA system is used for proof-of-concept, the approach should be general for almost any target with known binding partners, and by using the nanoparticle-based biobarcode approach [36], one can prepare a unique identifiable barcode for virtually every target of interest.

Example 9 demonstrates that the probes of the invention can be used to detect and measure directly the amount of target analyte in a sample. Thus, a step comprising *B*PCR

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to amplify the barcode DNA is not required to achieve excellent sensitivity and detection limits (Figs. 6B (step 4), 9 (inset), and 12).

Any suitable washing solution that removes unbound probes from the surface of the substrate after complex formation may be used. A representative example includes, without limitation, PBS (phosphate buffer solution).

In the presence of target analyte, nanoparticle aggregate complexes are produced as a result of the binding interactions between the nanoparticle complex probe and the target analyte. These aggregates are may isolated and subject to conditions effective to dehybridize the aggregate and to release the reporter oligonucleotide. The reporter oligonucleotide is then isolated. If desired, the reporter oligonucleotide may be amplified by any suitable means including PCR amplification. Analyte detection occurs indirectly by ascertaining for the presence of reporter oligonucleotide or biobarcode by any suitable means such as a DNA chip.

The DNA barcodes or reporter oligonucleotides may then be detected by any suitable means. Generally, the DNA barcodes are released via dehybridization from the complex prior to detection. Any suitable solution or media may be used that dehybridize and release the DNA barcode from the complex. A representative medium is water.

The DNA barcodes released by dehybridization of the aggregates can be directly detected using a substrate having capture oligonucleotides bound thereto. The oligonucleotides have a sequence complementary to at least one portion of the reporter oligonucleotides. Some embodiments of the method of detecting the DNA barcodes utilize a substrate having complementary oligonucleotides bound thereto to capture the reporter oligonucleotides. These captured reporter oligonucleotides are then detected by any suitable means. By employing a substrate, the detectable change (the signal) can be amplified and the sensitivity of the assay increased.

Any suitable method for attaching oligonucleotides to a substrate may be used. For instance, oligonucleotides can be attached to the substrates as described in, *e.g.*, Chrisey et al., *Nucleic Acids Res.*, **24**, 3031-3039 (1996); Chrisey et al., *Nucleic Acids Res.*, **24**, 3040-3047 (1996); Mucic et al., *Chem. Commun.*, **555** (1996); Zimmermann and Cox, *Nucleic Acids Res.*, **22**, 492 (1994); Bottomley et al., *J. Vac. Sci. Technol. A*, **10**, 591 (1992); and Hegner et al., *FEBS Lett.*, **336**, 452 (1993).

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The oligonucleotides attached to the substrate have a sequence complementary to a first portion of the sequence of reporter oligonucleotides to be detected. The reporter oligonucleotide is contacted with the substrate under conditions effective to allow hybridization of the oligonucleotides on the substrate with the reporter oligonucleotide. In this manner the reporter oligonucleotide becomes bound to the substrate. Any unbound reporter oligonucleotide is preferably washed from the substrate before adding a detection probe such as nanoparticle-oligonucleotide conjugates.

In one aspect of the invention, the reporter oligonucleotide bound to the oligonucleotides on the substrate is contacted with a first type of nanoparticles having oligonucleotides attached thereto. The oligonucleotides have a sequence complementary to a second portion of the sequence of the reporter oligonucleotide, and the contacting takes place under conditions effective to allow hybridization of the oligonucleotides on the nanoparticles with the reporter oligonucleotide. In this manner the first type of nanoparticles become bound to the substrate. After the nanoparticle-oligonucleotide conjugates are bound to the substrate, the substrate is washed to remove any unbound nanoparticle-oligonucleotide conjugates.

The oligonucleotides on the first type of nanoparticles may all have the same sequence or may have different sequences that hybridize with different portions of the reporter oligonucleotide to be detected. When oligonucleotides having different sequences are used, each nanoparticle may have all of the different oligonucleotides attached to it or, preferably, the different oligonucleotides are attached to different nanoparticles. Alternatively, the oligonucleotides on each of the first type of nanoparticles may have a plurality of different sequences, at least one of which must hybridize with a portion of the reporter oligonucleotide to be detected.

Optionally, the first type of nanoparticle-oligonucleotide conjugates bound to the substrate is contacted with a second type of nanoparticles having oligonucleotides attached thereto. These oligonucleotides have a sequence complementary to at least a portion of the sequence(s) of the oligonucleotides attached to the first type of nanoparticles, and the contacting takes place under conditions effective to allow hybridization of the oligonucleotides on the first type of nanoparticles with those on the second type of nanoparticles. After the nanoparticles are bound, the substrate is preferably washed to remove any unbound nanoparticle-oligonucleotide conjugates.

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The combination of hybridizations produces a detectable change. The detectable changes are the same as those described above, except that the multiple hybridizations result in an amplification of the detectable change. In particular, since each of the first type of nanoparticles has multiple oligonucleotides (having the same or different sequences) attached to it, each of the first type of nanoparticle-oligonucleotide conjugates can hybridize to a plurality of the second type of nanoparticle-oligonucleotide conjugates. Also, the first type of nanoparticle-oligonucleotide conjugates may be hybridized to more than one portion of the reporter oligonucleotide to be detected. The amplification provided by the multiple hybridizations may make the change detectable for the first time or may increase the magnitude of the detectable change. This amplification increases the sensitivity of the assay, allowing for detection of small amounts of reporter oligonucleotide.

If desired, additional layers of nanoparticles can be built up by successive additions of the first and second types of nanoparticle-oligonucleotide conjugates. In this way, the number of nanoparticles immobilized per molecule of target nucleic acid can be further increased with a corresponding increase in intensity of the signal.

Also, instead of using first and second types of nanoparticle-oligonucleotide conjugates designed to hybridize to each other directly, nanoparticles bearing oligonucleotides that would serve to bind the nanoparticles together as a consequence of hybridization with binding oligonucleotides could be used.

When a substrate is employed, a plurality of the initial types of nanoparticle-oligonucleotide conjugates or oligonucleotides can be attached to the substrate in an array for detecting multiple portions of a target reporter oligonucleotide, for detecting multiple different reporter oligonucleotides, or both. For instance, a substrate may be provided with rows of spots, each spot containing a different type of oligonucleotide designed to bind to a portion of a target reporter oligonucleotide. A sample containing one or more reporter oligonucleotides is applied to each spot, and the rest of the assay is performed in one of the ways described above using appropriate oligonucleotide-nanoparticle conjugates.

In yet another aspect, the methods of analyte detection by *B*PCR, as well as direct detection, can be adapted for use with methods that comprise analyte detection on a substrate, for example, glass, gold, silicon, nickel, plastics, and the like. These methods

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can also be adapted to detect other biological and chemical recognition events such as DNA-protein binding events, physiological protein-protein binding or dimerization, and other biomolecular interactions previously described above.

In one embodiment of this aspect, the method comprises attaching one or more types of capture probe for each target analyte to a substrate, contacting the substrate with a test solution, optionally washing the test solution from the substrate, subsequently contacting the substrate with one or more types of detection probe for each target analyte, removing any unbound detection probe, and detecting an observable signal, wherein the detection of an observable signal indicates the presence of the target analyte in the test solution.

In this aspect of the invention, the observable signal can be detected using any method described herein. For example, direct detection of barcode DNAs, detection of *B*PCR-amplified barcode DNAs, detection of aggregation of the one or more types of detection probe (e.g., by visual inspection, fluorescence, colorimetric, electrochemistry, electronic, densitometry, radioactivity, and the like), or by using reporter nucleotides that comprise a sequence that is complementary to at least a portion of the barcode DNAs and a detectable signal moiety (e.g., fluorescent label).

When a substrate is employed, a detectable change can be produced or further enhanced by staining such as silver or gold staining. Silver staining can be employed with any type of nanoparticles that catalyze the reduction of silver. Preferred are nanoparticles made of noble metals (*e.g.*, gold and silver). See Bassell, et al., *J. Cell Biol.*, **126**, 863-876 (1994); Braun-Howland et al., *Biotechniques*, **13**, 928-931 (1992). If the nanoparticles being employed for the detection of a nucleic acid do not catalyze the reduction of silver, then silver ions can be complexed to the nucleic acid to catalyze the reduction. See Braun et al., *Nature*, **391**, 775 (1998). Also, silver stains are known which can react with the phosphate groups on nucleic acids.

Silver staining can be used to produce or enhance a detectable change in any assay performed on a substrate, including those described above. In particular, silver staining has been found to provide a huge increase in sensitivity for assays employing a single type of nanoparticle so that the use of layers of nanoparticles can often be eliminated.

In assays for detecting reporter oligonucleotides performed on a substrate, the detectable change can be observed with an optical scanner. Suitable scanners include

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those used to scan documents into a computer which are capable of operating in the reflective mode (*e.g.*, a flatbed scanner), other devices capable of performing this function or which utilize the same type of optics, any type of greyscale-sensitive measurement device, and standard scanners which have been modified to scan substrates according to the invention (*e.g.*, a flatbed scanner modified to include a holder for the substrate) (to date, it has not been found possible to use scanners operating in the transmissive mode). The resolution of the scanner must be sufficient so that the reaction area on the substrate is larger than a single pixel of the scanner. The scanner can be used with any substrate, provided that the detectable change produced by the assay can be observed against the substrate (*e.g.*, a gray spot, such as that produced by silver staining, can be observed against a white background, but cannot be observed against a grey background). The scanner can be a black-and-white scanner or, preferably, a color scanner. Most preferably, the scanner is a standard color scanner of the type used to scan documents into computers. Such scanners are inexpensive and readily available commercially. For instance, an Epson Expression 636 (600 x 600 dpi), a UMAX Astra 1200 (300 x 300 dpi), or a Microtec 1600 (1600 x 1600 dpi) can be used. The scanner is linked to a computer loaded with software for processing the images obtained by scanning the substrate. The software can be standard software which is readily available commercially, such as Adobe Photoshop 5.2 and Corel Photopaint 8.0. Using the software to calculate greyscale measurements provides a means of quantifying the results of the assays. The software can also provide a color number for colored spots and can generate images (*e.g.*, printouts) of the scans which can be reviewed to provide a qualitative determination of the presence of a nucleic acid, the quantity of a nucleic acid, or both. The computer can be a standard personal computer which is readily available commercially. Thus, the use of a standard scanner linked to a standard computer loaded with standard software can provide a convenient, easy, inexpensive means of detecting and quantifying nucleic acids when the assays are performed on substrates. The scans can also be stored in the computer to maintain a record of the results for further reference or use. Of course, more sophisticated instruments and software can be used, if desired.

In another embodiment of the invention, a method is provided for detecting for the presence or absence of one or more target analytes in a sample, each target analyte having at least two binding sites, the method comprising:

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providing one or more types of capture probes bound to a substrate, each type of capture probe comprising a specific binding complement to a first binding site of a specific target analyte;

providing one or more types of detection probes, each type of detection probe comprising a nanoparticle having oligonucleotides bound thereto, one or more specific binding complements to a second binding site of the specific target analyte, and one or more DNA barcodes that serve as a marker for the particular target analyte, wherein at least a portion of a sequence of the DNA barcodes is hybridized to at least some of the oligonucleotides bound to the nanoparticles

contacting the sample, the capture probe, and the detection probe under conditions effective to allow specific binding interactions between the target analyte and the probes and to form an aggregate complex in the presence of the target analyte;

washing the substrate to remove any unbound detection probes;

detecting for the presence or absence of the DNA barcode in any aggregate complex on the substrate, wherein the detection of the presence or absence of the DNA barcode is indicative of the presence or absence of the target analyte in the sample.

In one aspect of this embodiment of invention, the detection probe comprises (i) one or more specific binding complements to the second binding site of a specific target analyte, (ii) at least one type of oligonucleotides bound to the nanoparticle, and a DNA barcode having a predetermined sequence that is complementary to at least a portion of at least one type of oligonucleotides, the DNA barcode bound to each type of detection probe serving as a marker for a specific target analyte;

In another aspect of this embodiment, prior to said detecting step, the method further comprising the steps of:

subjecting the aggregate complex to conditions effective to dehybridize the complex and release the DNA barcodes; and

optionally amplifying the DNA barcode prior to said detecting.

In another aspect of the invention, the capture probe is bound to a magnetic substrate such as a magnetic particle, e.g., a polystyrene MMP with a magnetic iron oxide. This allows for facile removal of complexes from solution. DNA barcodes can then be detected directly using the substrate-based detection technique described above or indirectly by amplification followed by a detection technique. In the Examples below, a

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method based on oligonucleotide-modified nanoparticles (NPs), magnetic microparticles (MMPs), and the subsequent detection of barcode DNA that serve as amplifiers of one or more target nucleic acid sequences is described. Preferably, the oligonucleotide-modified nanoparticles comprise gold nanoparticles. The detection of the presence or absence of target DNA signal (via detection of bar-code DNA) is preferably performed using a substrate-based detection method as discussed above.

In one aspect, the invention provides a target nucleic acid amplification method that does not rely on PCR methods, and is based on oligonucleotide-modified nanoparticles (NPs), magnetic microparticles (MMPs), and detection of amplified target nucleic acid in the form of barcode DNA. In one embodiment of this aspect, the oligonucleotide-modified nanoparticles (NPs) comprise gold nanoparticles. In another embodiment of this aspect, the detection of the amplified DNA signal (bar-code DNA specific for a target sequence) is performed using a chip-based detection method. In another embodiment of this aspect, the barcode DNA comprises a sequence specific for each target nucleic acid molecule of interest, allowing for specific detection of multiple target nucleic acid sequences in a test solution.

In another aspection of the invention, the barcode DNA comprises a sequence specific for each particular target analyte of interest in a test sample, allowing for the detection of multiple specific targets in a single assay/test solution. As shown in the Examples, detection limits as low as about 500 zeptomolar (zM) can be achieved (the "zepto" order of magnitude is 10^{-21}; *e.g.*, 10 copies in an entire 20 μL sample). Such detection limits represent a significant increase in the sensitivity of PCR-less detection of target nucleic acid molecules.

In this aspect, two types of probes are provided for target DNA detection (DNA-BCA). In certain embodiments the first type of probe is a polystyrene MMP with a magnetic iron oxide core, functionalized with oligonucleotides that are complementary to at least a portion of a target sequence. The complementary portion of the oligonucleotides of the MMP can have various lengths, depending on the particular assay conditions (e.g., buffer system, target nucleic acid sequence, temperature, etc.).

In another embodiment, the second probe comprises a nanoparticle modified with two types of oligonucleotides, one that comprises a sequence that is complementary to at least a portion of a target sequence that is different from the region on the target that is

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recognized by the MMP; and the other comprises a sequence that is complementary to at least a portion of barcode DNA sequence, which barcode DNA provides a unique identification tag for the particular target sequence. In certain embodiments the nanoparticle is a gold nanoparticle. In further embodiments the gold nanoparticle comprises 13, 20, or 30 nm gold nanoparticles.

The ratio of barcode DNA to target binding sequence on the nanoparticle surface can be varied to suit each individual assay. In order to provide for PCR-less detection of barcode DNA, the ratio of barcode DNA to target binding DNA should be greater than 1:1, preferably at least about 25:1, more preferably at least about 50:1, most preferably at least about 100:1. The higher ratios provide for PCR-less target amplification because the barcode DNA, not the target sequence, is identified and detected in the DNA-BCA methods. For example, a 13 nm gold nanoparticle can accomodate at least 100 thiolated DNA strands per particle.[73] For 20 and 30 nm nanoparticles, assuming comparable oligonucleotide loading and a spherical shape for each particle, the particles can accommodate approximately 240 and 530 immobilized oligonucleotides, respectively.

In another aspect of this embodiment, the specific binding complement bound to the nanoparticle is a monoclonal or polyclonal antibody.

In another aspect of this embodiment, the specific binding complement bound to the capture probe is a monoclonal antibody.

In another aspect of this embodiment, the antibody is an anti-PSA antibody.

In another aspect of this embodiment, prior to said washing step, the method further comprises the step of:

isolating the aggregated complex prior to washing by subjecting the aggregated complex bound to the magnetic particle to a magnetic field.

In another aspect of this embodiment, the method further comprises the step of: subjecting the isolated aggregated complex to conditions effective to dehybridize the aggregated complex and release the DNA barcode.

In another aspect of this embodiment, the released DNA barcode is amplified by any suitable technique such as PCR.

In another aspect of this embodiment, the target analyte is a nucleic acid having at least two portions.

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In another aspect of this embodiment, the target analyte is a target nucleic acid having a sequence of at least two portions, the detection probe comprises a nanoparticle having at oligonucleotides having a sequence that is complementary to the DNA bar code, the specific binding complement of the detection probe comprising a first target recognition oligonucleotide having a sequence that is complementary to a first portion of the target nucleic acid, and the specific binding complement of the capture probes comprises second target recognition oligonucleotide having a sequence that is complementary to at least a second portion of the target nucleic acid.

In another aspect of this embodiment, the target analyte is a target nucleic acid having a sequence of at least two portions, the detection probe comprising a nanoparticle having oligonucleotides bound thereto, the DNA barcode having a sequence that is complementary to at least a portion of the oligonucleotides bound to the detection probe, the specific binding complement comprises a target recognition oligonucleotide having a sequence of at least first and second portions, the first portion is complementary to a first portion of the target nucleic acid and the second portion is complementary to a least a portion of the oligonucleotides bound to the nanoparticles, the specific binding complement of the substrate comprising a target recognition oligonucleotide having at least a portion that is complementary to a second portion of the target nucleic acid.

In another aspect of this embodiment, the detection probe comprises a dendrimeric nanoparticle as described above.

In yet another embodiment of this invention, a method is provided for detecting for the presence or absence of one or more target analytes in a sample, each target analyte having at least two binding sites, the method comprising:

providing one or more types of capture probes, each type of capture probe comprising (i) a magnetic particle; and (ii) a first member of a first specific binding pair attached to the magnetic particle, wherein the first member of the first specific binding pair binds to a first binding site of a specific target analyte;

providing one or more types of detection probe for each target analyte, each type of detection probe comprising (i) a nanoparticle; (ii) a first member of a second specific binding pair attached to the nanoparticle, wherein the first member of the second specific binding pair binds to a second binding site of the target analyte; (iii) at least one type of oligonucleotides bound to the nanoparticle; and (iv) at least one type of DNA barcodes,

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each type of DNA barcode having a predetermined sequence that is complementary to at least a portion of a specific type of oligonucleotides and serves as a marker for a specific target analyte;

contacting the sample with the capture probe and the detection probe under conditions effective to allow specific binding interactions between the target analyte and the probes and to form an aggregated complex bound to the magnetic particle in the presence of the target analyte;

washing any unbound detection probes from the magnetic particle; and

detecting for the presence or absence of the DNA barcodes in the complex, wherein the detection of the DNA barcode is indicative of the presence of the target analyte.

In one aspect of this embodiment, the method further comprises, prior to said detecting step, the steps of:

isolating the aggregated complex by applying a magnetic field;

subjecting the aggregated complex to conditions effective to dehybridize and release the DNA barcodes from the aggregated complex;

isolating the released DNA barcodes.

In another aspect of this embodiment, the method further comprises amplifying the released DNA barcodes.

In another aspect of this embodiment, the method further comprises:

providing a substrate having oligonucleotides bound thereto, the oligonucleotides having a sequence complementary to at least a portion of the sequence of the DNA barcode;

providing a nanoparticle comprising oligonucleotides bound thereto, wherein at least portion of the oligonucleotides bound to the nanoparticles have a sequence that is complementary to at least a portion of a DNA barcode; and

contacting the DNA barcodes, the oligonucleotides bound to the substrate, and the nanoparticles under conditions effective to allow for hybridization at least a first portion of the DNA barcodes with a complementary oligonucleotide bound to the substrate and a second portion of the DNA barcodes with some of the oligonucleotides bound to the nanoparticles.

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In another aspect of this embodiment, the DNA barcode is amplified by PCR prior to detection.

In another aspect of this embodiment, the method further comprises isolating the aggregated complexes prior to analyzing the aggregated complex.

In another aspect of this embodiment, the aggregated complex is isolated by applying a magnetic field to the aggregated complex.

In another aspect of this embodiment, the nanoparticles are metal nanoparticles such as gold nanoparticles or semiconductor nanoparticles.

In another aspect of this embodiment , the specific binding pair is an antibody and an antigen; a receptor and a ligand; an enzyme and a substrate; a drug and a target molecule; an aptamer and an aptamer target; two strands of at least partially complementary oligonucleotides.

In another aspect of this embodiment, the DNA barcode may be biotinylated, radioactively labeled, or fluorescently labeled.

In any of the embodiments, at least two types of particle complex probes are provided, the first type of probe having a specific binding complement to a first binding site on the target analyte and the second type of probe having a specific binding complement to a second binding site on the probe. A plurality of particle complex probes are provided, each type of probe having a specific binding complement to different binding sites on the target analyte.

The specific binding complement and the target analyte are members of a specific binding pair which comprise nucleic acid, oligonucleotide, peptide nucleic acid, polypeptide, antibody, antigen, carbohydrate, protein, peptide, amino acid, hormone, steroid, vitamin, drug, virus, polysaccharides, lipids, lipopolysaccharides, glycoproteins, lipoproteins, nucleoproteins, oligonucleotides, antibodies, immunoglobulins, albumin, hemoglobin, coagulation factors, peptide and protein hormones, non-peptide hormones, interleukins, interferons, cytokines, peptides comprising a tumor-specific epitope, cells, cell-surface molecules, microorganisms, fragments, portions, components or products of microorganisms, small organic molecules, nucleic acids and oligonucleotides, metabolites of or antibodies to any of the above substances.

The nucleic acid and oligonucleotide comprise genes, viral RNA and DNA, bacterial DNA, fungal DNA, mammalian DNA, cDNA, mRNA, RNA and DNA

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fragments, oligonucleotides, synthetic oligonucleotides, modified oligonucleotides, single-stranded and double-stranded nucleic acids, natural and synthetic nucleic acids, and aptamers.

The target analyte is a nucleic acid and the specific binding complement is an oligonucleotide. Alternatively, the target analyte is a protein or hapten and the specific binding complement is an antibody comprising a monoclonal or polyclonal antibody. Alternatively, the target analyte is a sequence from a genomic DNA sample and the specific binding complements are oligonucleotides, the oligonucleotides having a sequence that is complementary to at least a portion of the genomic sequence. The genomic DNA may be eukaryotic, bacterial, fungal or viral DNA.

The specific binding complement and the target analyte are members of an antibody-ligand pair.

In addition to its first binding site, the target analyte has been modified to include a second binding site.

The methods may further comprise a filtration step to remove aggregate complexes, wherein the filtration is performed prior to analyzing the aggregated complex. The filtration step comprises a membrane that removes sample components that do not comprise DNA barcodes.

In another embodiment of the invention, a method is provided for detecting for the presence or absence of one or more target analytes in a sample, the method comprises:

providing at least one or more types of particle complex probes, each type of probe comprising oligonucleotides bound thereto, one or more specific binding complements of a specific target analyte, and one or more DNA barcodes that serves as a marker for the particular target analyte, wherein at least a portion of a sequence of the DNA barcodes is hybridized to at least some of the oligonucleotides bound to the nanoparticles;

contacting the sample with the particle complex probes under conditions effective to allow specific binding interactions between the target analytes and the particle complex probes and to form an aggregate complex in the presence of a target analyte; and

observing whether aggregate complex formation occurred.

In this aspect of the invention, the observable signal can be detected using any method described herein. For example, direct detection of barcode DNAs, detection of

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*B*PCR-amplified barcode DNAs, detection of aggregation of the one or more types of detection probe (e.g., by visual inspection, fluorescence, colorimetric, electrochemistry, electronic, densitometry, radioactivity, and the like), or by using reporter nucleotides that comprise a sequence that is complementary to at least a portion of the barcode DNAs and a detectable signal moiety (e.g., fluorescent label).

If sufficient complex is present, the complex can be observed visually with or without a background substrate. Any substrate can be used which allows observation of the detectable change. Suitable substrates include transparent solid surfaces (*e.g.*, glass, quartz, plastics and other polymers), opaque solid surface (*e.g.*, white solid surfaces, such as TLC silica plates, filter paper, glass fiber filters, cellulose nitrate membranes, nylon membranes), and conducting solid surfaces (*e.g.*, indium-tin-oxide (ITO)). The substrate can be any shape or thickness, but generally will be flat and thin. Preferred are transparent substrates such as glass (*e.g.*, glass slides) or plastics (*e.g.*, wells of microtiter plates).

In one aspect of the invention, a method for detecting for the presence of a target analyte, e.g., an antibody, in a sample is provided. An antibody such as immunoglobulin E (IgE) or immunoglobulin G1 (IgG1) shown in the Examples below can be detected with olignucleotide-modified probes prehybridized with oligonucleotide strands modified with the appropriate hapten (biotin in the case of IgG1 and dinitropheny1 (DNP) in the case of IgE; Figure 1A). [13,14] The DNA sequences in the proof-of-concept assays presented in the Examples below were designed in a way that would ensure that the two different aggregates formed from the probe reactions with IgG1 and IgE would melt at different temperatures, Figure 1B. The probes for IgG1 have longer sequences and greater G,C base contents than those for IgE. Therefore, the former sequences melt at a higher temperature than the latter ones. These sequence variations allow one to prepare probes with distinct melting signatures that can be used as codes to identify which targets have reacted with them to form nanoparticle aggregates. Three different systems have been studied: (1) two probes with one target antibody present (IgG1 or IgE); (2) two probes with the two different target antibodies present, and (3) a control where no target antibodies are present.

In this aspect of the invention, a method is provided for detecting the presence of a target analyte, e.g., an antibody, in a sample comprises contacting a nanoparticle probe

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having oligonucleotides bound thereto with a sample which may contain a target analyte. At least some of the oligonucleotides attached to the nanoparticle are bound to a first portion of a reporter oligonucleotide as a result of hybridization. A second portion of the reporter oligonucleotide is bound, as a result of hybridization, to an oligonucleotide having bound thereto a specific binding complement (e.g., antigen) to the analyte. The contacting takes place under conditions effective to allow specific binding interactions between the analyte and the nanoparticle probe. In the presence of target analyte, nanoparticle aggregates are produced. These aggregates may be detected by any suitable means.

In another aspect of the invention, particle complex probes, preferably nanoparticle complex probes, are used. These particle complexes may be generated prior to conducting the actual assay or in situ while conducting the assay. These complexes comprise a particle, preferably a nanoparticle, having oligonucleotides bound thereto, a reporter oligonucleotide bound to at least a portion of the oligonucleotides bound to the nanoparticle, and a specific binding complement of the target analyte. The specific binding complement may be directly or indirectly bound to the nanoparticle. For instance, the specific binding complement can be bound to a linker or oligonucleotide and the labeled linker or oligonucleotide is then bound to the nanoparticle. In one embodiment, the DNA barcode or reporter oligonucleotides has a sequence having at least two portions and joins via hybridization the nanoparticle having oligonucleotides bound thereto and the oligonucleotide having bound thereto the specific binding complement. The oligonucleotides bound to the nanoparticles have a sequence that is complementary to one portion of the reporter oligonucleotide and the oligonucleotide having bound thereto the specific binding complement having a sequence that is complementary to a second portion of the reporter oligonucleotide. The reporter oligonucleotides have at least two portions and joins via hybridization the nanoparticle having oligonucleotides bound thereto and the oligonucleotide having bound thereto the specific binding complement. When employed in a sample containing the target analyte, the nanoparticle complex binds to the target analyte and aggregation occurs. The aggregates may be isolated and subject to further melting analysis to identify the particular target analyte where multiple targets are present as discussed above. Alternatively, the aggregates can be dehybridized to release the reporter oligonucleotides.

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These reporter oligonucleotides, or DNA barcode can optionally be amplified, and then be detected by any suitable DNA detection system using any suitable detection probes.

In practicing the invention, a nanoparticle complex probes are prepared by hybridizing the nanoparticles having oligonucleotides bound thereto with an oligonucleotide modified with a specific binding complement to a target analyte, and a reporter oligonucleotide. At least some of the oligonucleotides attached to the nanoparticle have a sequence that is complementary to a first portion of a reporter oligonucleotide. The oligonucleotides having bound thereto a specific binding complement have a sequence that is complementary to a second portion of a reporter oligonucleotide. The reporter oligonucleotide hybridizes to the at least some of the oligonucleotides attached to the nanoparticle and to the oligonucleotides having bound thereto the specific binding complement, forming the nanoparticle complex probe under conditions sufficient to allow for hybridization between the components. Any suitable solvent medium and hybridization conditions may be employed in preparing the nanoparticle complex solution that allows for sufficient hybridization of the components. Preferably, the components are hybridized in a phosphate buffered solution (PBS) comprised of 0.3 M NaCl and 10 mM phosphate buffer (pH 7) at room temperature for about 2-3 hours. The concentration of nanoparticle-oligonucleotide conjugates in the hybridization mixture range between about 2 nM and about 50 nM, preferably about 13 nM. The concentration of hapten-modified oligonucleotides generally ranges between about 50 and about 900, preferably about 300 nM. The concentration of reporter oligonucleotide generally ranges between about 50 and about 900, preferably about 300 nM. Unreacted hapten-modified oligonucleotide and reporter oligonucleotides may be optionally, but preferably, removed by any suitable means, preferably via centrifugation (12,000 rpm, 20 minutes) of the hybridization mixture and subsequent decanting of the supernatant. The prepared complexes were stored in 0.3 M NaCl and 10 mM phosphate buffer (pH 7-7.4), 0.01% azide solution at 4-6 °C.

A typical assay for detecting the presence of a target analyte, e.g, antibody, in a sample is as follows: a solution containing nanoparticle complex probe comprising nanoparticles having oligonucleotides bound thereto, a reporter oligonucleotide, and an oligonucleotide having a specific binding complement to the target analyte, is admixed with an aqueous sample solution believed to contain target protein. The total protein

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content in the aqueous sample solution generally ranges between about 5 and about 100, usually about 43 ug/ml. The concentration of nanoparticles in the reaction mixture generally ranges between about 2 nM and about 20 nM, usually about ~13 nM. The total volume of the resulting mixture generally ranges between about 100 uL and about 1000 uL, preferably about 400 uL. Any suitable solvent may be employed in preparing the aqueous sample solution believed to contain target analyte, preferably PBS comprising 0.3 M NaCl and 10 mM phosphate buffer (pH 7-7.4).

The resulting assay mixture is then incubated at a temperature ranging between about 35 and about 40°C, preferably at 37 °C, for a time ranging between about 30 and about 60, preferably about 50 minutes, sufficient to facilitate specific binding pair, e.g., protein-hapten, complexation. If the target protein is present, particle aggregation takes place effecting a shift in the gold nanoparticle plasmon band and a red-to-purple color change along with precipitation. The hybridized products are centrifuged (e.g., 3000 rpm for 2 minutes), and the supernatant containing unreacted elements are decanted prior to analysis.

If desired, the nanoparticle complex probe may be prepared in situ within the assay mixture by admixing all the nanoparticles having oligonucleotides bound thereto, the reporter oligonucleotide, and the hapten-modified oligonucleotide with the sample suspected of containing a target analyte. To ensure complete hybridization among all the components, especially the complementary DNA strands, the assay mixture may be incubated to expedite hybridization at –15 °C for 20 minutes (Boekel Tropicooler Hot/Cold Block Incubator) and stored at 4 °C for 24 hours. In practicing the invention, however, it is preferred that the nanoparticle complex probe is prepared prior to conducting the assay reaction to increase the amount of DNA barcode within the nanoparticle complex probe.

To determine which proteins are present, a melting analysis of the aggregates which monitors the extinction at 260 nm as a function of temperature may carried out in the solution. See, for instance, Figure 2 in Example 3 which describes analysis of a sample containing one or two known target analytes: IgG1 and IgE. As discussed in Example 3, when IgG1 is treated with the probes via the aforementioned protocol, the solution turns pinkish-blue, indicating the formation of nanoparticle aggregates. In a control experiment where no target but background proteins are present, there is no

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discernible precipitation. A melting analysis of the solution shows a sharp transition with a melting temperature (Tm) of 55 °C. This is the expected transition for the IgG1 target, Figure 2A (---). If IgE is added to a fresh solution of probes, the same color change is observed but the melting analysis provides a curve with a Tm of 36 °C, the expected transition for this target, Figure 2A (——). Significantly, when both protein targets are added to the solution of probes, the solution turns dark purple, and the melting analysis exhibits two distinct transactions. The first derivative of this curve shows two peaks centered at 36 and 55 °C, respectively, Figure 2B. This demonstrates that two distinct assemblies form and their melting properties, which derive from the oligonucleotide barcodes, can be used to distinguish two protein targets.

In another aspect of the invention, a variation of the above aggregation method strategy can be used to increase the sensitivity of the aforementioned system and to increase the number of targets that can be interrogated in one solution. See, for instance, Figure 3 in Example 4. With this strategy, the protein targets can be detected indirectly via the DNA biobarcodes or unique reporter oligonucleotides assigned to specific target analytes. Generally, the suitable length, GC content, and sequence, and selection of the reporter oligonucleotide for the target analyte is predetermined prior to the assay. For instance, a 12-mer oligonucleotide has 4^{12} different sequences, many of which can be used to prepare a barcode for a polyvalent protein of interest as shown in Figure 1A. In this variation of the assay, the melting properties of the aggregates that form are not measured in solution but rather the reporter oligonucleotides or DNA biobarcodes within the aggregates are separated via centrifugation (e.g., 3000 rpm for 2 minutes) from the unreacted probes and target molecules. The aggregates are then denatured by any suitable means, e.g., by adding water to the solution, to free the reporter oligonucleotides or biobarcodes. If the reporter oligonucleotide is present in small amounts, it may be amplified by methods known in the art. See, *e.g.*, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (2nd ed. 1989) and B.D. Hames and S.J. Higgins, Eds., *Gene Probes 1* (IRL Press, New York, 1995). Preferred is polymerase chain reaction (PCR) amplification. The particles and proteins can be separated from the reporter oligonucleotides by any suitable means, e.g., a centrifugal filter device (Millipore Microcon YM-100, 3500 rpm for 25 min. Once the reporter oligonucleotides are isolated, they can be captured on an oligonucleotide array and can be identified using one of the

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many suitable DNA detection assays (Figure 3). For the examples described herein involving IgG1 and IgE, the reporter oligonucleotides are captured on a microscope slide that has been functionalized with oligonucleotides (250 μm diameter spots) that are complementary to one half of the barcode of interest (**A3** and **B3** in Figure 1). If the barcode is captured by the oligonucleotide array, a DNA-modified particle that is complementary to the remaining portion of the barcode can be hybridized to the array (see experimental section). When developed via the standard scanometric approach [11] (which involves treatment with photographic developing solution), a flat bed scanner can be used to quantify the results, Figure 4.[11] If IgG1 is present, only the spot designed for IgG1 shows measurable signal. Similarly if IgE is the only protein present, the spot designed for it only exhibits signal. Finally, if both proteins are present, both spots exhibit intense signals.

In one aspect of this embodiment, the DNA barcode in each type of particle complex probe has a sequence that is different and that serves as an identifier for a particular target analyte.

In another aspect of this embodiment, the method further comprises the steps of:

isolating aggregated complexes; and

analyzing the aggregated complexes to determine the presence of one or more DNA barcodes having different sequences.

In another aspect of this embodiment, the method further comprises the steps of:

isolating the aggregated complex;

subjecting the aggregated complex to conditions effective to dehybridize the aggregated complex and release the DNA barcode;

isolating the DNA barcode; and

detecting for the presence of one or more DNA barcodes having different sequences, wherein each DNA barcode is indicative of the presence of a specific target analyte in the sample.

In another aspect of this embodiment, the method further comprises the steps of:

isolating the aggregated complex;

subjecting the aggregated complex to conditions effective to dehybridize the aggregated complex and release the DNA barcode;

isolating the DNA barcode;

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amplifying the isolated DNA barcode; and

detecting for the presence of one or more amplified DNA barcodes having different sequences, wherein each DNA barcode is indicative of the presence of a specific target analyte in the sample.

In another aspect of this embodiment, target has more than two binding sites and at least two types of particle complex probes are provided, the first type of probe having a specific binding complement to a first binding site on the target analyte and the second type of probe having a specific binding complement to a second binding site on the probe. A plurality of particle complex probes may be provided, each type of probe having a specific binding complement to different binding sites on the target analyte.

In another aspect of this embodiment, the specific binding complement and the target analyte are members of a specific binding pair comprising nucleic acid, oligonucleotide, peptide nucleic acid, polypeptide, antibody, antigen, carbohydrate, protein, peptide, amino acid, hormone, steroid, vitamin, drug, virus, polysaccharides, lipids, lipopolysaccharides, glycoproteins, lipoproteins, nucleoproteins, oligonucleotides, antibodies, immunoglobulins, albumin, hemoglobin, coagulation factors, peptide and protein hormones, non-peptide hormones, interleukins, interferons, cytokines, peptides comprising a tumor-specific epitope, cells, cell-surface molecules, microorganisms, fragments, portions, components or products of microorganisms, small organic molecules, nucleic acids and oligonucleotides, metabolites of or antibodies to any of the above substances.

The nucleic acid and oligonucleotide comprise genes, viral RNA and DNA, bacterial DNA, fungal DNA, mammalian DNA, cDNA, mRNA, RNA and DNA fragments, oligonucleotides, synthetic oligonucleotides, modified oligonucleotides, single-stranded and double-stranded nucleic acids, natural and synthetic nucleic acids, and aptamers.

In one aspect, the target analytes may be a nucleic acid and the specific binding complement may be an oligonucleotide. Alternatively, the target analyte may be a protein or hapten and the specific binding complement may be an antibody comprising a monoclonal or polyclonal antibody.

In another aspect of this embodiment, the target analyte may be a sequence from a genomic DNA sample and the specific binding complements are oligonucleotides, the

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oligonucleotides having a sequence that is complementary to at least a portion of the genomic sequence.

In another aspect of this embodiment, the genomic DNA may be eukaryotic, bacterial, fungal or viral DNA.

In another aspect, the specific binding complement and the target analyte are members of an antibody-ligand pair.

In another aspect of this embodiment, detecting step for the presence of one or more DNA barcodes comprises:

providing a substrate having oligonucleotides bound thereto, the oligonucleotides having a sequence complementary to at least a portion of the sequence of the DNA barcode;

providing a nanoparticle comprising oligonucleotides bound thereto, wherein at least portion of the oligonucleotides bound to the nanoparticles have a sequence that is complementary to at least a portion of a DNA barcode; and

contacting the DNA barcodes, the oligonucleotides bound to the substrate, and the nanoparticles under conditions effective to allow for hybridization at least a first portion of the DNA barcodes with a complementary oligonucleotide bound to the substrate and a second portion of the DNA barcodes with some of the oligonucleotides bound to the nanoparticles; and

observing a detectable change.

In another aspect of this embodiment, substrate comprises a plurality of types of oligonucleotides attached thereto in an array to allow for the detection of one or more different types of DNA barcodes.

In another aspect of this embodiment, the detectable change is the formation of dark areas on the substrate.

In another aspect of this embodiment, the detectable change is observed with an optical scanner.

In another aspect of this embodiment, the substrate is contacted with a silver stain to produce the detectable change.

In another aspect of this embodiment, the DNA barcodes are contacted with the substrate under conditions effective to allow the DNA barcodes to hybridize with complementary oligonucleotides bound to the substrate and subsequently contacting the

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DNA barcodes bound to the substrate with the nanoparticles having oligonucleotides bound thereto under conditions effective to allow at least some of the oligonucleotides bound to the nanoparticles to hybridize with a portion of the sequence of the DNA barcodes on the substrate.

In another aspect of this embodiment, the DNA barcodes are contacted with the nanoparticles having oligonucleotides bound thereto under conditions effective to allow the DNA barcodes to hybridize with at least some of the oligonucleotides bound to the nanoparticles; and subsequently contacting the DNA barcodes bound to the nanoparticles with the substrate under conditions effective to allow at least a portion of the sequence of the DNA barcodes bound to the nanoparticles to hybridize with complementary oligonucleotides bound to the substrate.

In another aspect of this embodiment, the DNA barcode is amplified prior to the contacting step.

In another aspect of this embodiment, at least two types of particle complex probes are provided, a first type of probe having a specific binding complement to a first binding site of the target analyte and a second type of probe having a specific binding complement to a second binding site of the target analyte.

As discussedf above, the nucleic acid and oligonucleotide comprise genes, viral RNA and DNA, bacterial DNA, fungal DNA, mammalian DNA, cDNA, mRNA, RNA and DNA fragments, oligonucleotides, synthetic oligonucleotides, modified oligonucleotides, single-stranded and double-stranded nucleic acids, natural and synthetic nucleic acids, and aptamers.

In another embodiment of the invention, kits are provided which comprise the particle complex probe described above.

In another aspect of this embodiment, a kit is provided for detecting for the presence or absence of one or more target analytes in a sample, each target analyte having at least two binding sites. The kit comprising:

at least one type of detection probe for each target analyte, each type of detection probe comprising (i) a nanoparticle; (ii) a member of a specific binding pair bound to the nanoparticle; (iii) oligonucleotides bound to the nanoparticle; and (iv) a DNA barcode having a predetermined sequence that is complementary to a least a portion of the oligonucleotides.

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In another aspect of this embodiment, the kit comprises:

at least one type of capture probe comprising (i) a substrate; (ii) a first member of a first specific binding pair attached to the substrate, wherein the first member of the fist specific binding pair binds to a first binding site of the target analyte;

at least one type of detection probe comprising (i) a nanoparticle; (ii) a first member of a second specific binding pair attached to the nanoparticle, wherein the first member of the second specific binding pair binds to a second binding site of the target analyte; (iii) at least one type of oligonucleotides bound to the nanoparticle; and (iv) at least one type of DNA barcodes, each type having a predetermined sequence that is complementary to at least a portion of a specific type of oligonucleotides.

Any suitable substrate can be used in the kit. Preferably, the substrate is magnetic such as a magnetic microparticle.

In another aspect of this embodiment, the kit comprises:

at least one type of capture probe comprising (i) a magnetic particle; (ii) a first member of a first specific binding pair attached to the magnetic particle, wherein the first member of the first specific binding pair binds to a first binding site of the target analyte;

at least one type of detection probe comprising (i) a nanoparticle; (ii) a first member of a second specific binding pair attached to the nanoparticle, wherein the first member of the second specific binding pair binds to a second binding site of the target analyte; (iii) at least one type of oligonucleotides bound to the nanoparticle; and (iv) at least one type of DNA barcodes, each type having a predetermined sequence that is complementary to at least a portion of a specific type of oligonucleotides.

In another aspect of this embodiment, the kit comprises:

at least one container including particle complex probes comprising a particle having oligonucleotides bound thereto, a DNA barcode, and an oligonucleotide having bound thereto a specific binding complement to a target analyte, wherein the DNA barcode has a sequence having at least two portions, at least some of the oligonucleotides attached to the particle have a sequence that is complementary to a first portion of a DNA barcode, the oligonucleotides having bound thereto a specific binding complement have a sequence that is complementary to a second portion of a DNA barcode, and wherein the DNA barcode is hybridized to at least to some of the oligonucleotides attached to the

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particle and to the oligonucleotides having bound thereto the specific binding complement, and an optional substrate for observing a detectable change.

In another aspect of this embodiment, the kit comprises:

at least one or more containers, container holds a type of particle complex probe comprising a particle having oligonucleotides bound thereto, a DNA barcode, and an oligonucleotide having bound thereto a specific binding complement to a specific target analyte, wherein (i) the DNA barcode has a sequence having at least two portions, (ii) at least some of the oligonucleotides attached to the particle have a sequence that is complementary to a first portion of a DNA barcode,(iii) the oligonucleotides having bound thereto a specific binding complement have a sequence that is complementary to a second portion of a DNA barcode, and (iv) the DNA barcode in each type of particle complex probe has a sequence that is different and that serves as an identifier for a particular target analyte; wherein the kit optionally includes a substrate for observing a detectable change.

In another aspect of this embodiment, the kit comprises:

at least one pair of containers and an optional substrate for observing a detectable change,

the first container of the pair includes particle probe comprising a particle having oligonucleotides bound thereto and a DNA barcode having a sequence of at least two portions, wherein at least some of the oligonucleotides attached to the particle have a sequence that is complementary to a first portion of a DNA barcode;

the second container of the pair includes an oligonucleotide having a sequence that is complementary to a second portion of the DNA barcode, the oligonucleotide having a moiety that can be used to covalently link a specific binding pair complement of a target analyte.

In another aspect of this embodiment, the kit comprises:

at least two or more pairs of containers,

the first container of each pair includes particle complex probes having particles having oligonucleotides bound thereto and a DNA barcode having a sequence of at least two portions, wherein at least some of the oligonucleotides bound to the particles have a sequence that is complementary to a first portion of a DNA barcode having at least two portions; and

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the second container of each pair contains an oligonucleotide having a sequence that is complementary to a second portion of the DNA barcode, the oligonucleotide having a moiety that can be used to covalently link a specific binding pair complement of a target analyte,

wherein the DNA barcode for type of particle complex probe has a sequence that is different and that serves as an identifier for a target analyte and wherein the kit optionally include a substrate for observing a detectable change.

In another aspect of this embodiment, the kit comprises:

a first container and at least two or more pairs of containers,

the first container includes particle complex probes having particles having oligonucleotides bound thereto;

the first container of the pair includes a DNA barcode having a sequence of at least two portions, wherein at least some of the oligonucleotides bound to the particles have a sequence that is complementary to a first portion of the DNA barcode; and

the second container of each pair contains an oligonucleotide having a sequence that is complementary to a second portion of the DNA barcode, the oligonucleotide having a moiety that can be used to covalently link a specific binding pair complement of a target analyte,

wherein the DNA barcode present in the first container of each pair of containers serves as an identifier for a target analyte and has a sequence that is different from a DNA barcode in another pair of containers, and wherein the kite optionally include a substrate for observing a detectable change.

In another aspect of this embodiment, the kit comprises:

an oligonucleotide sequence that serves as an identifier for the presence of a specific target analyte.

The above kits can include instructions for assembling the assay and for conducting the assay.

It is to be noted that the term "a" or "an" entity refers to one or more of that entity. For example, "a characteristic" refers to one or more characteristics or at least one characteristic. As such, the terms "a" (or "an"), "one or more" and "at least one" are used interchangeably herein. It is also to be noted that the terms "comprising", "including", and "having" have been used interchangeably. The following examples are intended

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for illustration purposes only, and should not be construed as limiting the spirit or scope of the invention in any way.

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EXAMPLES

Example 1: Preparation of Oligonucleotide-Modified Gold Nanoparticles

A. Preparation Of Gold Nanoparticles

Oligonucleotide-modified 13 nm Au particles were prepared by literature methods (~110 oligonucleotides/particle)[18-20]. Gold colloids (13 nm diameter) were prepared by reduction of $HAuCl_4$ with citrate as described in Frens, *Nature Phys. Sci.*, **241**, 20 (1973) and Grabar, *Anal. Chem.*, **67**, 735 (1995). Briefly, all glassware was cleaned in aqua regia (3 parts HCl, 1 part HNO_3), rinsed with Nanopure H_2O, then oven dried prior to use. $HAuCl_4$ and sodium citrate were purchased from Aldrich Chemical Company. An aqueous solution of $HAuCl_4$ (1 mM, 500 mL) was brought to a reflux while stirring, and then 50 mL of a 38.8 mM trisodium citrate solution was added quickly, which resulted in a change in solution color from pale yellow to deep red. After the color change, the solution was refluxed for an additional fifteen minutes, allowed to cool to room temperature, and subsequently filtered through a Micron Separations Inc. 0.45 micron nylon filter. Au colloids were characterized by UV-vis spectroscopy using a Hewlett Packard 8452A diode array spectrophotometer and by Transmission Electron Microscopy (TEM) using a Hitachi 8100 transmission electron microscope. A typical solution of 13 nm diameter gold particles exhibited a characteristic surface plasmon band centered at 518 - 520 nm. Gold particles with diameters of 13 nm will produce a visible color change when aggregated with target and probe oligonucleotide sequences in the 10-72 nucleotide range.

B. Synthesis Of Oligonucleotides

Oligonucleotides were synthesized on a 1 micromole scale using a Milligene Expedite DNA synthesizer in single column mode using phosphoramidite chemistry. Eckstein, F. (ed.) *Oligonucleotides and Analogues: A Practical Approach* (IRL Press, Oxford, 1991). All solutions were purchased from Milligene (DNA synthesis grade). Average coupling efficiency varied from 98 to 99.8%, and the final dimethoxytrityl (DMT) protecting group was not cleaved from the oligonucleotides to aid in purification.

For 3'-thiol-oligonucleotides, Thiol-Modifier C3 S-S CPG support was purchased from Glen Research and used in the automated synthesizer. The final dimethoxytrityl

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(DMT) protecting group was not removed to aid in purification. After synthesis, the supported oligonucleotide was placed in 1 mL of concentrated ammonium hydroxide for 16 hours at 55 °C to cleave the oligonucleotide from the solid support and remove the protecting groups from the bases.

After evaporation of the ammonia, the oligonucleotides were purified by preparative reverse-phase HPLC using an HP ODS Hypersil column (5 um, 250 x 4 mm) with 0.03 M triethyl ammonium acetate (TEAA), pH 7 and a 1%/minute gradient of 95% CH_3CN/5% 0.03 M TEAA at a flow rate of 1 mL/minute, while monitoring the UV signal of DNA at 254 nm. The retention time of the DMT protected modified 12-base oligomer was 30 minutes. The DMT was subsequently cleaved by soaking the purified oligonucleotide in an 80 % acetic acid solution for 30 minutes, followed by evaporation; the oligonucleotide was redispersed in 500 uL of water, and the solution was extracted with ethyl acetate (3 x 300 uL). After evaporation of the solvent, the oligonucleotide (10 OD's) was redispersed in 100 uL of a 0.04 M DTT, 0.17 M phosphate buffer (pH 8) solution overnight at 50 °C to cleave the 3' disulfide. Aliquots of this solution (< 10 OD's) were purified through a desalting NAP-5 column. The amount of oligonucleotide was determined by absorbance at 260 nm. Purity was assessed by ion-exchange HPLC using a Dionex Nucleopac PA-100 column (250 x 4 mm) with 10 mM NaOH (pH 12) and a 2%/minute gradient of 10 mM NaOH, 1 M NaCl at a flow rate of 1 mL/minute while monitoring the UV signal of DNA at 254 nm. Three peaks with retention times (T_r) of 18.5, 18.9 and 22 minutes were observed. The main single peak at T_r = 22.0 minutes, which has been attributed to the disulfide, was 79 % by area. The two peaks with shorter retention times of 18.5 and 18.9 minutes were ~9 % and 12 % by area respectively, and have been attributed to oxidized impurity and residual thiol oligonucleotide.

5'-Alkylthiol modified oligonucleotides were prepared using the following protocol: 1) a CPG-bound, detritylated oligonucleotide was synthesized on an automated DNA synthesizer (Expedite) using standard procedures; 2) the CPG-cartridge was removed and disposable syringes were attached to the ends; 3) 200 uL of a solution containing 20 umole of 5-Thiol-Modifier C6-phosphoramidite (Glen Research) in dry acetonitrile was mixed with 200 uL of standard "tetrazole activator solution" and, *via* one of the syringes, introduced into the cartridge containing the oligonucleotide-CPG; 4) the solution was slowly pumped back and forth through the cartridge for 10 minutes and then

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ejected followed by washing with dry acetonitrile (2 x 1 mL); 5) the intermediate phosphite was oxidized with 700 uL of 0.02 M iodine in THF/pyridine/water (30 seconds) followed by washing with acetonitrile/pyridine (1:1; 2 x 1 mL) and dry acetonitirile. The trityloligonucleotide derivative was then isolated and purified as described by the 3'-alkylthiol oligonucleotides; then the trityl protecting group was cleaved by adding 15 uL (for 10 OD's) of a 50 mM $AgNO_3$ solution to the dry oligonucleotide sample for 20 minutes, which resulted in a milky white suspension. The excess silver nitrate was removed by adding 20 uL of a 10 mg/mL solution of DTT (five minute reaction time), which immediately formed a yellow precipitate that was removed by centrifugation. Aliquots of the oligonucleotide solution (< 10 OD's) were then transferred onto a desalting NAP-5 column for purification. The final amount and the purity of the resulting 5' alkylthiol oligonucleotides were assessed using the techniques described above for 3' alkylthiol oligonucleotides. Two major peaks were observed by ion-exchange HPLC with retention times of 19.8 minutes (thiol peak, 16 % by area) and 23.5 minutes (disulfide peak, 82 % by area).

C. Attachment Of Oligonucleotides To Gold Nanoparticles

A 1 mL solution of the gold colloids (17nM) in water was mixed with excess (3.68 uM) thiol-oligonucleotide (22 bases in length) in water, and the mixture was allowed to stand for 12-24 hours at room temperature. Then, the solution was brought to 0.1 M NaCl, 10 mM phosphate buffer (pH 7) and allowed to stand for 40 hours. This "aging" step was designed to increase the surface coverage by the thiol-oligonucleotides and to displace oligonucleotide bases from the gold surface. The solution was next centrifuged at 14,000 rpm in an Eppendorf Centrifuge 5414 for about 25 minutes to give a very pale pink supernatant containing most of the oligonucleotide (as indicated by the absorbance at 260 nm) along with 7-10% of the colloidal gold (as indicated by the absorbance at 520 nm), and a compact, dark, gelatinous residue at the bottom of the tube. The supernatant was removed, and the residue was resuspended in about 200 μL of buffer (10 mM phosphate, 0.1 M NaCl) and recentrifuged. After removal of the supernatant solution, the residue was taken up in 1.0 mL of buffer (10 mM phosphate, 0.3 M NaCl, 0.01% NaN_3). The resulting red master solution was stable (*i.e.*, remained red and did not aggregate) on standing for months at room temperature, on spotting on silica thin-layer

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chromatography (TLC) plates (see Example 4), and on addition to 1 M NaCl, 10 mM $MgCl_2$, or solutions containing high concentrations of salmon sperm DNA.

Example 2: Preparation of Hapten-modified oligonucleotides

Hapten-modified oligonucleotides were prepared with a biotin-triethylene glycol phosphoramidite for **A1** and 2, 4-dinitrophenyl-triethylene glycol phosphoramidite for **B1** (Glen research) using standard solid-phase DNA synthesis procedures.[21]

Biotin modified oligonucleotides were prepared using the following protocol: A CPG-bound, detritylated oligonucleotide was synthesized on an automated DNA synthesizer (Expedite) using standard procedures[21]. The CPG-cartridge was then removed and disposable syringes were attached to the ends. 200 uL of a solution containing 20 umole of biotin-triethylene glycol phosphoramidite in dry acetonitrile was then mixed with 200 uL of standard "tetrazole activator solution" and, *via* one of the syringes, introduced into the cartridge containing the oligonucleotide-CPG. The solution then was slowly pumped back and forth through the cartridge for 10 minutes and then ejected followed by washing with dry acetonitrile (2 x 1 mL). Thereafter, the intermediate phosphite was oxidized with 700 uL of 0.02 M iodine in THF/pyridine/ water (30 seconds) followed by washing with acetonitrile/pyridine (1:1; 2 x 1 mL) and dry acetonitirile with subsequent drying of the column with a stream of nitrogen. The trityl protecting group was not removed, which aids in purification. The supported oligonucleotide was placed in 1 mL of concentrated ammonium hydroxide for 16 hours at 55 °C to cleave the oligonucleotide from the solid support and remove the protecting groups from the bases. After evaporation of the ammonia, the oligonucleotides were purified by preparative reverse-phase HPLC using an HP ODS Hypersil column (5 um, 250 x 4 mm) with 0.03 M triethyl ammonium acetate (TEAA), pH 7 and a 1%/minute gradient of 95% CH_3CN/5% 0.03 M TEAA at a flow rate of 1 mL/minute, while monitoring the UV signal of DNA at 254 nm. The retention time of the DMT protected oligonucleotides was ~32 minutes. The DMT was subsequently cleaved by soaking the purified oligonucleotide in an 80% acetic acid solution for 30 minutes, followed by evaporation; the oligonucleotide was redispersed in 500 uL of water, and the solution was extracted with ethyl acetate (3 x 300 uL) and dried. The same protocol was used to

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synthesize DNP modified oligonucleotide using 2, 4-dinitrophenyl-triethylene glycol phosphoramidite.

Example 3: Assay Using Nanoparticle Complex Probes

The Oligonucleotide-modified 13 nm gold particles were prepared as described in Example 1. Hapten-modified oligonucleotides were prepared as described in Example 2 with a biotin-triethylene glycol phosphoramidite for **A1** and 2, 4-dinitrophenyl-triethylene glycol phosphoramidite for **B1** (Glen research) using standard solid-phase DNA synthesis procedures.[21] The PBS buffer solution used in this research consists of 0.3 M NaCl and 10 mM phosphate buffer (pH 7). IgE and IgG1 were purchased from Sigma Aldrich (Milwaukee, WI) and dissolved in 0.3 M PBS buffer with 0.05% Tween 20 (final concentration: 4.3×10^{-8} b/μl) and background proteins (10 ug/ml of lysozyme, 1% bovine serum albumin, and 5.3 ug/ml of anti-digoxin; 10 uL of each) prior to use.

To prepare the probes, the oligonucleotide modified particles (13 nM, 300 μL) were hybridized with hapten-modified complementary oligonucleotides (10 μL of 10 μM) and biobarcode DNA (10 μL of 10 μM) at room temperature for 2-3 h, sequences given in Figure 1. Unreacted hapten-modified oligonucleotide and biobarcodes were removed via centrifugation (12,000 rpm, 20 min) of the nanoparticle probes and subsequent decanting of the supernatant.

In a typical assay for IgE and/or IgG1, the target proteins (40 μl of 43 μg/ml for each) were added to the solution containing the probes (~13 nM), and the mixture was incubated at 37 °C for 50 minutes to facilitate protein-hapten complexation. To ensure complete reaction among all the components, especially the complementary DNA strands, the solution was incubated to expedite hybridization at –15 °C for 20 minutes (Boekel Tropicooler Hot/Cold Block Incubator) and stored at 4 °C for 24 hours. If the target protein is present, particle aggregation takes place affecting a shift in the gold nanoparticle plasmon band and a red-to-purple color change along with precipitation. The hybridized products were centrifuged (3000 rpm for 2 minutes), and the supernatant containing unreacted elements was decanted prior to analysis. To determine which proteins are present, a melting analysis, which monitors the extinction at 260 nm as a function of temperature is carried out in the solution, Figure 2. When IgG1 is treated with the probes via the aforementioned protocol, the solution turns pinkish-blue, indicating the

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formation of nanoparticle aggregates. In a control experiment where no target but background proteins are present, there is no discernible precipitation. A melting analysis of the solution shows a sharp transition with a melting temperature (Tm) of 55 °C. This is the expected transition for the IgG1 target, Figure 2A (---). If IgE is added to a fresh solution of probes, the same color change is observed but the melting analysis provides a curve with a Tm of 36 °C, the expected transition for this target, Figure 2A (——). Significantly, when both protein targets are added to the solution of probes, the solution turns dark purple, and the melting analysis exhibits two distinct transactions. The first derivative of this curve shows two peaks centered at 36 and 55 °C, respectively, Figure 2B. This demonstrates that two distinct assemblies form and their melting properties, which derive from the oligonucleotide barcodes, can be used to distinguish two protein targets.

Example 4: Assay Using Nanoparticle Complex Probes

A variation of this strategy can be used to increase the sensitivity of the aforementioned system and to increase the number of targets that can be interrogated in one solution (Figure 3). With this strategy, the protein targets can be detected indirectly via the DNA biobarcodes. A 12-mer oligonucleotide has 4^{12} different sequences, many of which can be used to prepare a barcode for a polyvalent protein of interest via Figure 1A. In this variation of the assay, the melting properties of the aggregates that form are not measured in solution but rather the DNA biobarcodes within the aggregates are separated via centrifugation (3000 rpm for 2 minutes) from the unreacted probes and target molecules. The aggregates are then denatured by adding water to the solution, freeing the complexed DNA. The particles and proteins can be separated from the DNA barcodes with a centrifugal filter device (Millipore Microcon YM-100, 3500 rpm for 25 min). Once the DNA barcodes are isolated, they can be captured on an oligonucleotide array and can be identified using one of the many DNA detection assays (Figure 3). For the examples described herein involving IgG1 and IgE, the barcodes are captured on a microscope slide that has been functionalized with oligonucleotides (250 μm diameter spots) that are complementary to one half of the barcode of interest (**A3** and **B3** in Figure 1). If the barcode is captured by the oligonucleotide array, a DNA-modified particle that is complementary to the remaining portion of the barcode can be hybridized to the array

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(see experimental section). When developed via the standard scanometric approach [11] (which involves treatment with photographic developing solution), a flat bed scanner can be used to quantify the results, Figure 4.[11] If IgG1 is present, only the spot designed for IgG1 shows measurable signal. Similarly if IgE is the only protein present, the spot designed for it only exhibits signal. Finally, if both proteins are present, both spots exhibit intense signals.

For scanometric DNA biobarcode detection, the DNA/Au nanoparticle assembly was centrifuged (3000 rpm for 2 min) in a polystyrene 1.5 mL vial, and the supernatant was removed. PBS buffer solution (700 μl) was added to the aggregate and the procedure was repeated to ensure isolation of the aggregate from unreacted protein and assay components. Then, 500 μl of water was added to the vial containing the aggregate to denature it. Microarrays were prepared and DNA hybridization methods were used according to literature methods.[11, 22] The isolated DNA biobarcodes were premixed with **A2**-modified nanoparticles or **B2**-modified nanoparticles (2nM), exposed to the DNA microarray, and incubated in a hybridization chamber (GRACE BIO-LABS) at room temperature for three hours. The array was then washed with 0.3M $NaNO_3$ and 10nM NaH_2PO_4/Na_2HPO_4 buffer (pH 7) and submerged in Silver Enhancer Solution (Sigma) for three minutes at room temperature. The slide was washed with water and then analyzed with a flat bed scanner.

Example 5: Assay using nanoparticle complex probe

The Biobarcode PCR (*B*PCR) protocol was performed to detect a protein target, free prostate specific antigen (PSA), at 3 aM sensitivity (Figure 6), which is six orders of magnitude more sensitive than the current conventional clinical assay for detecting PSA (46). The nanoparticle detection probes were prepared by adding polyclonal anti-PSA antibody (7 μg) to an aqueous solution of 13 nm Au nanoparticles (10 ml of 13 nM solution in colloidal suspension, citrate (~38 mM) stabilized) at pH 9.0. After 20 minutes, the anti-PSA modified nanoparticles were reacted with alkylthiol-capped barcode DNA capture strands (0.2 OD; 5' CAA CTT CAT CCA CGT TCA ACG CTA GTG AAC ACA GTT GTG T-A_{10}-SH 3' SEQ ID NO:9) for 12 hours and then salt-stabilized to 0.1 M NaCl/0.01 M phosphate buffer, pH 7. Next, the solution was treated with 1 ml of a 10 % BSA solution for 20 minutes to passivate and stabilize the gold nanoparticles. The final

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solution was centrifuged for 1 hour at 4 °C (20,000g), and the supernatant was removed. This centrifugation procedure was repeated for further purification. The PSA-specific barcode DNA strands (1 OD; 5' ACA CAA CTG TGT TCA CTA GCG TTG AAC GTG GAT GAA GTT G 3' SEQ ID NO:10) were then hybridized with the barcode DNA capture strands coordinated to the nanoparticles and purified using a similar centrifugation procedure.

Amino-functionalized 1 μm diameter magnetic particles were obtained from Polysciences, Inc. They were then linked to proteins using the commercial glutaraldehyde-amine coupling chemistry. Coupling efficiency was determined to be 90 % by UV-vis spectroscopy by comparing the absorbance at 270 nm before and after protein coupling to the particles. The particles, the magnetic capture probes, were suspended in 40 ml of 0.1 M PBS buffer (pH 7.4) prior to use.

In a typical PSA detection experiment (Figure 6B), an aqueous dispersion of magnetic capture probes functionalized with monoclonal anti-PSA antibodies (50 μl of 3 mg/ml magnetic probe solution) is mixed with an aqueous solution (0.1 M PBS) of free PSA (10 μl of PSA) and stirred at 37 °C for 30 minutes (Step 1 of Figure 6B). PSA bound magnetic detection probe can be easily separated from unbound PSA by applying a magnetic field. To effect magnetic separation, a 1.5 ml tube containing the assay solution is placed in a BioMag® microcentrifuge tube separator (Polysciences, Inc.) at room temperature. After 15 seconds, the magnetic capture probe-PSA hybrids are concentrated on the wall of the tube. The supernatant (solution of unbound PSA molecules) is removed, and the magnetic capture probes, now in the form of a pellet on the side of the tube, are re-suspended in 50 μl of 0.1 M PBS (repeated 2X). Nanoparticle detection probes (50 μl at 1 nM), functionalized with polyclonal anti-PSA antibodies and hybrid barcode DNA strands, are then added. The detection probes react with the PSA immobilized on the capture probes and provide DNA strands for signal amplification and protein identification (Step 2 of Figure 6B). The solution is vigorously stirred at 37 °C for 30 minutes. The magnetic particles were then washed with 0.3 M PBS using the magnetic separator to isolate the magnetic particles. This step is repeated 4 times, each time requiring one minute, removing everything but the magnetic capture probes (along with PSA bound nanoparticle detection probes). After the final wash step, the magnetic capture

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probes are resuspended in Nanopure (18 M Ohm) water (50 μl) to dehybridize barcode DNA strands from the nanoparticle detection probe surface for 2 minutes. Dehybridized barcode DNA is then easily separated and collected from the probes using the magnetic separator (Step 3 of Figure 6B).

For barcode DNA amplification (Step 4, figure 6B), isolated barcode DNA is added to a PCR reaction mixture (20 μl, final volume) containing the appropriate primers, and the solution is then thermally cycled according to the following procedure. An aliquot of free barcode DNA (8.9μl) is added to the top wax layer of an EasyStart™ Micro 20 PCR Mix-in-a-Tube (Molecular Bio-Products, San Diego, CA) along with 0.3 μl of the appropriate primers (each at 25 μM, Primer 1: 5'CAA CTT CAT CCA CGT TCA AC 3' SEQ ID NO:11, primer 2: 5'ACA CAA CTG TGT TCA CTA GC 3' SEQ ID NO:12), 0.4 μl DMSO (2 % final concentration), and 0.1 μL of Taq DNA polymerase, a polymerase shown to be compatible with the EasyStart™ system (5U/μl, Fisher Scientific), for a final volume of 20 μl. The final concentrations of the PCR reaction mix are as follows: Primers 1 and 2, 0.37 μM; dNTP mix, 0.2 mM; PCR buffer, 1X; and $MgCl_2$, 2 mM. The PCR tubes are then loaded into a thermal cycler (GeneAmp 9700, Applied Biosystems) and subjected to a 7 minute "hot start" at 94 °C, cycled 25 times at 94 °C for 30 seconds, 58 °C for 30 seconds and 72 °C for 1 minute, with a final extension of 72 °C for seven minutes followed by a 4 °C soak.

Control experiments were first performed to assess primer dimer formation after PCR amplification. Dimethyl sulfoxide (DMSO) was added to reduce the melting temperature of spurious hybridized primers and minimize the possibility of primer dimmer formation and amplification (Figure 7, 0 to 2 % from left to right in 0.5 % increments in lanes 1-5, and lanes 6-10). In addition, the number of thermal cycles was set at 25. As seen in Figure 7, there are clear bands with barcode DNA amplicon (lanes 1-5), while there are no observable bands when only primers are thermally cycled in the presence of Taq polymerase (lanes 6-10). Therefore, 2 % DMSO was added to all *B*PCR reactions since there is no observable band trace for that concentration (Figure 7, lane 10) while amplification was maintained for barcode DNA.

The barcode DNA amplicon was stained with ethidium bromide and mixed with gel loading dye. Gel electrophoresis was then performed to determine whether

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amplification has taken place (Figure 8, panels A and C). For all electrophoresis experiments, an aliquot (15 μl) of the PCR mixture is stained with ethidium bromide (1 mg), mixed with gel electrophoresis loading dye (3 μl, 6X, Promega, Madison, WI), and gel electrophoresis was performed (2 % agarose gel, 110 V, 35 minutes) in 1X TAE running buffer. A biobarcode standard (1 μl, 6 μM biobarcode duplex) was added to the gel for reference. The biobarcode standard (40-mer) was made by adding the biobarcode DNA to its complementary strand in 0.3 M PBS. All gel images and determinations of band intensities were done using a Kodak DC-120 digital camera and Kodak ID 2.0.2 imaging software. Gel bands were also stained with ethidium bromide after electrophoresis by soaking the gel in ethidium bromide for 35 minutes (0.5 μg/ml in 1X TAE running buffer) and qualitatively similar results to those where ethidium bromide was added to the PCR reaction prior to electrophoresis were obtained.

The relative intensity of the ethidium bromide stained bands allowed for an estimate of the relative concentration of PSA (Figure 8, panels B and D). The stained intensity of PCR amplicons represents PSA concentrations in lanes 3-8 of 300 aM, 3 fM, 30 fM, 300 fM, 3 pM, and 30 pM, respectively. The non-specific binding of the nanoparticle probe to the magnetic probe was negligible, as *B*PCR generated little signal when PSA was absent (Figure 8, panel A, lanes 1 and 2, and panel C, lane 1) According to panel B, the intensities for control bands are at least 8 times lower than the bands with PSA present. In the graph representing low concentration (Figure 8, panel B, 3 aM to 300 fM, lanes 2-7, respectively) PSA detection, the gel band corresponding to 3 aM (lane 2) has a relative intensity 2.5 times higher than the negative control (lane 1).

Example 6: Assay using nanoparticle complex probe

Although gel electrophoresis was routinely used to analyze the results of the assay, in general, the scanometric method provides higher sensitivity and is easier to implement than the gel-based method. Therefore, the results of the scanometric assay are reported herein. Chip-immobilized DNA 20-mers, which are complementary with half of the target barcode sequence (40-mer), were used to capture the amplified barcode DNA sequences, and gold nanoparticles were used to label the other half of the sequence in a sandwich assay format. The amplified duplex barcode DNA must be first denatured in order to effect hybridization between the barcode DNA, the chip surface, and gold

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nanoparticle probes. Thus, the barcode DNA amplicons were removed from the original PCR tube and added (5 μl) to a solution of gold nanoparticle probes (5 μl, 10 nM in 0.3 M PBS). The solution was diluted with 0.3 M PBS (90 μl) to a final volume of 100 μl in a clean 0.2 ml PCR tube. In order to hybridize barcode DNA single strands (40-mer) and nanoparticle bound complements (20-mer), the PCR tubes were added to a thermal cycler, heated to 95 °C for 3 minutes to denature the barcode DNA duplexes, and then cooled to a hybridization temperature of 45 °C for 2 minutes to bind nanoparticle probes to their complementary barcode DNA sequences. This mixture was removed from the PCR tube and added to the microarrayed (GMC 417 Arrayer, Genetic MicroSystems) chip with immobilized capture strands (20-mer). The test solution for each experiment was confined over the active region of the array with a 100 μl hybridization well (Grace BioLabs, Bend, OR) for 45 minutes in a humidity chamber. After hybridization, the chips were rinsed with 0.1 M $NaNO_3$/0.01 M phosphate buffer, pH 7.0 at 45 °C to remove excess gold particles (repeated 2X). Chips with hybridized nanoparticle probes were then subjected to silver amplification with silver enhancement solution (6 minute reaction time, Ted Pella Inc., Redding, CA), rinsed with Nanopure (18 M Ohm) water, and dried using a benchtop centrifuge. Gold nanoparticle binding followed by silver amplification results in gray spots that can be read with a Verigene ID system (Nanosphere, Inc.) that measures light scattered from the developed spots [11, 51]. Target PSA concentrations from 300 fM to 3 aM could be easily detected via this method (Figure 9). The 3 aM sample correlates with eighteen protein molecules in the entire sample. The selectivity for the barcode DNA sequence is excellent as evidenced by the lack of signal from the control spots with noncomplementary capture DNA (5'SH-C6-A10-GGCAGCTCGTGGTGA-3', SEQ ID NO:13) (Figure 9, Spotting template), and the observation that there is little discernible signal when PSA is absent (Figure 9, control).

Example 7: Theoretical limit of protein detection using *B*PCR

To examiner the theoretical lower-limit of protein detection using *B*PCR, PCR/gel electrophoresis was performed with a dilution series of barcode DNA concentrations for PCR amplification (Figure 10). The signal when barcode DNA amplicons were present is quite discernible from the control band (lane 10) even when only 30 copies of barcode DNA were added to the PCR reaction (lane 9). Assuming each nanoparticle probe has

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about 50 barcode DNA strands, *B*PCR can, in theory, generate a detection signal with a single bound nanoparticle probe.

Example 8: Detection of PSA in complex medium

To demonstrate the applicability of the *B*PCR amplification method in a sample solution more comparable to a clinical setting, the assays as described in Examples 5 and 6 were performed by dissolving the PSA target in goat serum, and the barcodes detected following a *B*PCR amplification step. PSA was successively diluted in 0.1 M PBS and then added to un-diluted goat serum (ICN Biomedicals, Inc., Aurora, Ohio). The goat serum effectively mimics 1X normal saline buffer, (e.g., any biological system, as far as ionic strength and pH).

The data demonstrates that in a complex medium the methods of the invention can detect target analytes (here PSA) at concentrations as low as 30 aM. The signal generated at this concentration is clearly discernible from control experiments (Fig. 11). Background signal can be reduced by introducing an optional step wherein the barcode DNA sample is filtered with a membrane that can remove a majority of contaminating components. This optional filtration step can separate barcode DNA from impurities by any known method, such as, for example, size (molecular weight), shape, charge, or hydrophobicity/hydrophilicity.

Example 9: Direct detection and measurement of barcode DNA (non-*B*PCR amplified)

The gold nanoparticle (NP) probes were prepared essentially as described above in Example 5. Briefly, polyclonal antibodies (Abs) to PSA (3.5 μg) were added to an aqueous solution of 30 nm gold NPs (1 ml of 40 pM NP solution) at pH 9.0. After 20 minutes, the Ab modified NPs were reacted with alkylthiol-capped barcode DNA capture strands (1 OD for 30 nm gold particles; 5'- CAA CTT CAT CCA CGT TCA ACG CTA GTG AAC ACA GTT GTGT-A_{10}-$(CH_2)_3$-SH 3') for 16 hours and then salt-stabilized to 0.1 M NaCl. This solution was treated with 0.3 ml of a 10 % BSA solution for 30 minutes to passivate and stabilize the gold NPs. The final solution was centrifuged for 1 hour at 4 °C (20,000g), and the supernatant was removed. This centrifugation procedure was repeated for further purification. The final NP probes were re-dispersed in 0.1 M

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NaCl/0.01 M phosphate buffer solution at pH 7.4. The PSA-specific barcode DNA strands (1 OD; 5'-ACA CAA CTG TGT TCA CTA GCG TTG AAC GTG GAT GAA GTT G-3') were then hybridized with the biobarcode DNA capture strands coordinated to the NPs and purified using a similar centrifugation procedure. The oligonucleotide loading was determined by the method of Demers *et al.*, see: L. M. Demers *et al. Anal. Chem.* **72**, 5535 (2000). Amino-functionalized 1 μm diameter MMPs were obtained from Polysciences, Inc. MMPs were linked to mAbs to PSA using the commercial glutaraldehyde-amine coupling chemistry. Coupling efficiency was determined to be 90% by UV-vis spectroscopy by comparing the absorbance at 270 nm before and after protein coupling to the MMPs. The MMPs were suspended in 40 ml of 0.1 M PBS buffer (pH 7.4) prior to use.

Chip-based assays were used to measure directly the amount of barcode DNA in solution, generally as described in Example 6, however without use of an amplification step by *B*PCR. Since PCR is not used, this method eliminates the need to denature the duplex DNA formed during PCR amplification. Thus, after protein detection and isolation of barcode DNA, an aliquot of the isolated barcode sample (10 μl) was mixed with 0.6 M PBS (85 μl) and the 13 nm gold detection probe (5 μl, 500 pM final concentration, same sequence as above). This mixture was added to the wells of a Verigene on-chip hybridization chamber under which the appropriate capture strands were arrayed as described above. The sample was incubated for 2 h at 42 °C. After incubation, the reaction mixture was removed and the chips were washed with 0.5 M $NaNO_3$/0.01 M phosphate buffer to remove excess gold nanoparticles. Surface immobilized gold particles were stained with silver enhancement solution (Ted Pella) for 6 min, washed with Nanopure water, and imaged with the Verigene ID system, all as described above.

Although 30 nm gold NPs were used in this instance, 13 nm gold NPs are contemplated for use with this procedure for direct detection of Barcode DNA. Nevertheless, by increasing the size of NP probes in the protein detection step, the number of DNA strands for each NP can be increased significantly, aiding in the direct detection (in theory, assuming 100 DNA strands are attached to a 13 nm gold NP, there could be as many as 532 DNA strands on each 30 nm gold NP). The size of the particular nanoparticle probe can be adjusted to optimize certain aspects

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The results (Fig. 12) demonstrate that the PSA target was directly detectable at concentrations as low as 30 attomolar, using 30 nm gold NP (at 20 pM) without *B*PCR amplification. Although this represents a loss in sensitivity of an order of magnitude relative to the *B*PCR amplification method, this method is still exquisitely sensitive and provides for significant decrease in cost, effort, and time, through elimination of the PCR step.

Example 10: Direct Detection of Target Nucleic Acid Sequence at ZeptoMolar Concentrations

For this experiment, the DNA sequence associated with the anthrax lethal factor (5′-GGA TTA TTG TTA AAT ATT GAT AAG GAT-3′; SEQ ID NO:14) was chosen as an initial target because this sequence is important for bio-terrorism and bio-warfare applications and is well studied in the literature.[63,67-69] To each 20 μL test sample, two control DNA sequences were added [1 μL of 10 pM (5′-**CT**A TTA **TA**A **TA**A AAT ATT **TAT ATA GCA**-3′; SEQ ID NO:15) and 1 μL of 10 pM for control 2 (5′-**GA**A TTA **TAG** TTA **ACT ATA GCT** AAG GAT-3′; SEQ ID NO:16)]. Prior to use, the nanoparticle probes were loaded with barcode DNA by hybridization (20 nm probes at 400 pM; 30 nm probes at 200 pM). Barcode DNA was introduced at 10 μM concentration to effect hybridization in an appropriate hybridization buffer. The particles were subsequently centrifuged and washed with PBS buffer. The probes were then suspended in appropriate storage buffer, and stored until use.

For the MMPs, the polyamine-functionalized polystyrene particles were linked with alkylthiol-capped DNA by reacting them with a sulfosuccinimidyl 4-[p-maleimidophenyl]butyrate (sulfo-SMPB) bifunctional linker that reacts with the primary amines on the MMPs and the thiol groups on the oligonucleotide which form the a specific recognition binding site for target sequence. The MMPs were passivated with bovine serum albumin prior to use by adding 10% BSA to the solution containing them. The probes were then centrifuged, washed with PBS buffer, and resuspended at 2 mg/mL to yield active probes (Figure **13A**).

The assay was performed by adding 50 μL of the MMP probes (at 2 mg/mL) to a solution that contained target DNA in single stranded form. The system was allowed to

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stand at room temperature for 10 min. Following the 10 min. standing period, 50 μL of the NP probes [50 μL at 400 pM (20 nm NP probe solution) or 50 μL at 200 pM (30 nm NP probe solution)] were added to the solution and allowed to hybridize for 50 min. After hybridization, a magnetic field was applied to the reaction vessel (BioMag multi-6 microcentrifuge tube separator, Polysciences, Incorporated, Warrington, PA), which pulled the target DNA strands sandwiched with MMPs and NPs, as well as unreacted MMPs, to the wall of the reaction vessel. Any remaining unreacted reaction solution components, especially NPs not specifically hybridized to MMPs, were washed away with several washes with PBS buffer. The magnetic field was then removed and 50 μl of NANOpure water (Barnstead International, Dubuque, IA) was added to the reaction vessel and the system was heated to 55°C for 3 min. to release the bar-code DNA. Reintroduction of the magnetic field removed all of the MMPs from solution, leaving barcode DNA for detection.

To analyze the amount and identity of the barcode DNA in the final reaction solution, scanometric methods were used.[67] Scanometric methods are chip-based DNA detection methods that rely on oligonucleotide-modified gold NP probes (5′-TCT CAA CTC GTA GCT-A_{10}-SH-3′-Au; SEQ ID NO:17) and NP-promoted reduction of silver(I) for signal amplification. For this particular assay, maleimide-modified glass chips were spotted with 5′ capture DNA strands (5′-SH-A_{10}-CGT CGC ATT CAG GAT-3′; SEQ ID NO:18) using a DNA microarrayer (spot diameter is 175 μm and the distance between two spots is 375 μm; GMS 417 Arrayer, Genetic MicroSystems, Woburn, Massachusetts). The non-spot area of the surface was passivated with a A_{10} sequence (10 μM of 5′-SH-AAA AAA AAA A-3′; SEQ ID NO:19) overnight. Once the chip surface was contacted with the barcode DNA solution, NP probes mixed with target sequence solution were added to the barcode/capture DNA-modified chip. The spots on a chip were labeled with NP probes and target DNA strands. The spotted chip was then exposed to silver enhancement solution (Ted Pella, Redding, CA) for further signal enhancement. The developed spots were then read with a Verigene ID (identification) system (Nanosphere, Incorporated, Northbrook, IL). The Verigene ID system measures the scattered light from the developed spots and provides a permanent record for the assay. Figure 14A illustrates 20-nm NP probes used in the detection of "amplified" barcode DNA. The spot intensities for target DNA concentrations from 5 fM to 50 aM are clearly

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stronger than control spots. To measure spot intensity of each concentration, three spots were patterned on a chip and imaged with the Verigene ID system. The gray levels of the spots were calculated with graphic software (Adobe Photoshop) and the spot intensity graph is shown in Figure 15. This graph shows that 20-nm NP probes can clearly detect target DNA at about 50 aM, but cannot differentiate signal from background at a concentration of 5 aM.

When 30 nm NP probes were used for DNA-BCA detection, target DNA concentration as low as 500 zM was detected (Figure 14B). This difference in detection limit for the 20 and 30 nm NP probes may be due to the difference in the absolute number of barcode DNA strands on NP probes of different surface areas. The intensity graph suggests that 30-nm NP probe system provides a more intense spot signal than 20-nm NP probe system at all sample concentrations (Figure 15). A sample volume of 20 μL at 500 zM represents a total sample number of approximately 10 target DNA strands, providing a sensitivity comparable to assays that employ PCR-based methods coupled with molecular fluorophore probes.[58-60]

Example 11

Simplified Reporter-Based Bio-barcode Assay

The detection of prostate-specific antigen (PSA) will be used as an illustrative example for consistency with Example 8. PSA was chosen for this example as clinically significant levels of PSA are close to the detection limits of the tests that are presently used for this cancer marker and, thus, a method of increased sensitivity is desirable. The generalization of this example to the detection of multiple target analytes in the same medium can be implemented by mixing substrates and detection probes prepared as described below to form the corresponding reagent "cocktails" that are then used as described below. Alternative implementations such as forming spatially separated binding zones for different target analytes on the same substrate are also practical and may be desirable in specific uses of this invention. A magnetic substrate will be described for consistency with the previous Examples, but other types of substrates may be more appropriate for specific uses of this invention.

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a) Preparation of Substrates

Magnetic microparticle detection probes (MMPs) were prepared by linking a monoclonal antibody specific for one epitope of PSA (Maine Biotechnologies, Maine, MA) to amino-functionalized magnetic microparticles (Polysciences, Inc., Warrington, PA) using a standard glutaraldehyde-amine coupling chemistry. Amino-functionalized MMPs (5 mg) were activated with 5 ml of 5% glutaraldehyde in pyridine wash buffer (PWB) for 3 hrs at room temperature. The activated MMPs were then magnetically separated, and the supernatant was removed. This magnetic separation step was repeated twice, and the MMPs were re-suspended in PWB. The monoclonal antibody (750 μg) was then added to MMPs, and the solution was mixed for 10 hrs at room temperature. Non-specific binding sites were blocked by adding 1 mg of bovine serum albumin (BSA) to the MMP solution and mixing for 10 additional hrs at room temperature. The magnetic separation step was repeated twice, and the MMPs were re-suspended in 5 ml of PWB. Then 3 ml of glycine solution (1 M at pH 8.0) was added to the resulting solution to quench all the unreacted glutaraldehydes and stirred for 30 min. After the magnetic separation step (repeated twice), 5 ml of wash buffer was mixed vigorously with monoclonal antibody-functionalized MMPs and the MMPs were magnetically separated again (this washing step was repeated three times). Finally, the MMP probes were re-suspended in 0.15 M phosphate-buffered saline (PBS) solution. The coupling efficiency was determined to be 90% by UV-Vis spectroscopy by comparing the absorbance at 280 nm before and after protein coupling to the MMPs.

b) Preparation of Probes

Detection probes were prepared using one micron diameter amino-functionalized polystyrene microparticles (Polysciences, Inc., Warrington, PA). One milliliter of a 1.25% aqueous suspension of the amino-functionalized polystyrene microparticles was centrifuged for 5 min at 10,000 rpm, and the supernatant was removed. The pellet was then resuspended in PBS and the centrifugation step was repeated once more. The resulting polystyrene particle pellet was re-suspended in 1 ml of 8% glutaraldehyde in PBS at pH 7.4 and mixed for 5 hr on a rocking shaker. The particles were then sedimented for 5 minutes at 10,000 rpm, and the supernatant was discarded (this step was repeated twice). The resulting pellet was re-suspended in PBS and 10 μg of polyclonal antibody for PSA (Maine Biotechnologies, Maine, MA) was added and allowed to react

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over night on a rocking shaker at room temperature. The amount of antibody added is 20-30 times less than the amount of antibody that is needed to fully modify the particle surface so that surface sites are available for the attachment of large numbers of oligonucleotides to the same particle in the next step. These particles were sedimented for 5 minutes at 10,000 RPM and washed twice with PBS by suspension and sedimentation as before.

Oligonucleotide-functionalized microparticles were prepared by resuspending the pelleted activated microparticles from the previous step in 1 ml of a 100 μM solution of the appropriate 3' amino-functionalized oligonucleotide and allowing them to react over night on a rocking shaker at room temperature. "Barcode" detection probes analogous to those of the previous invention were prepared using the oligonucleotides and methods described in the following paragraph. For detection probes where the identification of the target analyte was not encoded in the nucleotide sequence, fluorescently labeled 3' amino-functionalized 30-base oligonucleotides having arbitrary sequences were used. The reacted particles were sedimented for 5 minutes at 10,000 RPM and washed twice with PBS by suspension and sedimentation as before. The resulting pellet was re-suspended in 1 ml of 0.2 M ethanolamine to passivate all unreacted glutaraldehyde sites on the microparticles for 30 min at room temperature, sedimented for 5 minutes at 10,000 RPM, and the supernatant was removed by decantation. Bovine serum albumin (10% BSA) solution was subsequently added to further passive the protein-inactive regions of the particle surface. The centrifugation step was repeated twice to remove the supernatant, and the pellet was re-suspended in 1 ml of 0.15 M PBS solution. Disulfide-linked, fluorescently labeled chemically cleavable oligonucleotides were attached to the activated microparticles in a similar manner except that the initial reaction with an amino-functionalized oligonucleotide was omitted; thioethanolamine was substituted for ethanolamine in the passivation step; and a fluorescently labeled, 3' thiol-functionalized oligonucleotide was coupled to the resulting thiol groups by oxidation to the disulfide.

"Barcode" detection probes analogous to those of the previous invention were prepared by resuspending the pelleted microparticles in 1 ml of a 100 μM solution of the 3' amino-functionalized oligonucleotide 5' CGTCGCATTCAGGATTCTCAACTCGTAGCT-A_{10}-C6-amine 3' (SEQ ID NO:20) to the glutaraldehyde-activated microparticles of the previous step and allowed to react over

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night on a rocking shaker at room temperature. The same oligonucleotide pair or single oligonucleotide may be used for all target analytes. These particles were sedimented for 5 minutes at 10,000 RPM and washed twice with PBS by suspension and sedimentation as before. The resulting pellet was re-suspended in 1 ml of 0.2 M ethanolamine to passivate all unreacted glutaraldehyde sites on the microparticles for 30 min at room temperature, sedimented for 5 minutes at 10,000 RPM, and the supernatant was removed by decantation. Bovine serum albumin (10% BSA) solution was subsequently added to further passive the protein-inactive regions of the particle surface. The centrifugation step was repeated twice to remove the supernatant, and the pellet was re-suspended in 1 ml of 0.15 M PBS solution. 200 µl of 5 mg/ml polystyrene probe particle suspension was then combined with 200 µl of a 33 µM solution of the compelmentary Alexa Fluor® 647-labeled (Molecular Probes, Eugene OR) labeled oligonucleotide 5' AGCTACGAGTTGAGAATCCTGA-ATGCGACG 3' (SEQ ID NO:21, purchased from Integrated DNA Technologies, Coralville, IA, or synthesized and purified using literature methods.[35]) and hybridized to the complementary oligonucleotide strands on the polystyrene particles. The particles were then washed by suspension and sedimentation prior to use. The oligonucleotide loading for each particle was determined by sedimenting 100 µl of 500 µg/ml particle suspension, discarding the supernate and dehybridizing by resuspending the pellet in 100 µl of NANOpure water. The particles were removed by sedimentation and the fluorescence of the released oligonucleotides in solution was measured in a 50 µl fluorescence micro-cuvette (Starna Cells, Inc., Atascadero, CA) using a SPEX Fluorolog-3 spectro-fluorometer (JOBIN YVON). Quantitation was via comparison of the measured sample fluorescence against a calibration curve for the pure Alexa Fluor® 647-labeled bar-code DNA solution. The average number of bar-code DNA strands for each polystyrene particle was determined to be ~ $1.1x10^5$.

c) PSA Test (Reaction)

10 µl of a clinical sample or a calibration standard containing PSA (Sigma Chemical Company, Milwaukee, WI) was added to 50 µl of MMP probes functionalized with monoclonal anti-PSA antibodies (500 µg/ml) in a 1.5 mL BioMag® microcentrifuge tube and the solution was shaken on an orbital shaker at 37° C for 23 min (Step 1 in

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Figure 18). The tube containing the assay solution was then placed in a BioMag® microcentrifuge tube separator (Polysciences, Inc.) at room temperature. After 15 seconds, the MMP-PSA hybrids are concentrated on the wall of the tube. The supernatant was removed, and the MMPs are re-suspended in 50 µl of 0.1 M PBS (repeated twice). Detection probes (50 µl at 1.25 mg/ml) of either type described above were then added to the solution (Step 1 in Figure 18) and vigorously stirred at 37° C for 23 minutes. The magnetic particles were then separated from the reaction mixture and washed twice with 0.15 M PBS using the magnetic separator as described above (Step 2 in Figure 18).

d) PSA Test (Barcode Detection Version)

The magnetic particles were re-suspended in NANOpure (18 MΩ) water (50 µl) to dehybridize the fluorescently labeled bar-code oligonucleotides from the complementary oligonucleotides on the polystyrene probe surface. The magnetic particles were removed from the suspension using the magnetic separator (Step 3 in Figure 18) and the integrated fluorescence emission intensity of the released oligonucleotides over the 660-670nm spectral range was measured using a SPEX Fluorolog-3 spectro-fluorimeter (650nm excitation/665nm emission for Alexa Fluor®647). Integrated emission intensity over the 660-670nm spectral range was determined and used as a measure of PSA concentration in the initial medium.

e) PSA Test (Chemical Release Version)

The magnetic particles were re-suspended in PBS (50 µl) containing dithiothreitol (DTT) and heated for 10 minutes to 50°C to release the fluorescently labeled oligonucleotides from the polystyrene probe surface. The magnetic particles were removed from the suspension using the magnetic separator (Step 3 in Figure 18) and the integrated fluorescence emission intensity of the released oligonucleotides over the 660-670nm spectral range was measured using a SPEX Fluorolog-3 spectro-fluorimeter (650nm excitation/665nm emission for Alexa Fluor®647). Integrated emission intensity over the 660-670nm spectral range was determined and used as a measure of PSA concentration in the initial medium.

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f) PSA Test (In-Situ Detection Version)

The magnetic particles were re-suspended in PBS and the integrated fluorescence emission intensity of the particle-bound oligonucleotides was measured using a SPEX Fluorolog-3 spectro-fluorimeter (650nm excitation/665nm emission for Alexa Fluor®647). The fluorescence of the magnetic microparticles could also be determined in a flow cytometer according to standard procedures.

g) PSA Test (Results)

All target analyte concentrations over the 30 aM to 300 fM concentration range tested could be easily differentiated from the corresponding negative control (Figure 19A). Importantly, the improvements of the present inventions described above retained the 30 aM target concentration sensitivity of the previous invention, but allowed the reduction of the total assay time from over four hours to less than 50 minutes and significantly reduced the number of steps and the complexity of the instrumentation required.

Example 12

Chemical Release of Particle Bound Molecules

A fluorescent assay was constructed as demonstrated in Figures 20 through 22. Messenger RNA (mRNA) served as the target analyte for detection. Poly-T-labeled magnetic beads captured the 3'-Poly A target analytes to immoblize them against a magnetic support and separate them from the sample solution. The detection reagent used was composed of gold nanoparticles functionalized with an oligonucleotide complimentary to the 5' end of the mRNA targets. The complimentary oligonucleotides functioned as both the reporter moiety and the selective binding compound and were bound to the gold nanoparticles through gold-thiol bonds. The detection reagent was added to the solution allowing binding of the mRNA targets to the complementary reporter moieties to form a sandwich of the Poly-T-labeled beads-the target mRNA analyte-the complementary oligonucleotide binding compound-the gold nanoparticle. After washing, dithiothreitol (DTT) was added to the water containing the sandwich structure formed and heated for 10 minutes to 50°C. The water and heat dehybridized the magnetic and gold nanoparticle structures held together by the target mRNA molecule.

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The DTT exchanged with the thiolated DNA reporter moieties on the surface of the 13 nm gold nanoparticles releasing the latter into solution for subsequent detection after removal of the magnetic and gold particles. A fluorescent molecule (Alexa 488) was added adjacent to the thiol so that solution liberated probe sequences could be detected using a fluorimeter. As shown in Figure 23, target mRNA concentrations down to 6 pM were detected, representing liberated Alexa 488-labeled-mRNA probe strands at approximately 1 nM concentration; the limit for the fluorimetric detection of the Alexa 488 dye in a 50 microliter volume. This example demonstrates that the use of the presently claimed detection methodology for the detection of nucleic acid targets can be as sensitive as the polymerase chain reaction, without the need for enzymatic amplification of the target sequence.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

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WHAT IS CLAIMED:

1. A method for detecting for the presence of one or more target analytes in a sample, each target analyte having at least two binding sites for specific binding interactions with specific binding complements, in a sample comprising the steps of:

a) providing at least one type of capture substrates, the capture substrates having bound thereto at least one specific binding complement of the specific target analyte that binds to at least a first binding site of the specific target analyte;

b) providing at least one type of microparticle detection probes, the microparticle detection probes comprising a microparticle having bound thereto (i) at least one specific binding complement to a specific target analyte and (ii) a plurality of DNA barcodes, the DNA barcodes are optionally labeled with a reporter label, wherein the specific binding complement bound to the microparticle detection probes binds to at least a second binding site of the target analyte;

c) contacting the capture substrates with a sample believed to contain target analytes under conditions effective to allow for binding of the specific target analyte to the specific binding complement bound to the capture substrate so as to immobilize the target analytes onto the capture substrates;

d) contacting the immobilized target analytes with at least one type of microparticle detection probes under conditions effective to allow for binding between the target analytes and the specific binding complement bound to the microparticle detection probes to the analyte and to form a complex in the presence of the target analyte on the capture substrate;

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e) optionally isolating and washing the capture substrate to remove unbound microparticle detection probes; and

f) detecting for the presence of the DNA barcode wherein the presence of the DNA barcode is indicative of the presence of a specific target analyte in the sample.

2. The method of claim 1, wherein the target analyte is a protein or hapten and its specific binding complement is an antibody comprising a monoclonal or polyclonal antibody.

3. The method of claim 1, wherein the target analyte is a nucleic acid molecule.

4. The method of claim 1, wherein each DNA barcode is labeled with a detectable reporter group.

5. The method of claim 1, wherein the detectable reporter group comprises a fluorophore, a chromophore, a redox-active group, a group with an electrical signature, radioactive group, a catalytic group, or Raman label.

6. The method of claim 1, wherein the microparticle is labeled with a plurality of DNA barcodes.

7. The method of claim 1, further comprising the step of washing the capture substrate after the target analytes are immobilized thereon and prior to contacting the capture substrate with microparticle detection probes.

8. The method of claim 1, wherein, prior to said detecting step and subsequent to said isolating and washing step, further comprising the steps of:

a) subjecting the complex to conditions effective to release the DNA barcodes from the microparticle detection probes; and

b) optionally amplifying the DNA barcode prior to said detecting.

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9. The method of claim 8, wherein released DNA barcodes are immobilized on a substrate prior to said detecting.

10. The method of claim 1, wherein the DNA barcodes are directly bound to the microparticles.

11. The method of claim 10, wherein the DNA barcodes are released from the microparticle detection probes by a chemical releasing agent.

12. The method of claim 1, wherein the microparticles further comprise oligonucleotides bound thereto and the DNA barcodes are hybridized to at least a portion of the oligonucleotides bound to the microparticles.

13. The method of claim 12, wherein the DNA barcodes are released by dehybridization.

14. The method of claim 1, wherein the capture substrate is a magnetic substrate.

15. The method of claim 14, wherein said isolating comprises subjecting the magnetic substrate to a magnetic field.

16. The method of claim 15, wherein subsequent to said isolating and washing step and prior to said detecting step, further comprising subjecting the complex to conditions effective to release the DNA barcodes.

17. The method of claim 1, wherein said isolating comprises filtration, sedimentation, flotation, or hydrodynamics.

18. The method of claim 17, wherein the filtration step comprises a membrane that removes sample components that do not comprise DNA barcodes.

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19. The method of claim 1, wherein the microparticles are polymeric, glass, metallic, semiconductor, or ceramic.

20. The method of claim 19, wherein the polymeric comprises polystyrene.

21. The method of claim 1, wherein the specific binding pair is an antibody and an antigen.

22. The method of claim 1, wherein the specific binding pair is a receptor and a ligand.

23. The method of claim 1, wherein the specific binding pair is an enzyme and a competitive inhibitor.

24. The method of claim 1, wherein the specific binding pair is a drug and a target molecule.

25. The method of claim 1, wherein the specific binding pair is two strands of at least partially complementary oligonucleotides.

26. The method of claim 1, wherein the DNA barcode is biotinylated.

27. The method of claim 1, wherein the DNA barcode is radioactively labeled.

28. The method of claim 1, wherein the DNA barcode is fluorescently labeled.

29. The method of claim 1, wherein the target has more than two binding sites.

30. The method of claim 1 wherein at least two types of microparticle detection probes are provided, the first type of probe having a specific binding complement to a first binding site on the target analyte and the second type

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of probe having a specific binding complement to a second binding site on the target analyte.

31. The method of claim 30, wherein a plurality of microparticle detection probes are provided, each type of probe having a specific binding complement to different binding sites on the target analyte.

32. The method of claim 1, wherein the specific binding complement and the target analyte are members of a specific binding pair.

33. The method of claim 32, wherein members of a specific binding pair comprise nucleic acid, oligonucleotide, peptide nucleic acid, polypeptide, antibody, antigen, carbohydrate, protein, peptide, amino acid, hormone, steroid, vitamin, drug, virus, polysaccharides, lipids, lipopolysaccharides, glycoproteins, lipoproteins, nucleoproteins, oligonucleotides, antibodies, immunoglobulins, albumin, hemoglobin, coagulation factors, peptide and protein hormones, non-peptide hormones, interleukins, interferons, cytokines, peptides comprising a tumor-specific epitope, cells, cell-surface molecules, microorganisms, fragments, portions, components or products of microorganisms, small organic molecules, nucleic acids and oligonucleotides, metabolites of or antibodies to any of the above substances.

34. The method of claim 33, wherein nucleic acid and oligonucleotide comprise genes, viral RNA and DNA, bacterial DNA, fungal DNA, mammalian DNA, cDNA, mRNA, RNA and DNA fragments, oligonucleotides, synthetic oligonucleotides, modified oligonucleotides, single-stranded and double-stranded nucleic acids, and natural and synthetic nucleic acids.

35. The method according to claim 1, wherein the target analyte is a nucleic acid and the specific binding complement is an oligonucleotide.

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36. The method according to claim 1, wherein the target analyte is a protein or hapten and the specific binding complement is an antibody comprising a monoclonal or polyclonal antibody.

37. The method according to claim 1, wherein the target analyte is a sequence from a genomic DNA sample and the specific binding complements are oligonucleotides, the oligonucleotides having a sequence that is complementary to at least a portion of the genomic sequence.

38. The method of claim 37, wherein the genomic DNA is eukaryotic, bacterial, fungal or viral DNA.

39. The method according to claim 1, wherein the target analyte is a sequence from episomal DNA sample and the specific binding complements are oligonucleotides, the oligonucleotides having a sequence that is complementary to at least a portion of the episomal DNA sequence.

40. The method according to claim 1, wherein the specific binding complement and the target analyte are members of an antibody-ligand pair.

41. The method according to claim 1, wherein in addition to its first binding site, the target analyte has been modified to include a second binding site.

42. A method for detecting for the presence of one or more target analytes in a sample, each target analyte having at least two binding sites for specific binding interactions with specific binding complements, in a sample comprising the steps of:

a) providing at least one type of capture substrates, the capture substrates having bound thereto at least one specific binding complement of the specific target analyte that binds to at least a first binding site of the specific target analyte;

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b) providing at least one type of microparticle detection probes, the microparticle detection probes comprising a microparticle having bound thereto (i) at least one specific binding complement to a specific target analyte and (ii) a plurality of DNA barcodes, the DNA barcodes are fluorescently labeled, wherein the specific binding complement bound to the microparticle detection probes binds to at least a second binding site of the target analyte;

c) contacting the capture substrates with a sample believed to contain target analytes under conditions effective to allow for binding of the specific target analyte to the specific binding complement bound to the capture substrate so as to immobilize the target analytes onto the capture substrates;

d) optionally washing the capture substrate to remove unbound materials; and

e) contacting the immobilized target analytes with at least one type of microparticle detection probes under conditions effective to allow for binding between the target analytes and the specific binding complement bound to the microparticle detection probes to the analyte and to form a complex in the presence of the target analyte on the capture substrate;

f) optionally isolating and washing the capture substrate to remove unbound microparticle detection probes; and

g) detecting for the presence of a fluorescent signal from the DNA barcodes wherein the presence of a fluorescent signal is indicative of the presence of a specific target analyte in the sample.

43. A method for detecting for the presence of one or more target analytes in a sample, each target analyte having at least two binding sites for specific

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binding interactions with specific binding complements, in a sample comprising the steps of:

a) providing at least one type of capture substrates, the capture substrates having bound thereto at least one specific binding complement of the specific target analyte that binds to at least a first binding site of the specific target analyte;

b) providing at least one type of microparticle detection probes, the microparticle detection probes comprising a microparticle having bound thereto (i) at least one specific binding complement to a specific target analyte and (ii) a plurality of DNA barcodes, the DNA barcodes are fluorescently labeled, wherein the specific binding complement bound to the microparticle detection probes binds to at least a second binding site of the target analyte;

c) contacting the capture substrates with a sample believed to contain target analytes under conditions effective to allow for binding of the specific target analyte to the specific binding complement bound to the capture substrate so as to immobilize the target analytes onto the capture substrates;

d) optionally washing the capture substrate to remove unbound materials; and

e) contacting the immobilized target analytes with at least one type of microparticle detection probes under conditions effective to allow for binding between the target analytes and the specific binding complement bound to the microparticle detection probes to the analyte and to form a complex in the presence of the target analyte on the capture substrate;

f) optionally isolating and washing the capture substrate to remove unbound microparticle detection probes;

g) subjecting the isolated washed capture substrate to conditions effective to release the DNA barcodes; and

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h) detecting for the presence of the fluorescent signal of the DNA barcodes wherein the presence of a fluorescent signal of the DNA barcode is indicative of the presence of a specific target analyte in the sample.

44. A method for detecting for the presence of one or more target analytes in a sample, each target analyte having at least two binding sites for specific binding interactions with specific binding complements, in a sample comprising the steps of:

a) providing at least one type of capture substrates, the capture substrates having bound thereto at least one specific binding complement of the specific target analyte that binds to at least a first binding site of the specific target analyte;

b) providing at least one type of microparticle detection probes, the microparticle detection probes comprising a microparticle having bound thereto (i) at least one specific binding complement to a specific target analyte and (ii) a plurality of DNA barcodes, the DNA barcodes comprising a reporter label, wherein the specific binding complement bound to the microparticle detection probes binds to at least a second binding site of the target analyte;

c) contacting the capture substrates with a sample believed to contain target analytes under conditions effective to allow for binding of the specific target analyte to the specific binding complement bound to the capture substrate so as to immobilize the target analytes onto the capture substrates;

d) optionally washing the capture substrate to remove unbound materials; and

e) contacting the immobilized target analytes with at least one type of microparticle detection probes under conditions effective to allow for binding between the target analytes and the specific binding complement bound to the

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microparticle detection probes to the analyte and to form a complex in the presence of the target analyte on the capture substrate;

f) optionally isolating and washing the capture substrate to remove unbound microparticle detection probes;

g) subjecting the isolated washed capture substrate to conditions effective to release the DNA barcodes; and

h) detecting for the presence of the reporter label of the DNA barcodes wherein the presence of the reporter label of the DNA barcode is indicative of the presence of a specific target analyte in the sample.

45. A method for detecting for the presence of one or more target analytes in a sample, each target analyte having at least two binding sites for specific binding interactions with specific binding complements, in a sample comprising the steps of:

a) providing at least one type of capture substrates, the capture substrates having bound thereto at least one specific binding complement of the specific target analyte that binds to at least a first binding site of the specific target analyte;

b) providing at least one type of particle detection probes, the particle detection probes comprising a particle having bound thereto (i) at least one specific binding complement to a specific target analyte and (ii) a plurality of DNA barcodes, the DNA barcodes are optionally labeled with a reporter label, wherein the specific binding complement bound to the particle detection probes binds to at least a second binding site of the target analyte;

c) contacting the capture substrates with a sample believed to contain target analytes under conditions effective to allow for binding of the specific target analyte to the specific

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binding complement bound to the capture substrate so as to immobilize the target analytes onto the capture substrates;

d) optionally washing the capture substrate to remove unbound materials; and

e) contacting the immobilized target analytes with at least one type of particle detection probes under conditions effective to allow for binding between the target analytes and the specific binding complement bound to the particle detection probes to the analyte and to form a complex in the presence of the target analyte on the capture substrate;

f) optionally isolating and washing the capture substrate to remove unbound particle detection probes;

g) releasing the DNA barcodes from the particle detection probes by a chemical releasing agent; and

h) detecting for the presence of the DNA barcode wherein the presence of the DNA barcode is indicative of the presence of a specific target analyte in the sample.

46. The method of claim 45, wherein the particle is a nanoparticle or a microparticle.

47. A method for detecting for the presence of one or more target analytes in a sample, each target analyte having at least two binding sites for specific binding interactions with specific binding complements, in a sample comprising the steps of:

a) providing at least one type of capture substrates that are separable from the sample, the capture substrates having bound thereto at least one specific binding complement of the specific target analyte that binds to at least a first binding site of the specific target analyte;

b) contacting the capture substrates with a sample believed to contain target analytes under conditions effective to allow for binding of the specific target analyte to the specific binding complement bound to the

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capture substrate so as to immobilize the target analytes onto the capture substrates;

c) optionally separating the capture substrate and any target analytes bound thereto from the sample;

d) providing at least one type of particle detection probes, the particle detection probes comprising a particle having bound thereto (i) at least one specific binding complement to a specific target analyte and (ii) a plurality of oligonucleotides that are optionally labeled with a reporter label, wherein the specific binding complement bound to the particle detection probes binds to at least a second binding site of the target analyte;

e) contacting the immobilized target analytes with at least one type of particle detection probes under conditions effective to allow for binding between the target analytes and the specific binding complement bound to the particle detection probes to the analyte and to form a complex in the presence of the target analyte on the capture substrate, wherein the complex comprises the target analyte and capture substrate and particle detection probe;

f) optionally separating the complex from unbound particle detection probes; and

g) detecting for the presence of the complex wherein the presence of the complex is indicative of the presence of a specific target analyte in the sample.

48. The method of claim 47, wherein the specific binding complements corresponding to the first binding site of two or more target analytes are bound to the same substrate.

49. The method of claim 48, wherein the specific binding complements corresponding to the first binding sites of one target analyte that is bound to the substrate are localized in a different physical region of the substrate than

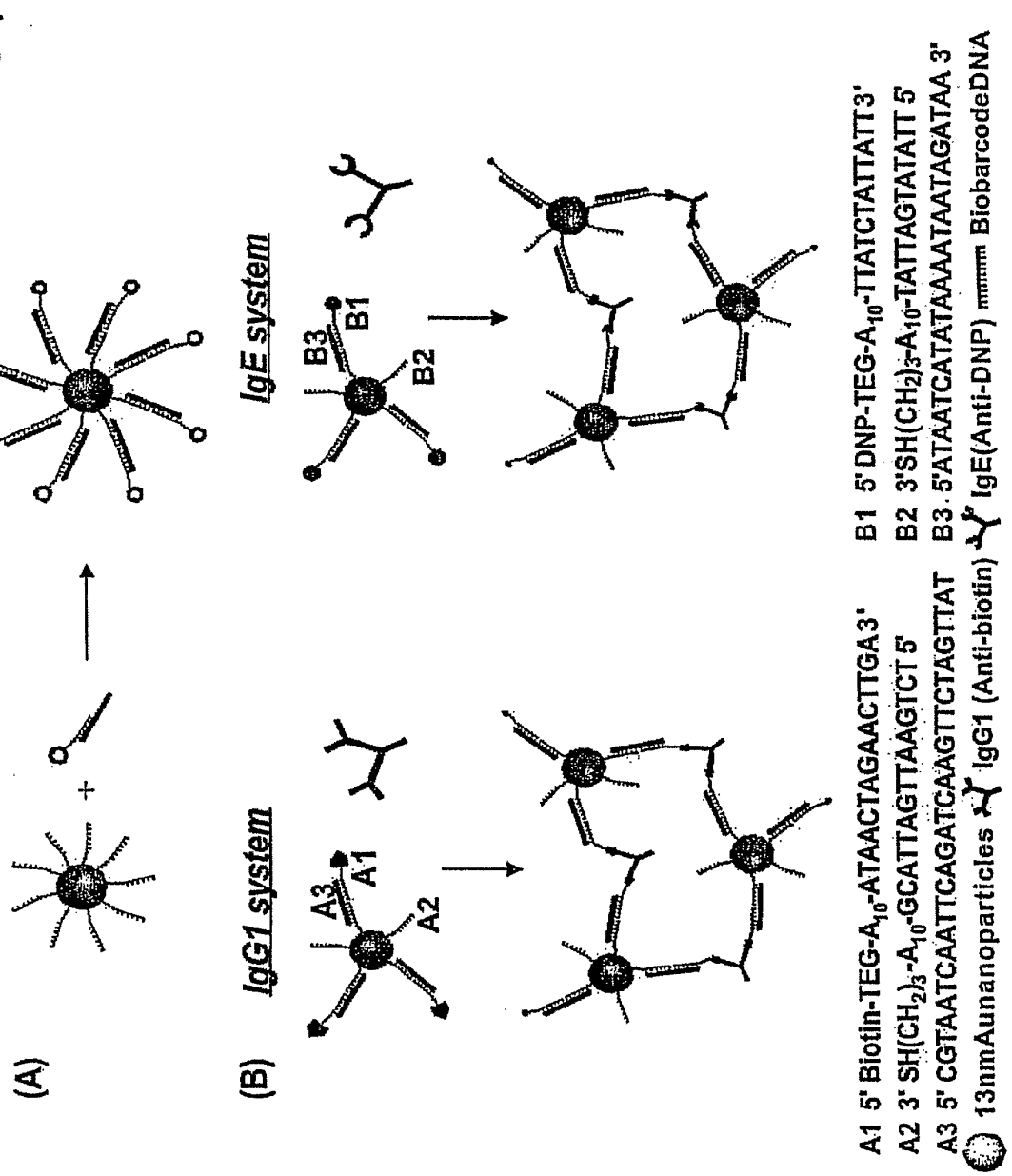
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are the specific binding complements corresponding to the first binding sites of a different target analyte.

50. The method of claim 49, wherein the oligonucleotides are released from the detection probe prior to said detecting.

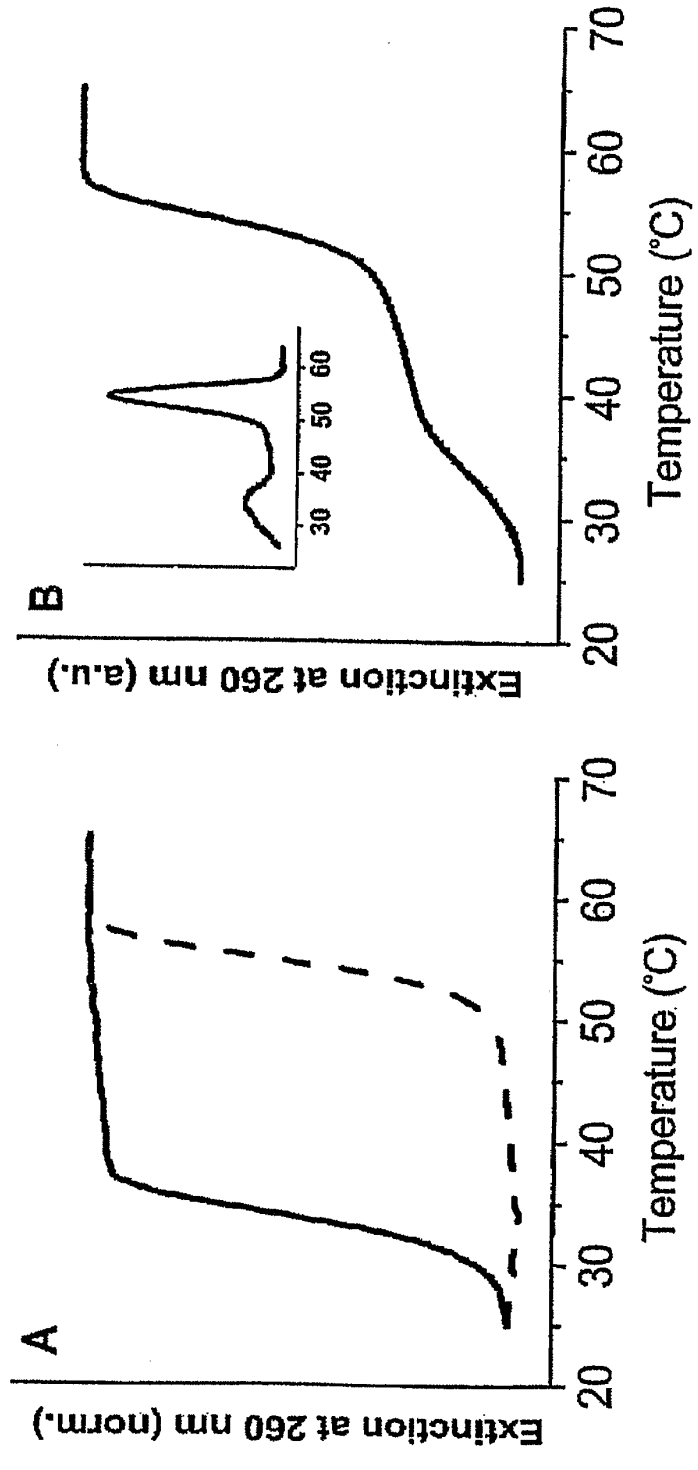
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FIGURE 1



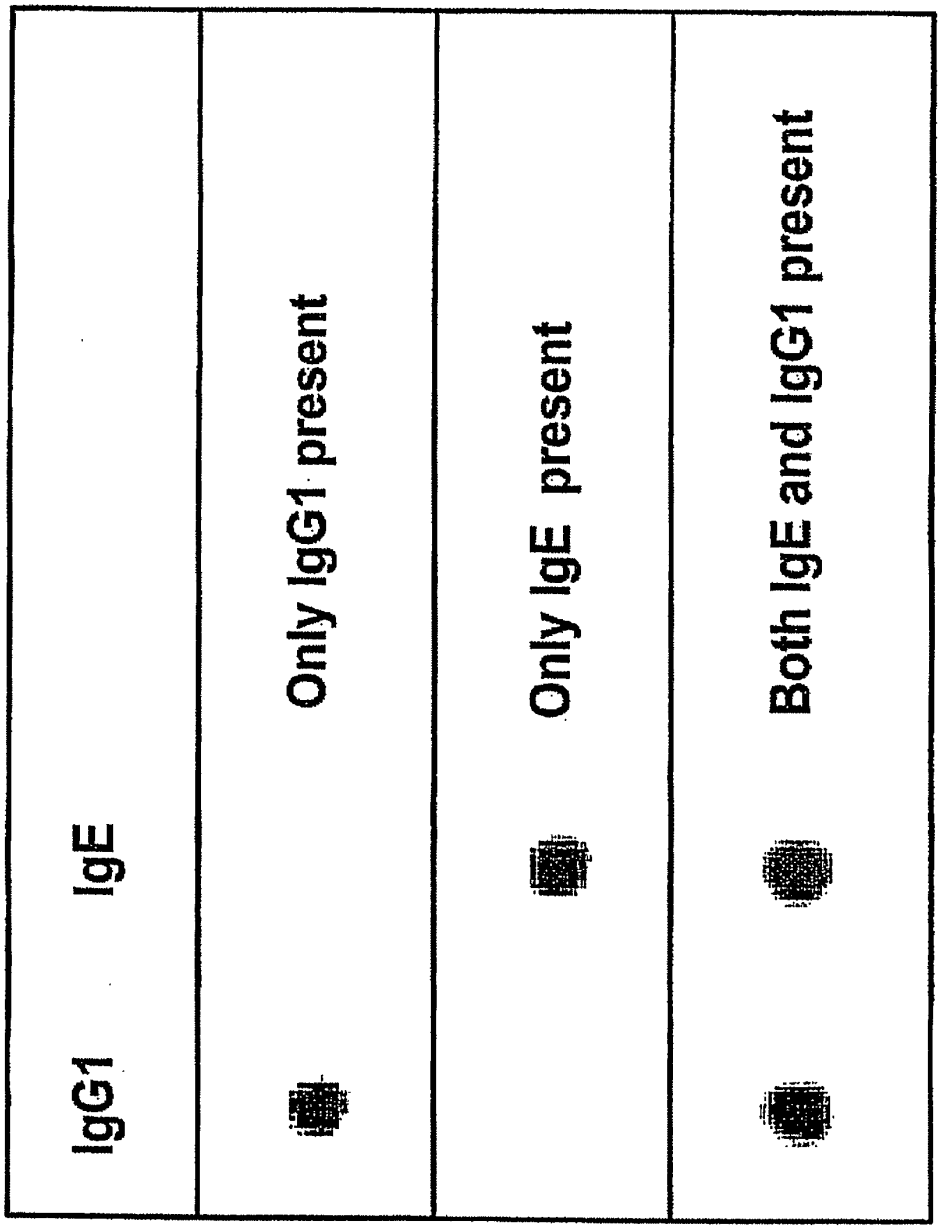
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FIGURE 2



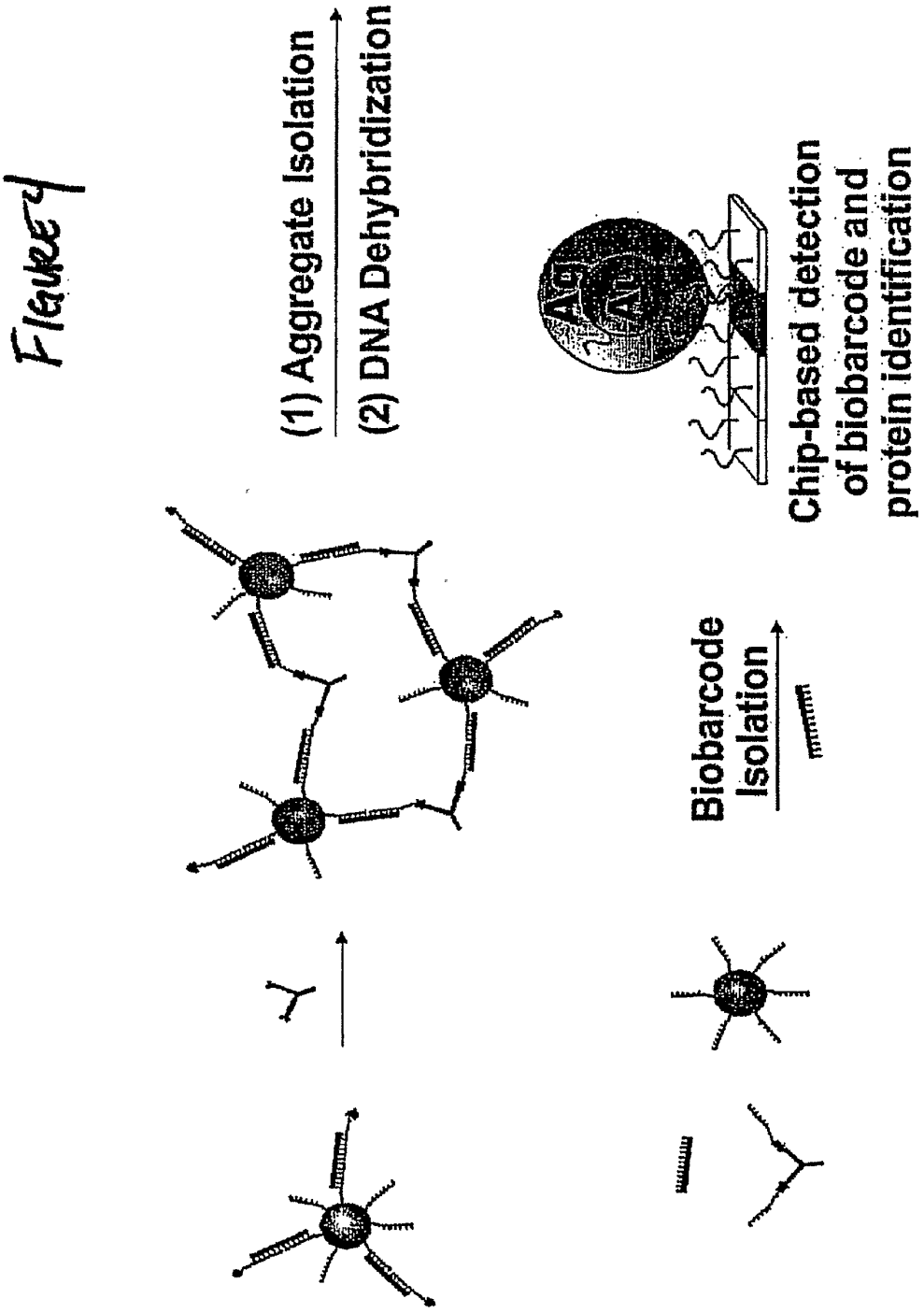
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FIGURE 3



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Figure 4



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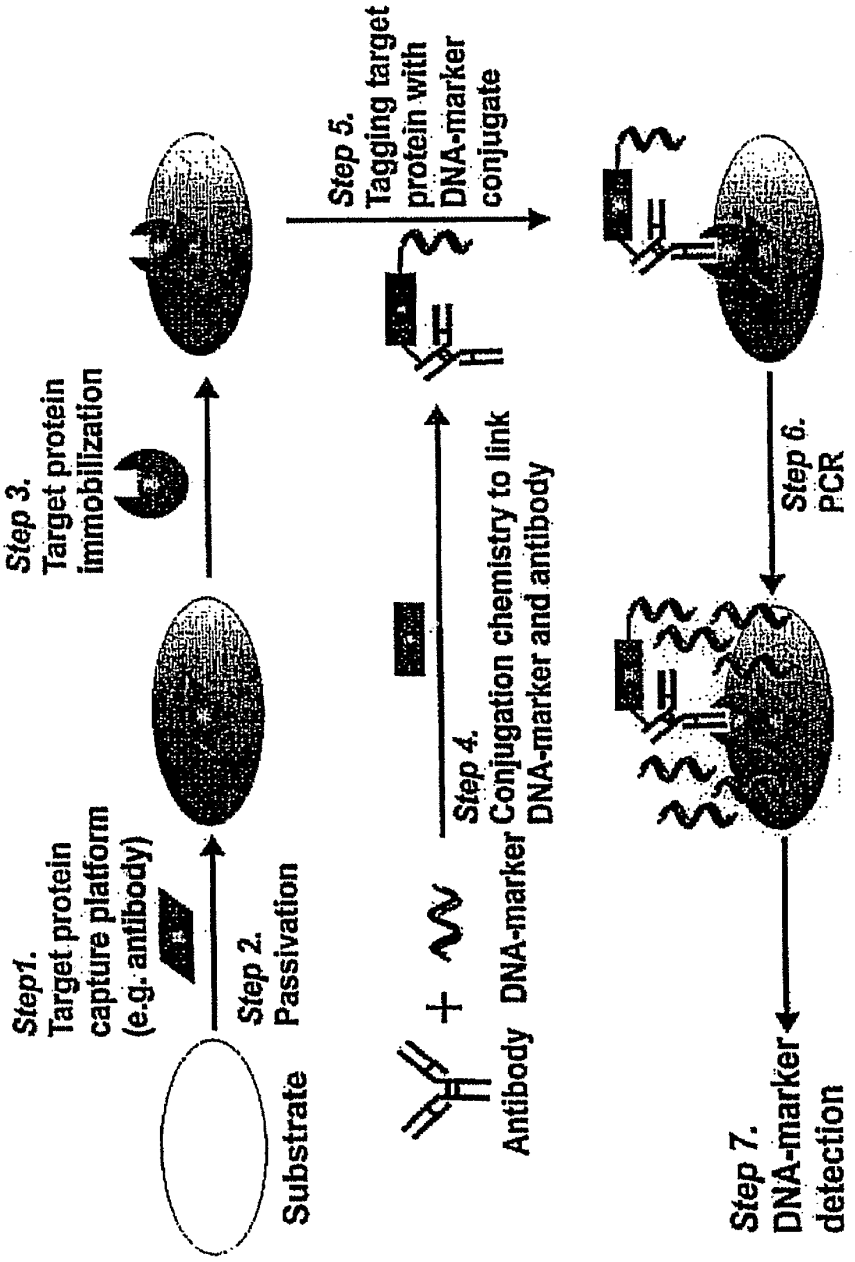
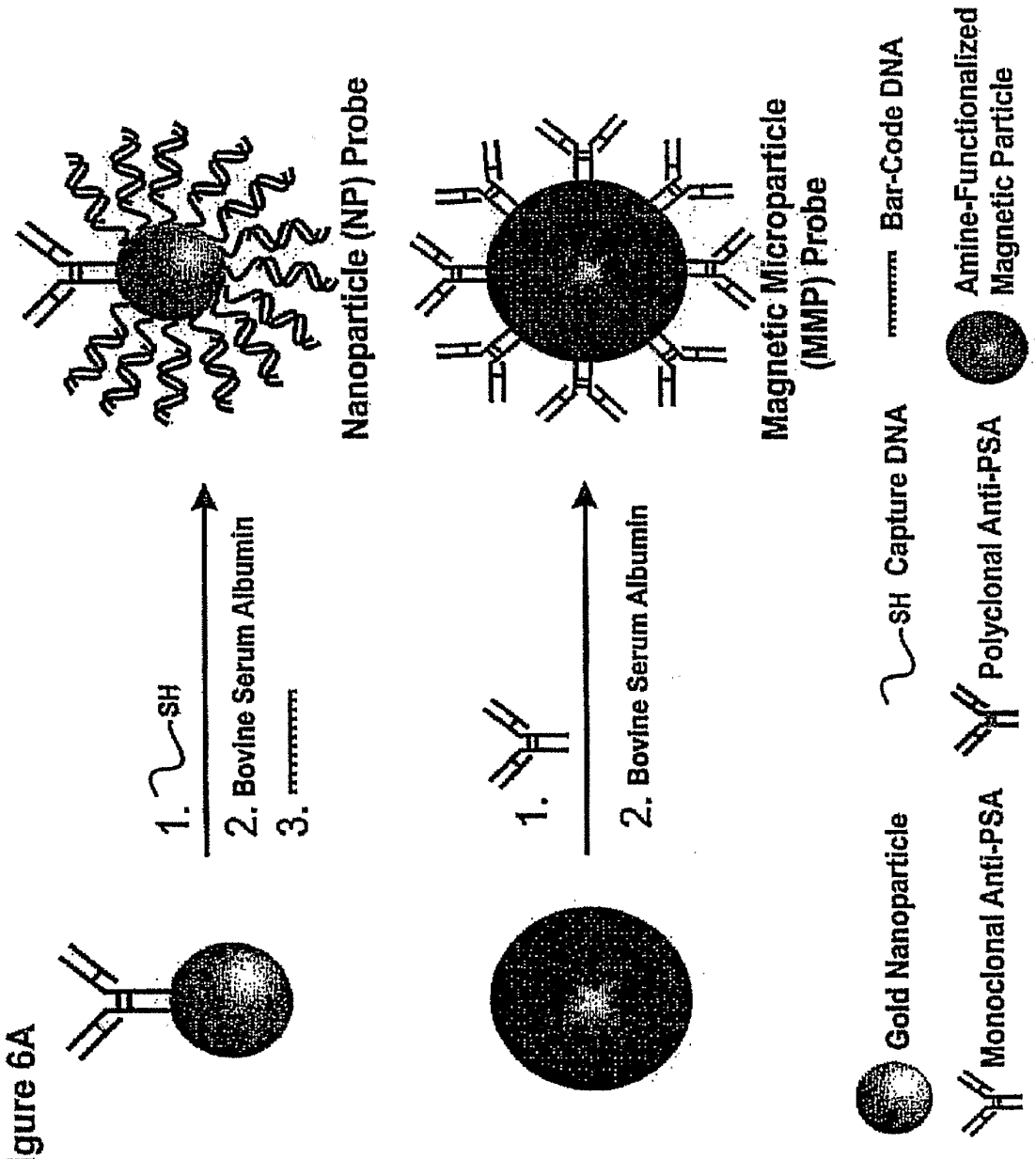


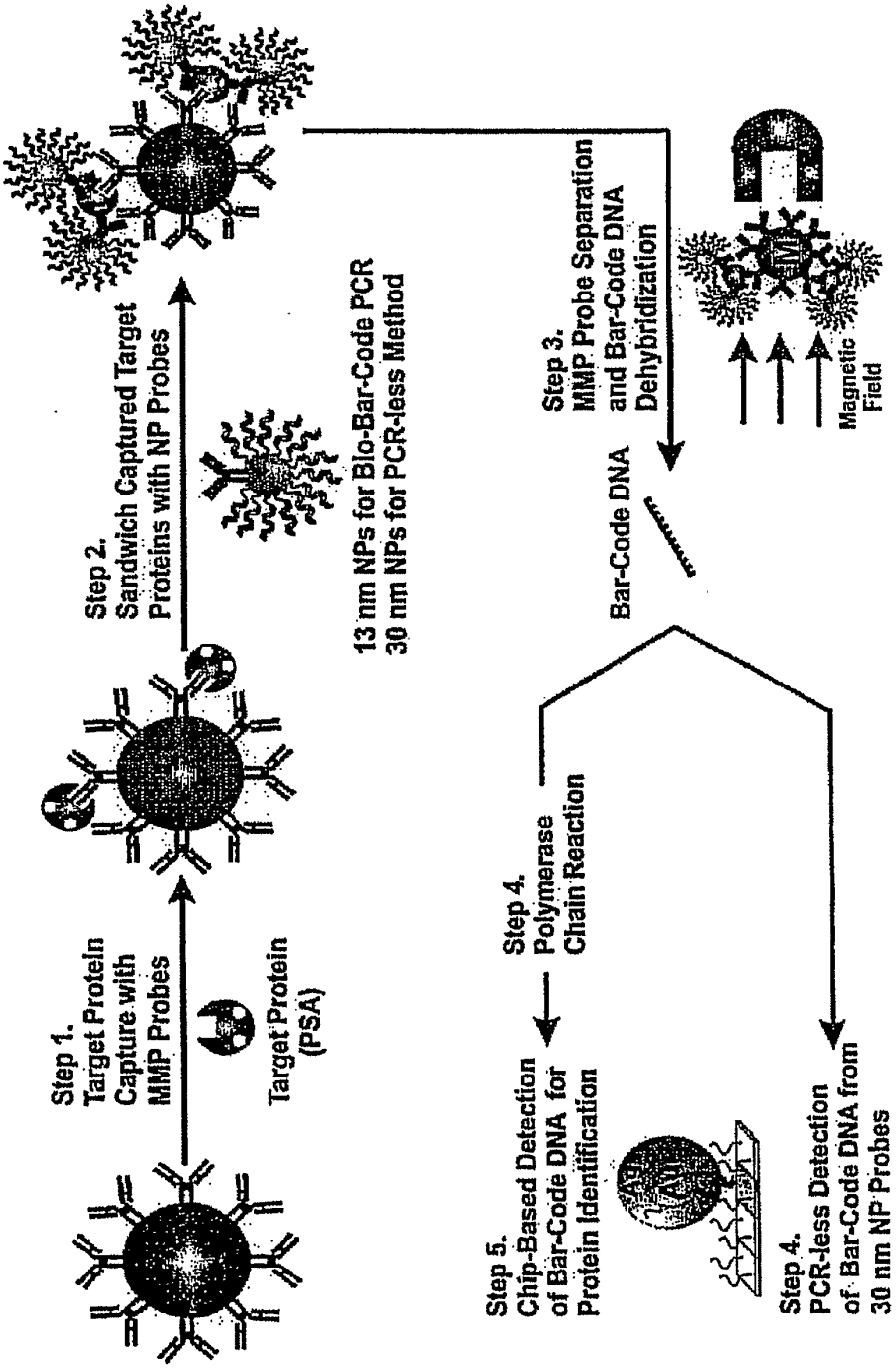
Figure 5

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Figure 6B



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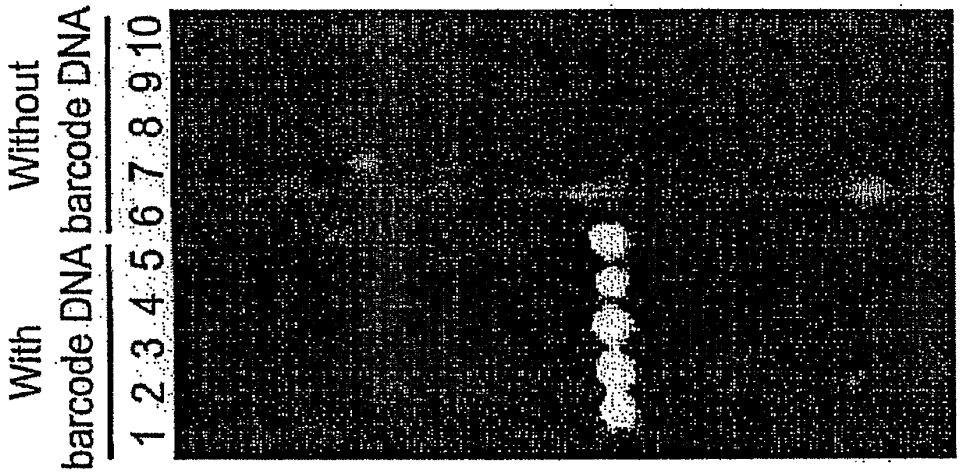


Figure 7

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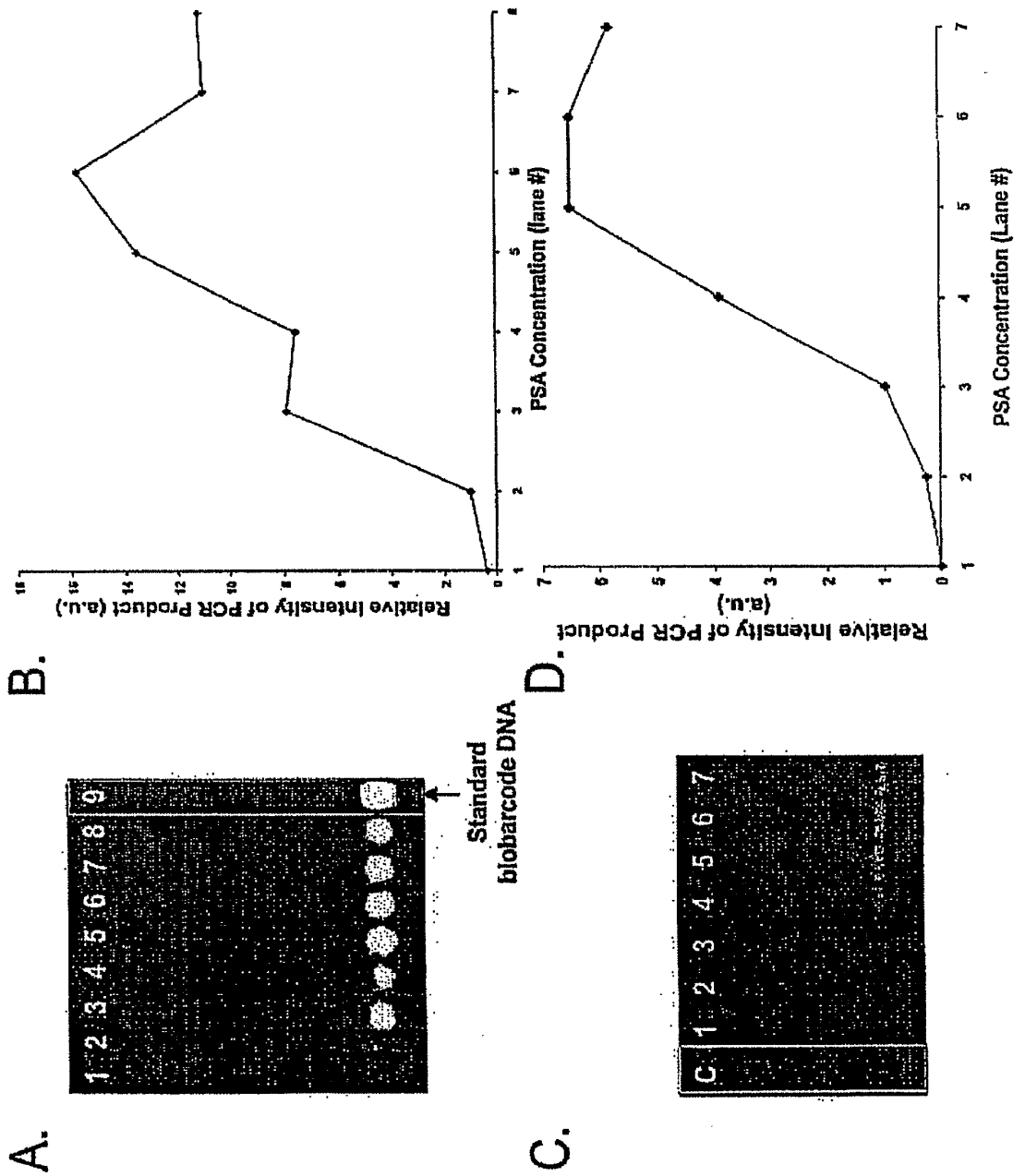


Figure 8

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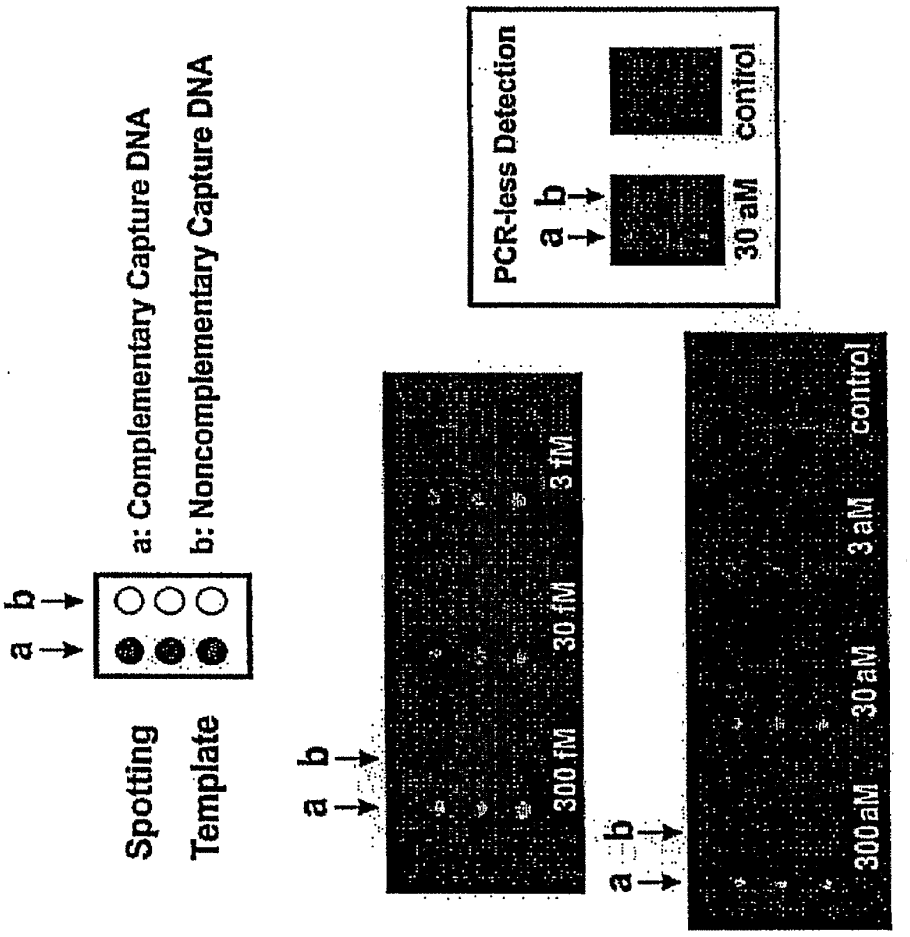
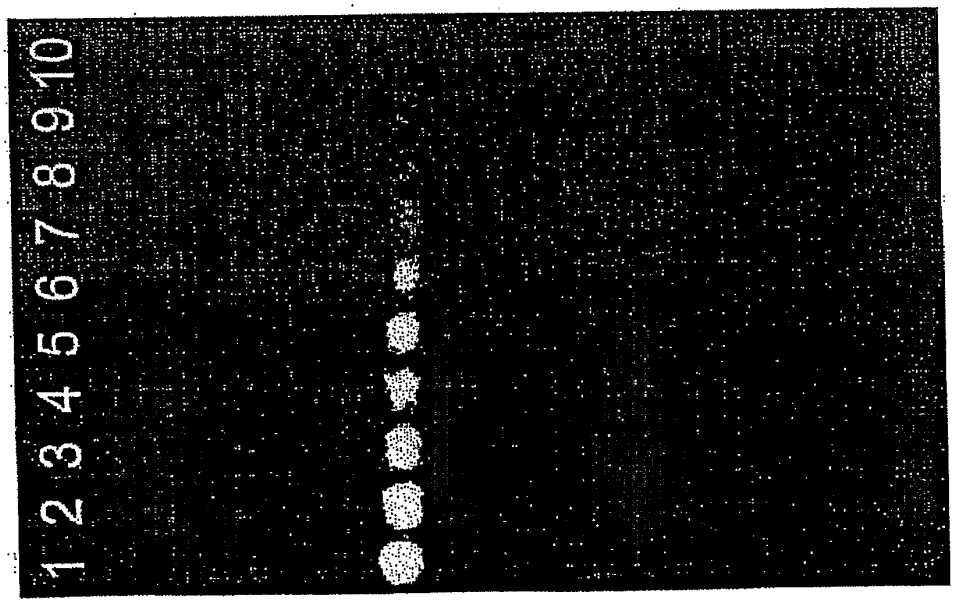


Figure 9

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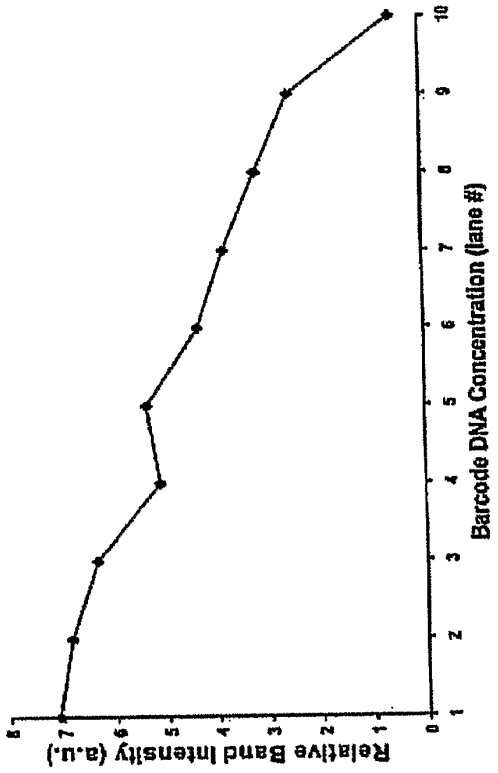


Figure 10

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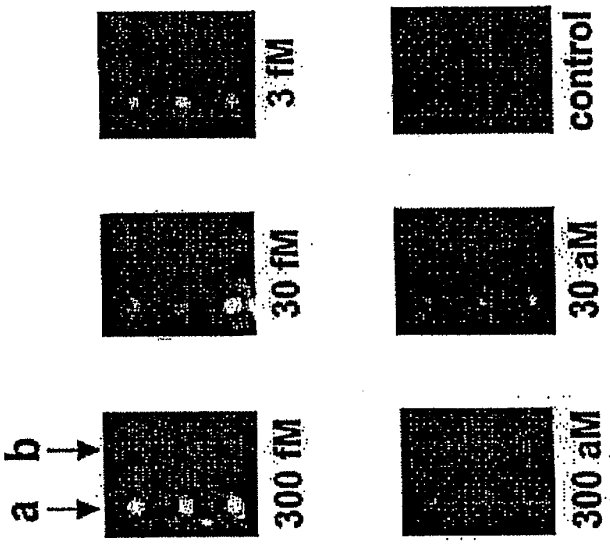


Figure 11

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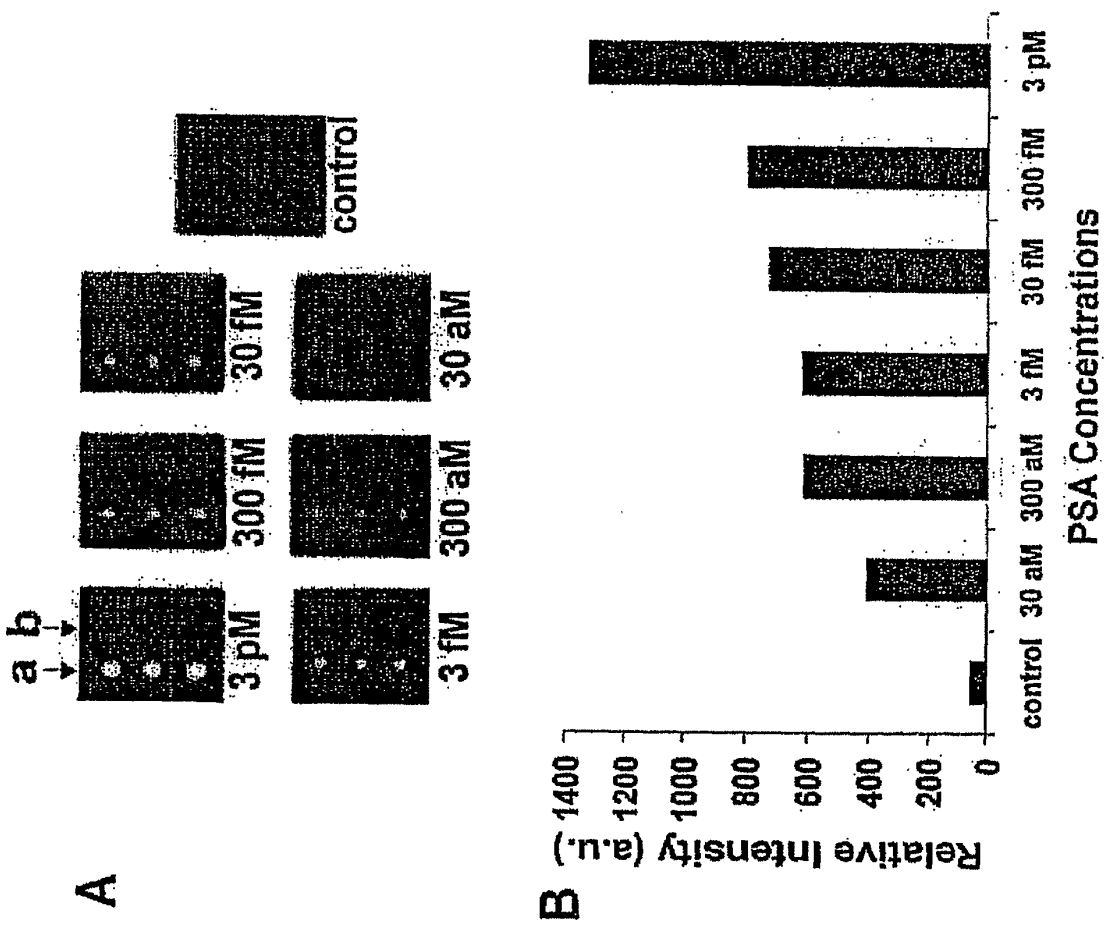


Figure 12

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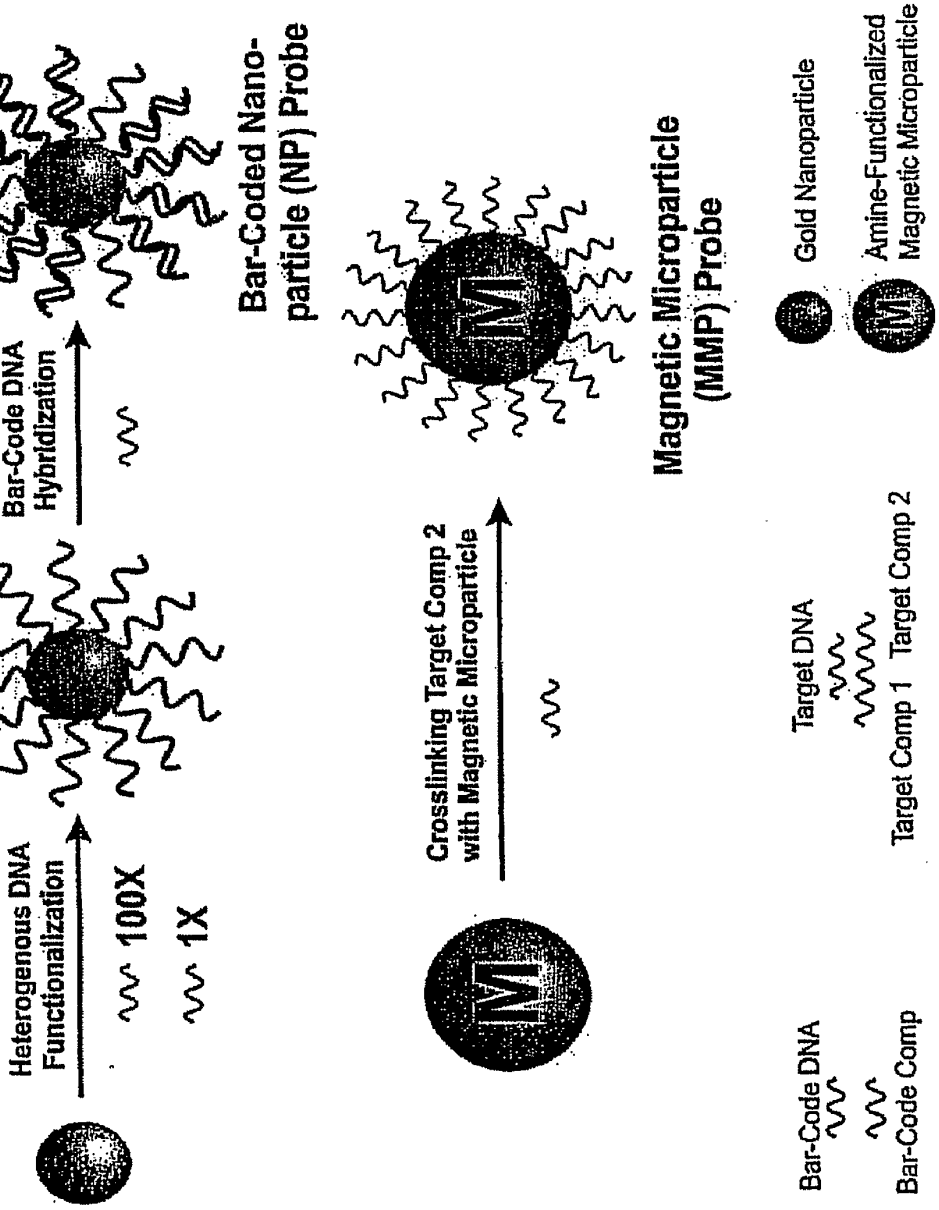


Figure 13A.

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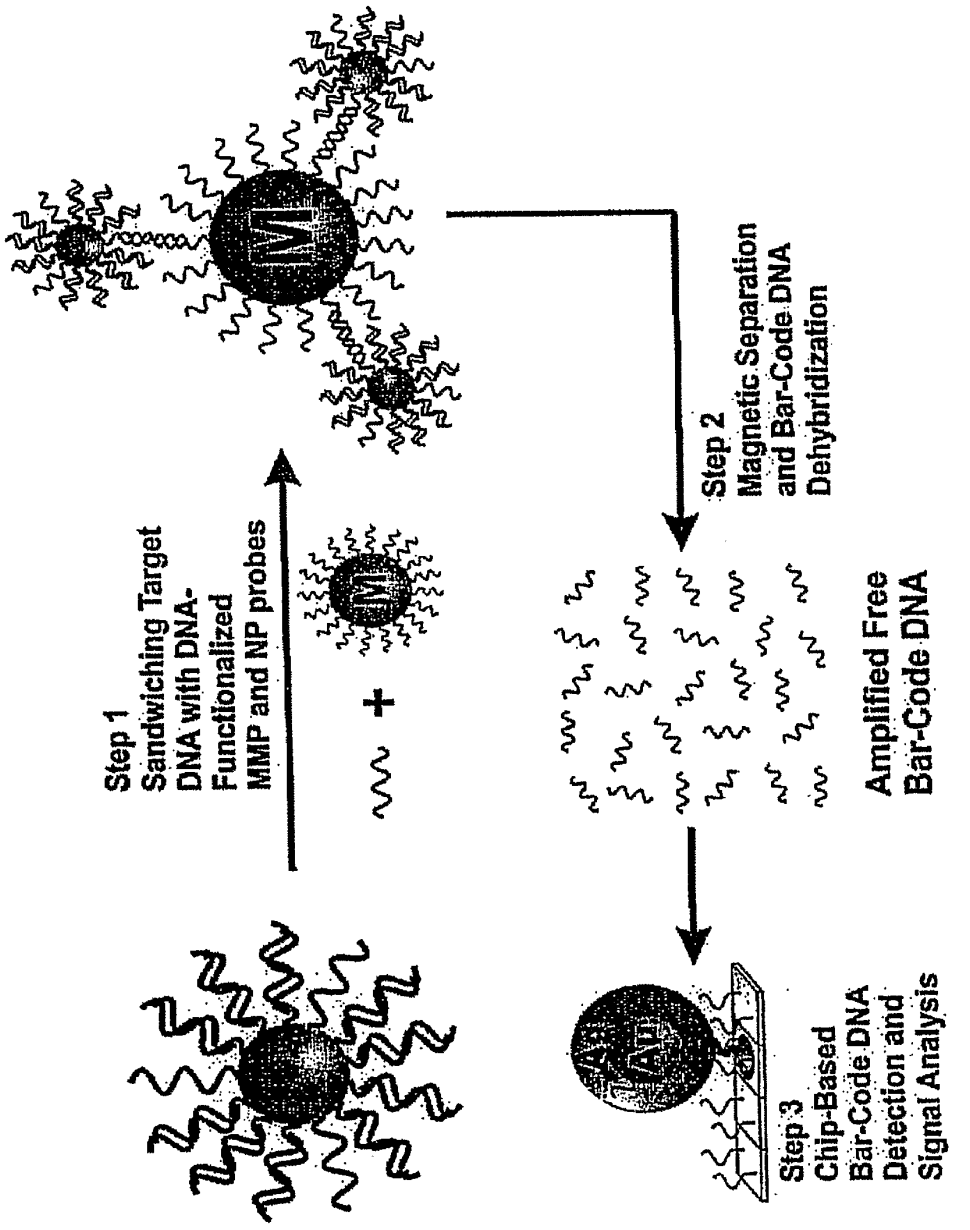


Figure 13B.

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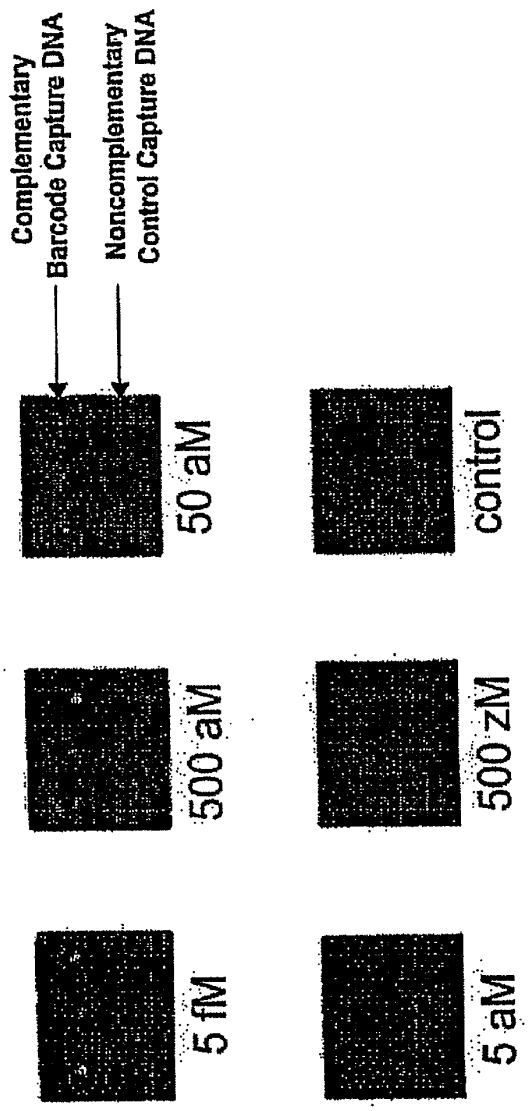


Figure 14A.

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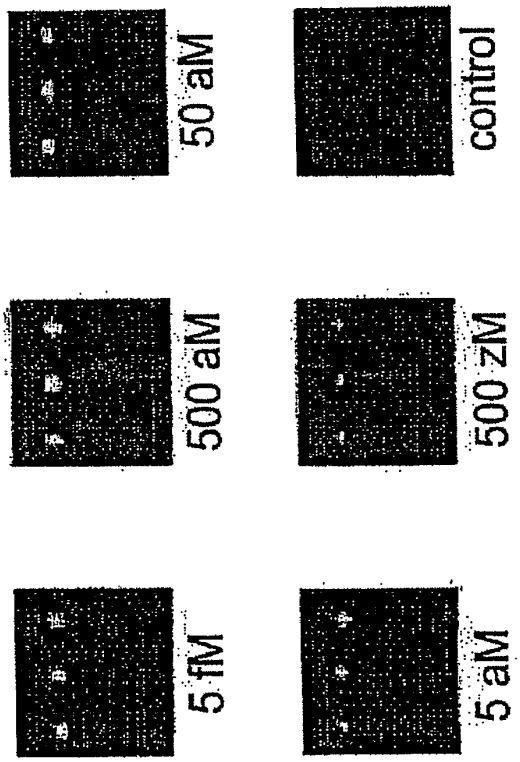


Figure 14B.

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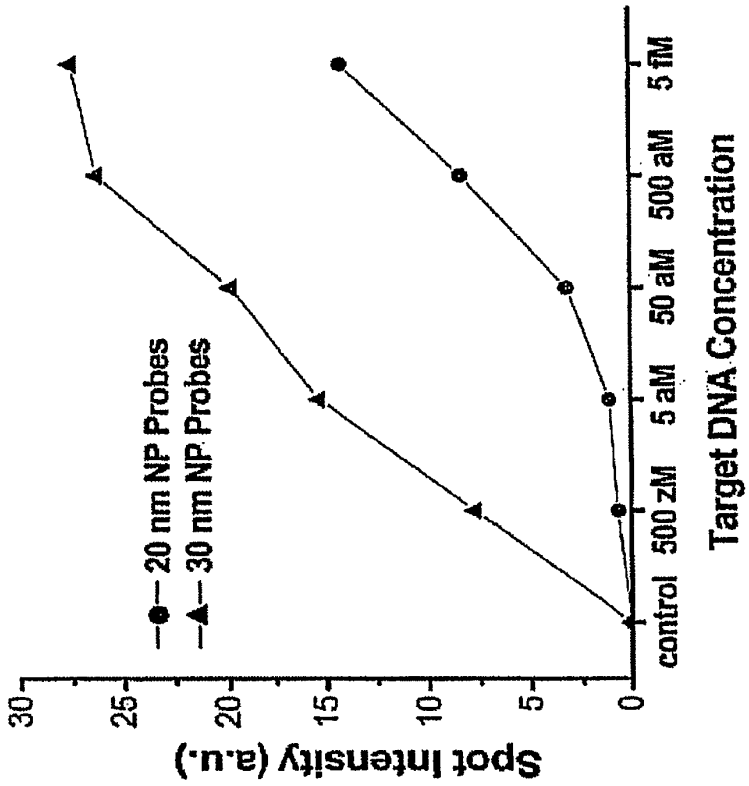
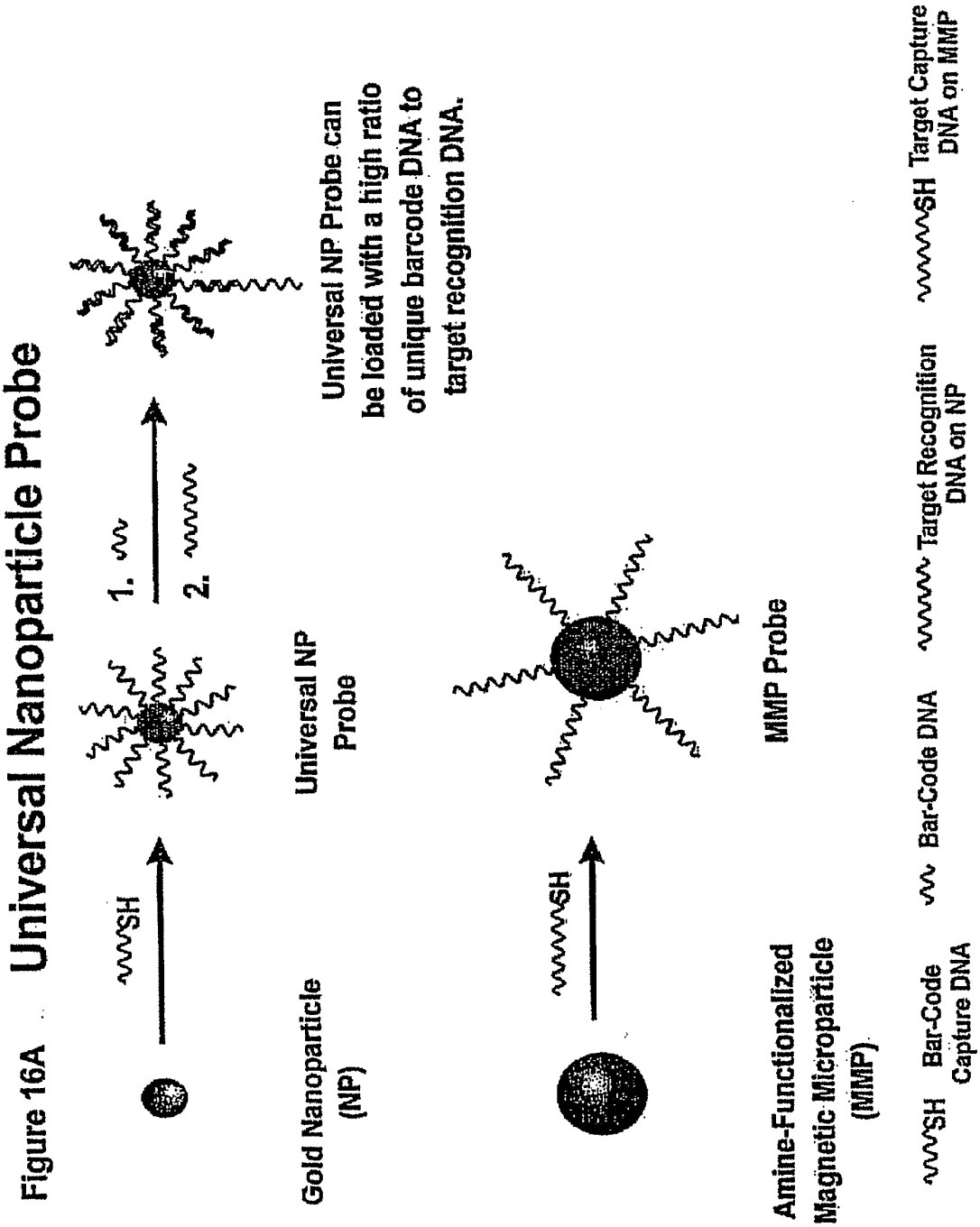


Figure 15

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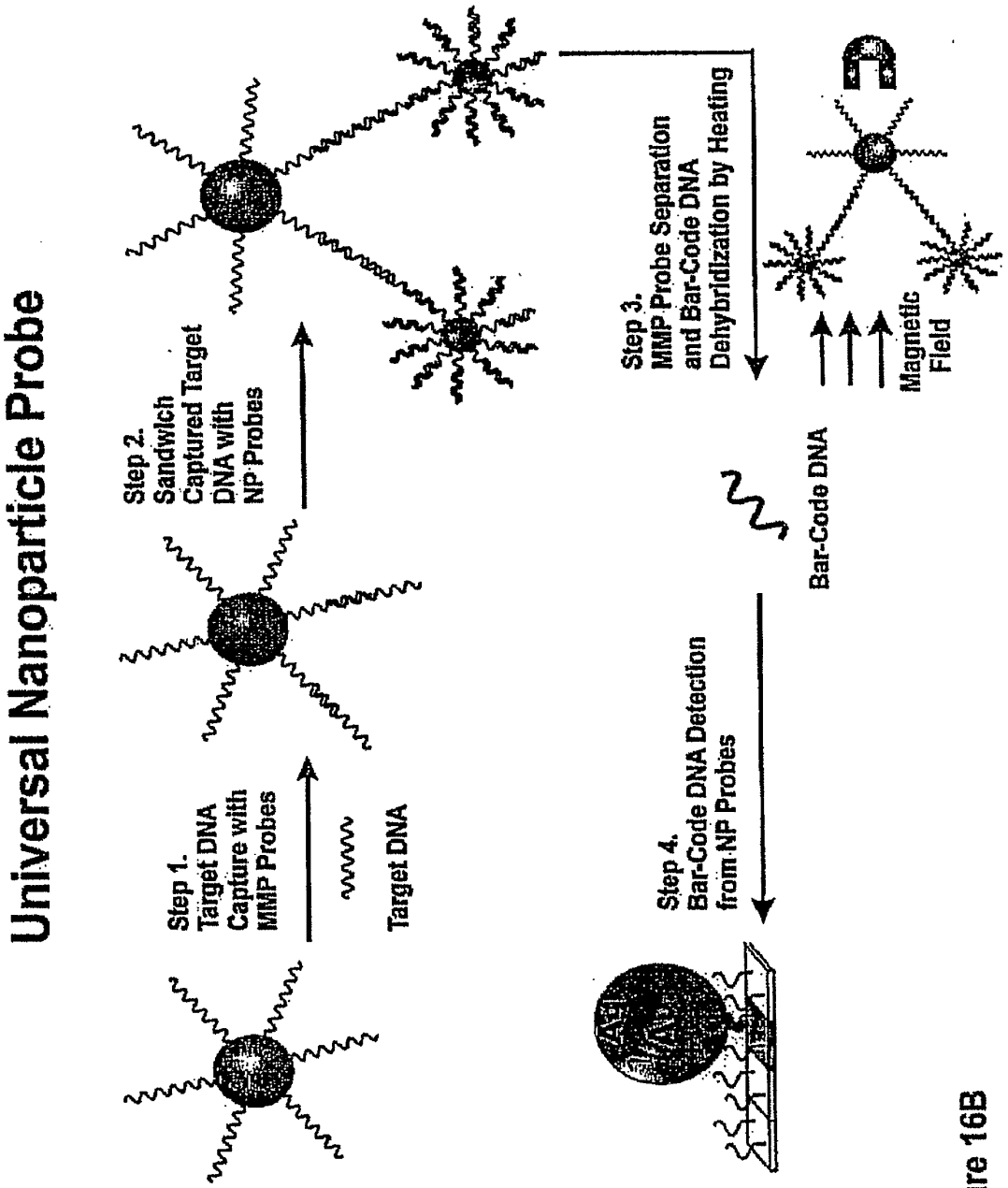


Figure 16B

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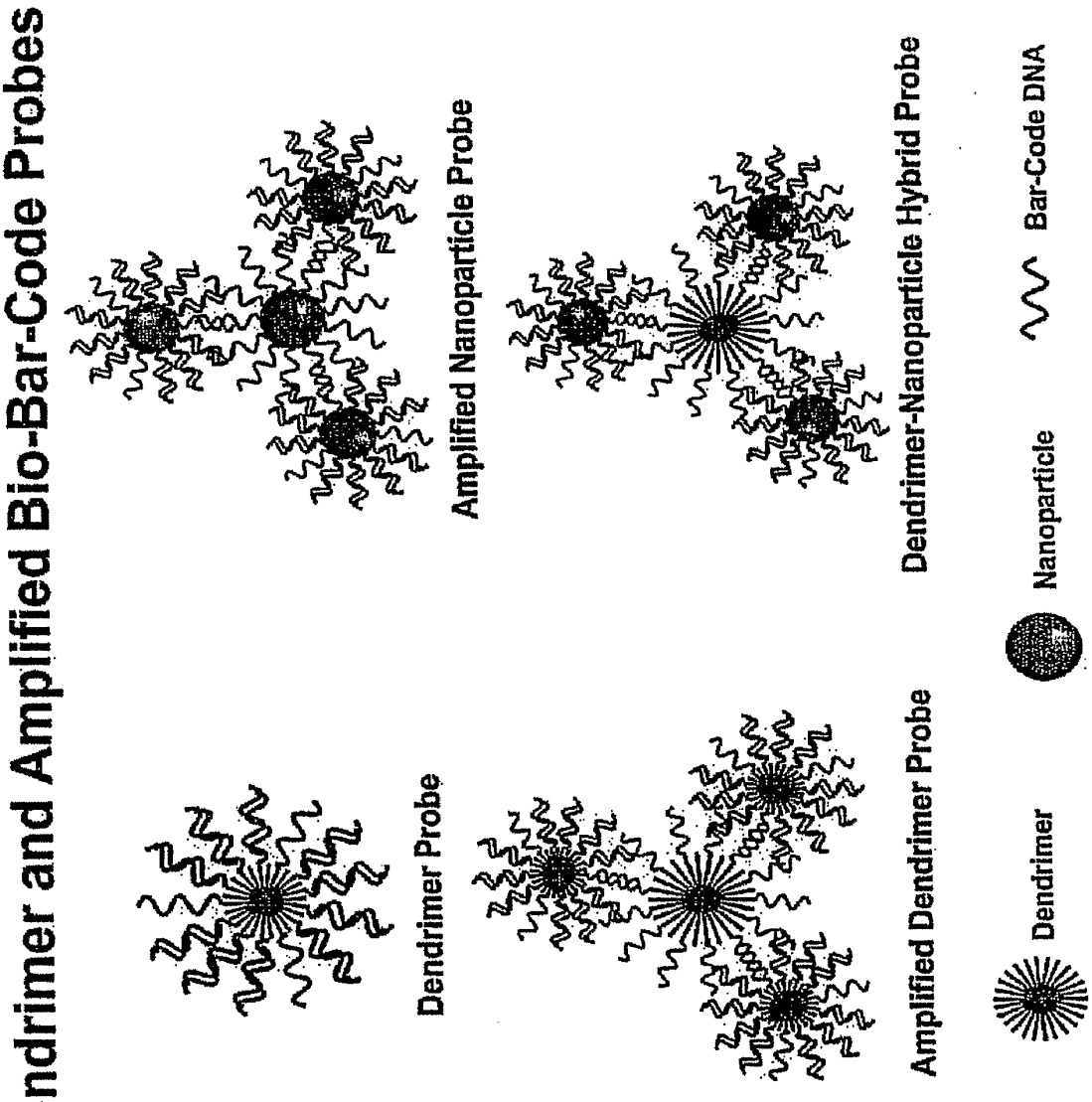


Figure 17

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Figure 18

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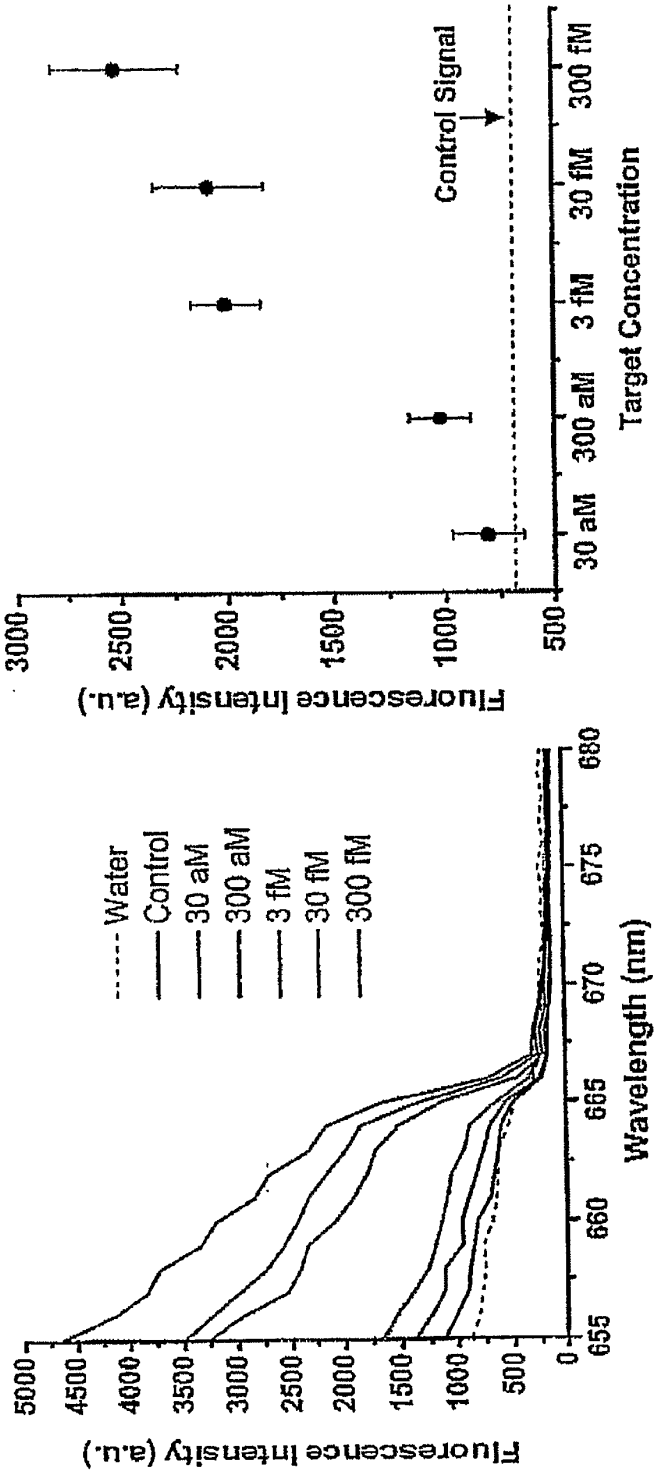
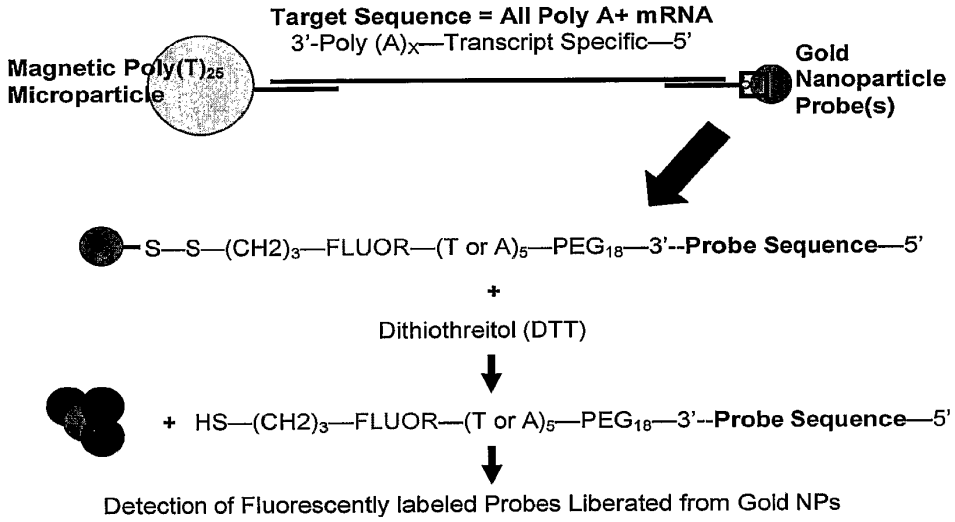


Figure 19

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Figure 20



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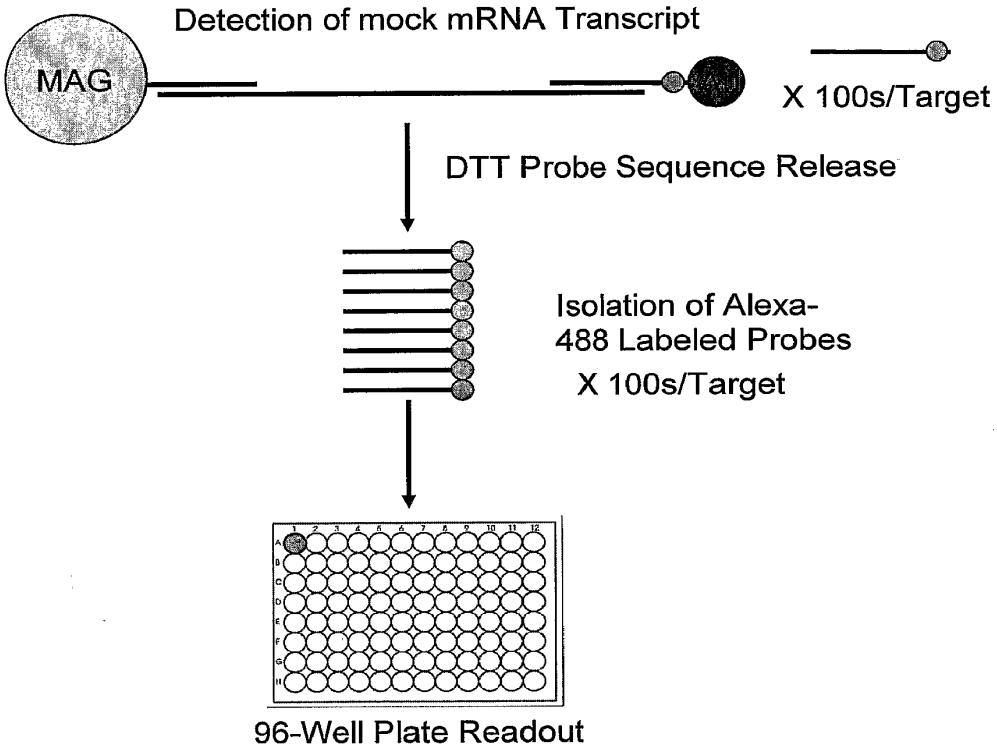
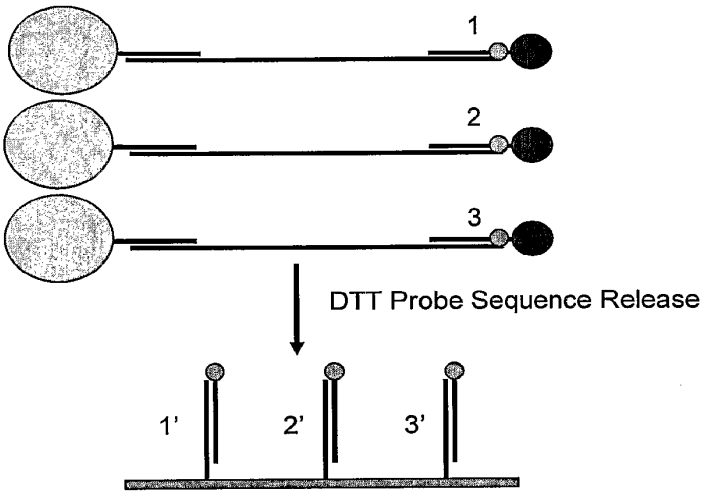


Figure 21

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Microarray Readout Options: 1) One or Two (Ratioing) Fluor System, 2) B-SA Systems (Enzymatic Amplification), 3) Maleimide-Fluor System, 4) Gold Nanoparticle System (Universal Probe, Raman, etc.)

Figure 22

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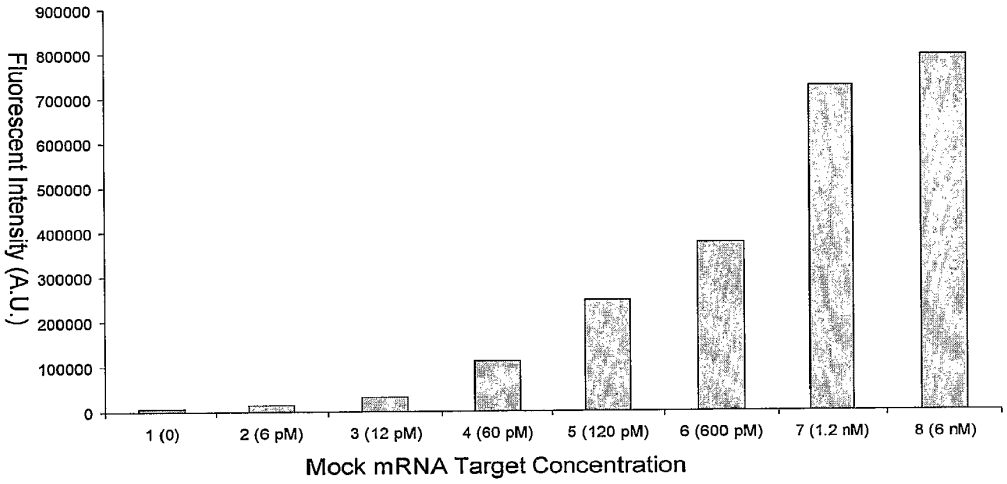


Figure 23

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(74) Agent: **POMIANEK, Michael, J.**; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210-2206 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,

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(54) Title: ULTRA-SENSITIVE DETECTION OF MOLECULES USING DUAL DETECTION METHODS

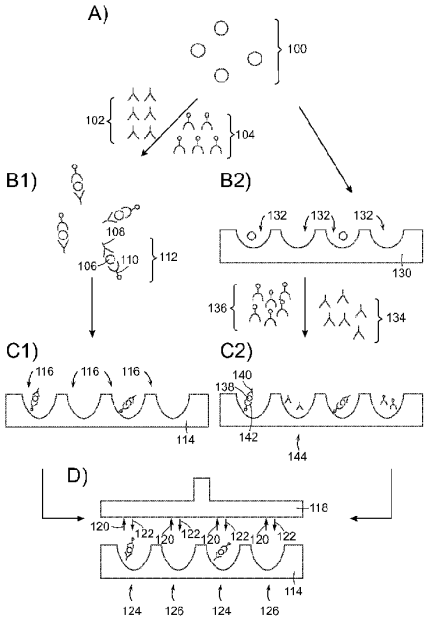


FIG. 1

(57) Abstract: Described herein are systems and methods for the detection of and/or determination of a measure of the concentration of analyte molecules or particles in a fluid sample. In some cases, the systems and methods employ techniques to reduce or limit the negative effects associated with non-specific binding events. Certain methods of the present invention involve associating the analyte molecules at least a first type of binding ligand and at least a second type of binding ligand, and spatially segregating the analyte molecules into a plurality of locations on a surface. The presence of an analyte molecule at or in a location may be determined by determining the presence of both the first type of binding ligand and the second type of binding ligand.

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EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

ULTRA-SENSITIVE DETECTION OF MOLECULES USING DUAL DETECTION METHODS

Field of the Invention

Described herein are systems and methods for the detection and/or determination of a measure of the concentration of analyte molecules or particles in a fluid sample. In some cases, the systems and methods employ techniques to reduce or limit negative effects associated with non-specific binding.

Related Applications

This application is a continuation-in-part of U.S. Patent Application Serial No. 12/731,135, filed March 24, 2010, entitled "Ultra-Sensitive Detection of Molecules Using Dual Detection Methods," by Duffy et al., which claims the benefit of U.S. Provisional Patent Application Serial No. 61/309,170, filed March 1, 2010, entitled "Ultra-Sensitive Detection of Molecules Using Dual Detection Methods," by Duffy et al. Each of the above-indicated applications is incorporated herein by reference.

Background of the Invention

Methods and systems that are able to quickly and accurately detect and, in certain cases, quantify a target analyte molecule in a sample are the cornerstones of modern analytical measurements. Such systems and methods are employed in many areas such as academic and industrial research, environmental assessment, food safety, medical diagnosis, and detection of chemical, biological, and/or radiological warfare agents. Advantageous features of such techniques may include specificity, speed, and sensitivity.

Many of the known methods and techniques are plagued with problems of non-specific binding, which is the binding of analyte molecules or particles to be detected or reporter species non-specifically to sites other than those expected. Non-specific binding events can lead to an increase in the background signal, and can affect the accuracy of the concentration determination and/or limit the lowest concentration that may be accurately or reproducibly detected.

Accordingly, improved methods for reducing the effects of non-specific binding events are needed, especially in samples where such molecules or particles are present at very low concentration.

Summary of the Invention

Described herein are systems and methods for the detection and/or determination of a measure of the concentration of analyte molecules or particles in a fluid sample, and in some cases, the systems and methods employ techniques to reduce or limit the negative effects associated with non-specific binding events. The subject matter of the present invention involves, in some cases, interrelated products, alternative solutions to a particular problem, and/or a plurality of different uses of one or more systems and/or articles.

In some embodiments, a method for determining a measure of the concentration of analyte molecules or particles in a fluid sample comprises providing the analyte molecules or particles immobilized with respect to a binding surface having affinity for at least one type of analyte molecule or particle, the binding surface forming or contained within one of a plurality of locations on a substrate, such that at least some of the plurality of locations contain at least one analyte molecule or particle and a statistically significant fraction of the plurality of locations do not contain any analyte molecules or particles, exposing the analyte molecules or particles to a first type of binding ligand having an affinity for a first epitope on the analyte molecules or particles and a second type of binding ligand having an affinity for a second epitope on the analyte molecules or particles, addressing at least a portion of the plurality of the locations and determining the number of said locations containing a first type of binding ligand and a second type of binding ligand, and determining a measure of the concentration of analyte molecules or particles in the fluid sample based at least in part on the number of locations containing both a first type of binding ligand and a second type of binding ligand.

In some embodiments, a method for determining a measure of the concentration of analyte molecules or particles in a fluid sample comprises providing a fluid sample containing or suspected of containing a plurality of analyte molecules or particles, exposing the plurality of analyte molecules or particles to a first type of binding ligand having an affinity for a first epitope on the analyte molecules or particles and a second type of binding ligand having an affinity for a second epitope on the analyte molecules or particles, spatially separating at least a portion of the analyte molecules or particles into a plurality of locations, such that at least some of the plurality of locations contain at least one analyte molecule or particle and a statistically significant fraction of the plurality of locations do not contain any analyte molecules or particles, addressing at least a portion of the plurality of locations and determining the number of said locations containing both

a first type binding ligand and a second type of binding ligand, and determining a measure of the concentration of analyte molecules or particles in the fluid sample based at least in part on the number of locations containing both a first type of binding ligand and a second type of binding ligand.

Brief Description of the Drawings

Other aspects, embodiments, and features of the invention will become apparent from the following detailed description when considered in conjunction with the accompanying drawings. The accompanying figures are schematic and are not intended to be drawn to scale. For purposes of clarity, not every component is labeled in every figure, nor is every component of each embodiment of the invention shown where illustration is not necessary to allow those of ordinary skill in the art to understand the invention. All patent applications and patents mentioned in the text are incorporated by reference in their entirety. In case of conflict between the description contained in the present specification and a document incorporated by reference, the present specification, including definitions, will control.

FIG. 1 is a schematic flow diagram depicting embodiments of steps (A-B1-C1-D) and (A-B2-C2-D) for performing exemplary methods of the present invention;

FIG. 2 illustrates an exemplary method of indirect detection;

FIG. 3 is a schematic flow diagram depicting one embodiment of steps (A-C) for performing an exemplary method of the present invention;

FIG. 4 is a schematic flow diagram depicting one embodiment of steps (A-D) for performing an exemplary method of the present invention;

FIG. 5 illustrates an exemplary method of indirect detection;

FIGS. 6A and 6B show schematic diagrams depicting some embodiments of steps for crosslinking a binding ligand and an analyte molecule, according to some methods of the present invention;

FIG. 7 is a schematic flow diagram depicting an embodiment of a method (steps A-D) for the formation of a plurality of reaction vessels through mating of a substrate and a sealing component and depicting examples of the size (E, F) of a sealing component relative to a substrate;

FIG. 8A depicts an experimental set-up for detection using light, according to one embodiment of the present invention;

FIG. 8B shows a fiber optic array that has been sealed with a sealing component, according to one embodiment;

FIGS. 9A and 9B shows plots of dissociation of immunocomplexes over time, according to some embodiments; and

FIG. 10 shows plot of average analyte molecules per bead versus concentration of analyte molecules, according to some embodiments.

Detailed Description

Described herein are systems and methods for the detection and/or determination of a measure of the concentration of analyte molecules or particles (such as, for example, cells, cell organelles and other biological or non-biological particulates) in a fluid sample that, in some cases, employ techniques to reduce or limit negative effects associated with non-specific binding events. The subject matter of the present invention involves, in some cases, interrelated products, alternative solutions to a particular problem, and/or a plurality of different uses of one or more systems and/or articles. It should be understood, that while much of the discussion below is directed to analyte molecules, this is by way of example only, and other materials may be detected and/or quantified, for example, analytes in particulate form. Some exemplary analyte molecules and particles are described herein.

The systems and methods of the present invention, in certain instances, may help reduce or limit certain negative effects of non-specific binding events on detection sensitivity and/or accuracy when compared to typical conventional systems and methods for performing similar assays. Non-specific binding is the binding or association in a non-specific fashion of one component of an assay with another component of the assay with which it is not desirable that it interact. For example, association, binding, or immobilization of a binding ligand with a substrate or assay material as opposed to with an analyte molecule or particle to which it has binding specificity. Non-specific binding may lead to false positive signals and may not only affect the accuracy of the assay measurement, but may also limit the lowest level of detection. Therefore, certain methods and/or systems of the present invention that provide improvements in reducing the negative effects of non-specific binding may allow for the detection and/or quantification of analyte molecules in a sample at a lower detection limit and/or with greater accuracy as compared to typical conventional technologies. In addition, certain embodiments of the methods and/or systems of the present invention may also allow for

the detection and/or quantification of analyte molecules in certain samples in which such analyte molecules have previously been undetected and/or unquantifiable because of the very low concentration at which they are present.

In some embodiments, the methods of the present invention may be used in conjunction with methods or systems used for determining a measure of the concentration of analyte molecules in a fluid sample. In some cases, the methods and systems may comprise the detection and/or quantification of single analyte molecules. In certain such embodiments, the analyte molecules in the fluid sample may be spatially segregated into a plurality of locations (e.g., on a surface) such that each analyte molecule can be individually interrogated. In some cases, the methods and systems involve immobilizing the plurality of analyte molecules with respect to a plurality of locations, wherein at least some, and in certain embodiments substantially each, location comprises either zero or one analyte molecules. A measure of the concentration of analyte molecules in the fluid sample may be determined using a binary read-out system, wherein the measure of the concentration is determined at least in part based on the number of locations which are determined to contain an analyte molecule. That is, a binary read-out method comprises determining a measure of the concentration of analyte molecules in a fluid sample at least in part on the number of "on" locations (e.g., locations with a positive signal). For examples of non-limiting methods and systems, see U.S. Patent Application Serial No. 12/731,130, entitled "Ultra-Sensitive Detection of Molecules or Particles using Beads or Other Capture Objects," by Duffy et al., filed March 24, 2010; International Patent Application No. (not yet determined), entitled "Ultra-Sensitive Detection of Molecules or Particles using Beads or Other Capture Objects," by Duffy et al., filed March 1, 2011 (Attorney Docket No. Q0052.70011WO00); U.S. Patent Application No. 20070259448, entitled "Methods and arrays for target analyte detection and determination of target analyte concentration in solution," by Walt et al., filed Feb. 16, 2007; U.S. Patent Application No. 20070259385, entitled "Methods and arrays for detecting cells and cellular components in small defined volumes," by Walt et al., filed Feb. 16, 2007; U.S. Patent Application No. 20070259381, entitled "Methods and arrays for target analyte detection and determination of reaction components that affect a reaction," by Walt et al., filed Feb. 16, 2007; International Patent Application No. PCT/US07/019184, entitled "Methods for Determining The Concentration of an Analyte In Solution," by Walt et al., filed August 20, 2007; International Patent Application No. PCT/US09/005428, entitled "Ultra-Sensitive

Detection of Molecules or Enzymes," by Duffy et al., filed September 9, 2009; U.S. Patent Application Serial No. 12/731,136, filed March 24, 2010, entitled "Methods and systems for extending dynamic range in assays for the detection of molecules or particles," by Rissin, et al. (Attorney Docket No. Q0052.70013US01); and International Patent Application No. (not yet determined) entitled "Methods and systems for extending dynamic range in assays for the detection of molecules or particles," by Rissin et al., filed March 1, 2011 (Attorney Docket No. Q0052.70013WO00), each herein incorporated by reference.

The methods of the present invention may reduce certain negative effects of non-specific binding by employing detection methods which involve the association of at least two components (e.g., binding ligands) with each analyte molecule for detection of the analyte molecule. Certain such methods can reduce the number of false positive (e.g., non-specific events) as compared to methods which require only a single component for detection. This can be easily understood when comparing two methods which either employ one component or two components for detection. For methods which require only a single component to associate with an analyte molecule for detection, generally, a significant number of these single components may bind non-specifically to locations. Each instance of non-specific binding of a single component with a location results in the determination of an "on" location (e.g., a false positive), thus increasing the effective number locations determined to contain an analyte molecule, and thereby directly affecting a parameter (i.e., the number of "on" locations) used to calculate a measure of the concentration of analyte molecules in the fluid sample when using a binary read-out digital detection mode of quantification. In a two component system, only locations which contain both components are determined to be "on" locations. The probability of both components binding non-specifically in a single location, which would result in a false positive, may be significantly smaller as compared to the number of false positives that would be observed for a single component system, when assuming that the non-specific binding affinity for the types of components are the same. Thus a two component system can result in a decrease in the number of false positives caused by non-specific binding of a binding ligand, thereby effectively reducing effects of non-specific binding events on the concentration determination.

For example, in one embodiment, a plurality of analyte molecules is spatially separated into a plurality of locations. Two types of components are provided to the assay under conditions such that substantially all analyte molecules associate with (e.g.,

specifically bind to) at least one of each type of component. The analyte molecules are then individually interrogated. Only locations which contain at least one of each type of component are determined to be a location which comprises an analyte molecule. Locations that only contain a single component are considered to be locations which do not contain an analyte molecule (e.g., location in which a single component has non-specifically bound are treated as "off" locations), and do not affect the determination of the measure of the concentration of analyte molecules in a fluid sample. As a comparison, consider a method conducted using the same steps as outline above, but with only a single component being used to facilitate detection. In such a method, locations which contain a single component due to non-specific binding are indistinguishable from locations which contain an analyte molecule and a single component. Thus, locations with only a single binding ligand are considered to be "on" locations (e.g., location in which a single component has non-specifically bound resulting in a false positive signal) and would undesirably affect the determination of the measure of the concentration of analyte molecules in a fluid sample.

In some embodiments, the methods of the present invention comprise the use of a plurality of types of binding ligands (examples of components for detection). A "binding ligand," as used herein, is any molecule, particle, or the like, which specifically binds to or otherwise specifically associates with an analyte molecule or another binding ligand and may aid in the detection of the analyte molecule. In some cases, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten, or more, binding ligands may be employed. A plurality of analyte molecules may be exposed to the differing types of binding ligands substantially simultaneously and/or substantially sequentially.

Binding ligands may aid in the detection of analyte molecules. For example, certain binding ligands comprise an entity which is able to facilitate detection, either directly (e.g., via a detectable moiety) or indirectly. In the case of direct detection, the binding ligands may comprise a molecule or moiety that may be directly interrogated and/or detected (e.g., a fluorescent entity). In the case of indirect detection, an additional component is used for determining the presence of the binding ligand. For example, a binding ligand associated with an analyte molecule may be exposed to a precursor labeling agent, wherein the precursor labeling agent is converted into a labeling agent upon exposure to the binding ligand associated with an analyte molecule, and the labeling agent is directly detected. As another example, a component (e.g., a

third type of binding ligand) may be provided which associates with a first type of binding ligand and a second type of binding ligand which are both immobilized with respect to an analyte molecule. The third type of binding ligand may be detected (e.g., directly or indirectly). The binding ligands which are to be detected may comprise or create a measurable entity or parameter (e.g., fluorescence) which makes it distinguishable from all other types of binding ligands employed in an assay which are to be detected, thus allowing for the determination of which binding ligands are present at/in a location.

Binding ligands may be selected to interact with other assay components (e.g., analyte molecules, other types of binding ligands, precursor labeling agents, etc.), in a variety of ways, as is described in more detail below. In certain embodiments, at least one type of binding ligand in the assay is selected such that it becomes immobilized with respect to an analyte molecule. In some embodiments, at least two types of binding ligands become immobilized with respect to an analyte molecule. The at least two types of binding ligands may either associate with the same or different components or moieties (e.g., epitopes) of an analyte molecule.

As an exemplary method employing two types of binding ligands, the method may first comprise exposing a plurality of analyte molecules to a plurality of a first type of binding ligand and plurality of a second type of binding ligand. The conditions may be selected such that substantially all of the analyte molecules are immobilized with respect to at least one of the first type of binding ligand and at least one of the second type of binding ligand. The analyte molecules (immobilized with respect to a plurality of binding ligands) are then spatially segregated into a plurality of locations on a surface, and in some cases, are spatially segregated such that each location contains zero or one analyte molecules. Alternatively, the analyte molecules may first be spatially segregated into a plurality of locations such that each location contains zero or one analyte molecules prior to being exposed to a plurality of a first type of binding ligand and a second type of binding ligand such that substantially all of the analyte molecules associate with at least a first type of binding ligand and at least a second type of binding ligand. At least a portion of the locations (e.g., comprising zero or one analyte molecules, the analyte molecules associates with at least one of a first type of binding ligand and at least one of a second type of binding ligand) may be interrogated/analyzed to determine the number of locations which contain both a first type of binding ligand and a second type of binding ligand (e.g., either by direct or indirect detection, as

described herein). The first type of binding ligand may possess, create or be made to create a first measurable parameter and the second type of binding ligand may possess, create or be made to create a second measurable parameter, wherein the first measurable parameter is distinguishable from the second measurable parameter. The number of locations which contain both binding ligands can be related to the number of locations which contain an analyte molecule. A measure of the concentration of analyte molecules in the fluid sample may be based at least in part on the number of locations which contain both a first type and a second type of binding ligand. Locations which contain only a first type of binding ligand or only a second type of binding ligand would be indicative of locations in which non-specific binding of a binding ligand had occurred, and therefore, these locations would not be considered in determining the total number of locations which comprise an analyte molecule. The first type of binding ligand and the second type of binding ligand may associate with the same or different epitopes of an analyte molecule.

Exemplary embodiments of inventive assay methods involving two types of binding ligands are illustrated in FIG. 1. A plurality of analyte molecules 100 are provided, as illustrated in step (A). In a first method, plurality of analyte molecules 100 are exposed to plurality of a first type of binding ligand 102 and plurality of second type of binding ligand 104. The conditions are selected such that substantially each of the analyte molecules associate with a first type of binding ligand and a second type of binding ligand. For example, as illustrated in step (B1), analyte molecule 106 is associated with one of a first type of binding ligand 108 and one of a second type of binding ligand 110, to form complex 112. The plurality of analyte molecules from step (B1) may then be spatially segregated into a plurality of locations. As shown in step (C1), the plurality of locations is illustrated as substrate 114 comprising a surface containing a plurality of wells/reaction vessels 116. In this example, each reaction vessel comprises either zero or one analyte molecules. At least some of the reaction vessels may then be addressed (e.g., optically or via other detection means) to determine the number of locations containing an analyte molecule by determining the number of locations which contain a first type of binding ligand and a second type of binding ligand. For example, as shown in step (D), the plurality of reaction vessels are interrogated optically using light source 118, wherein each reaction vessel is exposed to electromagnetic radiation (represented by arrows 122) from light source 118. The light emitted (represented by arrows 120) from each reaction vessel is detected (and/or

recorded) by detector 118 (in this example, housed in the same system as light source 118). The number of reaction vessels containing a first type of binding ligand and a second type of binding ligand (e.g., reaction vessels 124), and hence an analyte molecule, are determined, for example, based on the light emitted from the reaction vessels. In some cases, the number of reaction vessels which do not contain both a first type of binding ligand and a second type of binding ligand (e.g., reaction vessels 126), and/or the total number of wells addressed may also be determined. In some cases, at least one wash step may be performed (e.g., to remove any unbound binding ligands (e.g., prior to or following step (C1)).

Alternatively, following providing a plurality of analyte molecules as shown in step (A), the analyte molecules may be first spatially segregated into a plurality of locations, as shown in step (B2). The plurality of locations is illustrated as substrate 130 comprising a surface containing a plurality of wells/reaction vessels 132. In this example, each reaction vessel comprises either zero or one analyte molecules. The plurality of locations may be exposed to plurality of a first type of binding ligand 134 and plurality of a second type of binding ligand 136 under conditions such that substantially all of the analyte molecules associate with a first type of binding ligand and a second type of binding ligand. For example, as shown in step (C2), analyte molecule 138 is associated with first type of binding ligand 140 and second type of binding ligand 142. The reaction vessels which do not contain an analyte molecule may contain at least one binding ligand (e.g., reaction vessels 144). Following step (C2), in some embodiments, at least one wash step may be performed to aid in the removal of the binding ligand from the reaction vessels which do not contain an analyte molecule (e.g., reaction vessels 144 in step (C2)). Additionally, in some cases, the analyte molecules may be contained and/or immobilized in the reaction vessels such that the wash step does not cause the analyte molecules to be washed from the reaction vessels (e.g., via immobilization of the analyte molecule with respect to a surface of the reaction vessels, via aid of a surface comprising or contained in (e.g., on a capture object such as a bead) that has affinity for the analyte molecule). Following step (C2) and/or at least one wash step, at least some of the reaction vessels may then be addressed (e.g., optically or via other excitation/detection means) to determine the number of locations containing an analyte molecule by determining the number of locations which contain a first type of binding ligand and a second type of binding ligand. This may be carried out in a manner similar to that described above for FIG. 1, step (D).

In some embodiments, the first type of binding ligand and the second type of binding ligand may each comprise a component which work in synergy, and the detection of both types of binding ligands may occur only in cases where the first type of binding ligand and the second type of binding ligand are within close proximity of each other (e.g., immobilized with respect to the same analyte molecule). For example, the first type of binding ligand may comprise a component which upon stimulation, releases an excitation moiety. The excitation moiety may interact with a component on the second type of binding ligand. Provided the second type of binding ligand is within close proximity to the first type of binding ligand, the excitation moiety may interact with the component on the second type of binding ligand, allowing for the detection of the both the first and the second type of binding ligand. For example, as shown in FIG. 2, bead 300 contains a plurality of capture components 302. Analyte molecule 304 is associated with bead 300. First type of binding ligand 306 comprising first component 310 and second type of binding ligand 308 comprising second component 312 are both associated with analyte molecule 304. First component 310 is stimulated, in this example, with electromagnetic radiation 314. Upon stimulation, first component 310 releases an excitation moiety which excites second component 312, as indicated by arrow 315. Second component releases energy 316 upon interaction with the excitation moiety, the energy 316 being detected. As a specific embodiment, the first component may release a singlet oxygen donor that can diffuse about 200 nm before become quenched. Thus, if the second component associated with the second type of binding ligand is within 200 nm and the second component comprises a dye that is sensitive to singlet oxygen, the second component can be induced to release a signal which is detectable (e.g., fluorescence). For a non-limiting example of potentially compatible proximity sensitive detection facilitating components see Clinical Chemistry, 42(9), 1996, 1518-1526.

As another example, both the first type of binding ligand and the second type of binding ligand may comprise an enzymatic component as a precursor labeling agent/labeling agent, and the labeling agent produced from the conversion of a first type of precursor labeling agent may act as the precursor labeling agent for the enzymatic component on the second type of binding ligand converting it to the detected labeling agent. A "precursor labeling agent" is any molecule, particle, or the like, that can be converted to a labeling agent upon exposure to a suitable converting agent (e.g., an

enzymatic component). A "labeling agent" is any molecule, particle, or the like, that facilitates detection, by acting as the detected entity, using a chosen detection technique. In one non-limiting example, the product of one enzymatic conversion may be the substrate for a second enzymatic component (e.g., a coupled enzyme system). As a specific example, the coupled assay for the enzyme hexokinase (enzymatic component comprised in first type of binding ligand), which can be detected by coupling its production of glucose-6-phosphate to NADPH production, using glucose-6-phosphate dehydrogenase (enzymatic component comprised in second type of binding ligand). The NADPH may be detected to determine the presence of both the first type of binding ligand and the second type of binding ligand in a reaction vessel.

In some cases, both the first type of binding ligand and the second type of binding ligand comprise enzymatic components, and the labeling agent produced in one conversion reaction optically couples with the labeling agent produced in the second conversion reaction. For example, the product of one enzyme reaction optically couples to the detection of the second, e.g., the two enzymes produce a FRET pair where the fluorescence emission from product (e.g., donor) one excites fluorescence in the second product (e.g., acceptor). The fluorescence from the second product is detected to determine the presence of both the first type of binding ligand and the second type of binding ligand in a reaction vessel. Non-limiting examples of such donor/acceptor products include fluorescein/tetramethylrhodamine, IAEDANS/fluorescein, EDANS/dabcyl; fluorescein/fluorescein; BODIPY FL/BODIPY FL; fluorescein/QSY 7 and QSY 9, and the like.

In some cases, both the first type of binding ligand and the second type of binding ligand may be DNA labeled. When both types of binding ligands are in close proximity (e.g., associated with an analyte molecule), a connecting piece of DNA may be provided which is able to bridge the two DNA labels. A ligase can then be provided which connects the two DNA labels. Once connected, the contiguous piece of DNA can be replicated into many copies using PCR. Real time PCR in the reaction vessels may be used to detect the presence of both the first type of binding ligand and the second type of binding ligand in the reaction vessel. For non-limiting examples of DNA labels which may potentially be useful for the above detection scheme, see Nature Methods, 4(4), 2007, 327-329, and Nature Methods, 4(2), 2007, 135-137.

In some cases, the first type of binding ligand may comprise a first portion of an enzyme and the second type of binding ligand may comprise a second portion of an

enzyme. When the binding ligands are in close proximity to each other (e.g., two binding ligands are associated with a single analyte molecule), the two enzyme portions may interact with each other. When the two binding ligands are close together, the full enzyme structure may form and be detected (e.g., via exposure to a precursor labeling agent). For non-limiting examples of enzymes which may be partitioned and used in the present invention as described above, see Biochemical and Biophysical Research Communications 370, 2008, 164–168, and Journal of Immunological Methods, 279, 2003, 209– 218.

In some embodiments, more than two types of binding ligands may be employed. In some cases, three types of binding ligands may be employed. In some cases, the third type of binding ligand may function in a similar manner as the first type of binding ligand and the second type of binding ligand as described in the assays illustrated in FIG. 1. That is, the third type of binding ligand may associate with a component (e.g., an epitope) of the analyte molecule, and the presence of the first, second, and third types of binding ligands at a location may be used as an indicator of the presence of an analyte molecule at/in that location. The epitope of the analyte molecule with which the third binding ligand associates may be the same and/or different than the epitopes the first type of binding ligand and the second type of binding ligand associate with.

In other cases, the third type of binding ligand may function in a different manner as compared to the first type of binding ligand and the second type of binding ligand as described herein. For example, the first type of binding ligand and the second type of binding ligand may associate with an analyte molecule (e.g., as described in the assay method illustrated in FIG. 1) and the third type of binding ligand may associate with the first type of binding ligand and the second type of binding ligand. For the third type of binding ligand to associate with both the first type of binding ligand and the second type of binding ligand, the first and second types of binding ligands may be required to be in substantially close proximity to each other (e.g., via association with the same analyte molecule), therefore facilitating detection of the analyte molecule (e.g., by association and detection of the third type of binding ligand). The number of locations which contain the third type of binding ligand can be related to the number of locations containing both a first type of binding ligand and a second type of binding ligand in close proximity, and hence, the number of analyte molecules. In some cases, the first type of binding ligand and the second type of binding ligand may each incorporate a component (e.g., such as a DNA label) and a third type of binding ligand may comprise two

complimentary components to the components of the first type and second type of binding ligand (e.g., two types of complimentary DNA labels).

An exemplary embodiment of steps of an inventive assay method involving three types of binding ligands is illustrated in FIG. 3. An analyte molecule 70 (illustrated schematically as comprising two distinct epitopes) is provided, as shown in step (A). For the sole purpose of simplifying this illustrative example, only a single analyte molecule is illustrated, however generally a plurality of analyte molecules is provided. Analyte molecule 70 is exposed to a first type of binding ligand 74 and a second type of binding ligand 72 under conditions such that analyte molecule 70 associates with both types of binding ligand, as shown in step (B). First type of binding ligand 74 comprises component 78 for association with a third type of binding ligand and second type of binding ligand 72 comprises component 76 for association with a third type of binding ligand. In this embodiment, neither the first type of binding ligand nor the second type of binding ligand is directly detected in the assay. In step (C), the complex from step (B) is exposed to third type of binding ligand 80 under conditions such that third type of binding ligand 80 associates with first type of binding ligand 74 and second type of binding ligand 72. Third binding ligand comprises components 82 and 84 for association with and affinity for component 78 of first type of binding ligand 74 and association with and affinity for component 76 of second type of binding ligand 72, respectively. The presence of an analyte molecule at/in a location may be detected by detecting the third type of binding ligand at the location.

Those of ordinary skill in the art will be able to use this exemplary embodiment of the use of three types of binding ligands with various other steps of assay methods described herein. For example, as describe in relation to FIG. 1, the analyte molecules may be spatially segregated into a plurality of locations prior to and/or after exposure to the types of binding ligands. The different types binding ligands may be provided substantially simultaneously and/or sequentially. Additionally, at least one wash step may be performed. The third binding ligand may be detected directly or indirectly. For example, a fourth type, etc. of binding ligand may be provided, wherein the fourth type of binding ligand associates with the third type of binding ligand. The fourth type of binding ligand may be detected directly (e.g., a fluorescent entity) or indirectly (e.g., using a precursor labeling agent which is converted to a labeling agent upon exposure to the fourth type of binding ligand).

An exemplary embodiment of steps of an inventive assay method involving four types of binding ligands is illustrated in FIG. 4. Analyte molecule 130 is provided, as shown in step (A). Analyte molecule 130 is exposed to first type of binding ligand 132 and second type of binding ligand 138 such that analyte molecule 130 associates with at least one of each type, as shown in step (B). First type of binding ligand 132 comprises component 134 for associating with analyte molecule 130 and component 136 for associating with a third type of binding ligand. Second type of binding ligand 138 comprises component 140 for associating with analyte molecule 130 and component 142 for associating with a fourth type of binding ligand. In step (C), the analyte molecule from step (B) is exposed to third type of analyte molecule 145 and fourth type of binding ligand 149. Third type of binding ligand comprises component 144 for associating with component 136 of first type of binding ligand and component 146 for detection. Fourth type of binding ligand comprises component 148 for associating with component 138 of second type of binding ligand and component 150 for detection. In some cases, components 146 and 150 are distinguishable from each other and may be detected directly (e.g., a fluorescent entity). Following spatial segregation into a plurality of locations, locations which are determined to contain third type of binding ligand 145 and fourth type of binding ligand 149 (e.g., via detection of components 146 and 150), can be related to the number of locations which contain a first type and a second type of binding ligand, respectively, and hence, an analyte molecule.

In other cases, as shown in step (D), components 146 and 150 may be detected indirectly via exposure to first type of precursor labeling agent 152 and second type of precursor labeling agent 158, wherein first type of precursor labeling agent 152 is converted to labeling agent 156 upon exposure to component 146, as indicated by arrow 154 and second type of precursor labeling agent 158 is converted to labeling agent 162 upon exposure to component 150 as indicated by arrow 160, and wherein first type of labeling agent 156 is distinguishable from second type of labeling agent 162. Following spatial segregation of the analyte molecules into a plurality of locations, locations which are determined to contain first type of labeling agent 156 and second type of labeling agent 162 are determined to contain a third and fourth type of binding ligand, thus a first and second type of binding ligand, and hence an analyte molecule.

Non-limiting examples of first type of binding ligand components for associating with a third type of binding ligand (e.g., component 136 of first binding ligand in FIG. 4; or component 138 or second binding ligand) and third type of binding ligand components

for associating with the component from the first type of binding ligand (e.g., component of third binding ligand in FIG. 4; or component 148 of fourth binding ligand) are biotin/streptavidin, biotin/anti-biotin, fluorescein/anti-fluorescein, digoxin/anti-digoxin. and dinitrophenol (DNP)/anti-DNP. The third binding ligand may further comprise an enzymatic component (e.g., component 146 of third binding ligand in FIG. 4), as described herein.

Those of ordinary skill in the art will be able to determine, based on the teachings of this specification, numerous other combinations of types of binding ligands and detection methods to carry-out an assay of the present invention. For example, in the case of an assay method involving two types of binding ligands, the first type of binding ligand may comprise a component which is directly detected, and the second type of binding ligand may be indirectly detected (e.g., via exposure to a precursor labeling agent). In another example, three types of binding ligands may be employed, wherein the first type of binding ligand associates with an analyte molecule and is directly or indirectly detectable, the second type of binding ligand associates with the analyte molecule, and the third type of binding ligand associates with the second type of binding ligand and is directly or indirectly detectable. The presence of both the first type of binding ligand and the third type of binding ligand in a location can be read as an "on" location.

At any point during an assay method of the present invention, at least one wash step may be carried out. The wash step may aid in the removal of any unbound binding ligands from the solution. For example, referring to FIG. 1, following association of the first type of binding ligand and the second type of binding ligand with respect to an analyte molecule (e.g., following step (B1) or step (C2), a wash step may be performed to remove any unbound binding ligands not immobilized with respect to an analyte molecule. The wash step may be performed using any suitable technique known to those of ordinary skill in the art. In embodiments where the assay employs magnetic beads (e.g., as capture objects to immobilize the analyte molecules), the beads may be isolated from the bulk solution with aid of a magnet.

The analyte molecules may be exposed to the at least two types of binding ligands under conditions such that a substantial fraction (e.g., at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 97%, at least about 99%, or more) of the analyte molecules associate with at least one of each type of binding ligand. Non-limiting conditions which may be

varied include the ratio of binding ligands to analyte molecules, the period of time of the exposure, the existence and/or type of physical confinement during incubation (e.g., exposure in bulk solution versus exposure at/in locations), agitation of the solution, and the like. In some cases, the binding ligands may be added to a solution comprising the plurality of analyte molecules. The ratio of binding ligands provided to analyte molecules in solution may be at least about 1:1, at least about 2:1, at least about 4:1, at least about 5:1, at least about 10:1, at least about 20:1, at least about 30:1, at least about 50:1, at least about 100:1, or greater. In some embodiments, the number/concentration of analyte molecules in the fluid sample is unknown and the number of binding ligands provided may be based on the maximum suspected number of analyte molecules in the fluid sample. In some cases, the solution may be agitated (e.g., stirred, shaken). A single analyte molecule may associate with more than one of each type of binding ligand (e.g., at least two, three, four, five, six, etc., of each type of binding ligand) in certain embodiments.

In some embodiments, the concentration of binding ligand used in an assay may be selected as to minimize certain events which may occur when an excess of binding ligand is present, for example, non-specific binding of the binding ligand. In some cases, if the concentration of binding ligand is too high, an increase in background readings may occur due to non-specific interactions (e.g., with the capture objects, reaction vessels, etc.). In some cases, the concentration of binding ligand may be selected (or estimated, in the case of an unknown concentration of analyte molecule) such that a only a fraction of the analyte molecules immobilized with respect to a capture object associate with a binding ligand (e.g., about 0.1%, about 1%, about 2%, about 3%, about 4%, about 5%, about 10%, about 20%, about 30%, about 40%, about 50%, or more). This may be especially useful in embodiments where the percentage of capture objects which associate with at least one analyte molecule is relatively high (e.g., greater than about 20%, greater than about 30%, greater than about 40%, greater than about 50%, greater than about 60%, greater than about 70%, greater than about 80%, greater than about 90%, or more). By providing the binding ligand at a lower concentration, in some cases, not every analyte molecule immobilized with respect to a capture object will associate with a binding ligand, which can be advantageous for quantification, for example when the presence of a binding ligand is required for detection, and especially when using a digital/binary read-out technique. For example, if the percentage of capture objects associated with an analyte molecule is about 50% or greater, a reduced number of

binding ligands may be provided such that less than all of the immobilized analyte molecules associate with a binding ligand. In other cases, the percentage of binding ligands that associate with an analyte molecule may be reduced by decreasing the incubation time with the analyte molecule (e.g., limit the time of exposure such that only a fraction of the immobilized analyte molecules associate with an analyte molecule).

In some embodiments, a method for determining a measure of the concentration of analyte molecules in a fluid sample comprises providing the analyte molecules immobilized with respect to a binding surface having affinity for at least one type of analyte molecule, the binding surface forming (e.g., a surface of a well/reaction vessel on a substrate) or contained within (e.g., a surface of a capture object, such as a bead, contained within a well) one of a plurality of locations (e.g., a plurality of wells/reaction vessels) on a substrate (e.g., plate, dish, chip, optical fiber end, etc). The analyte molecules may be spatially segregated such that at least some of the plurality of locations contain at least one analyte molecule and a statistically significant fraction of the plurality of locations do not contain any analyte molecules, as described herein. At some point in the assay (e.g., prior to, concurrent with, or following the analyte molecules being immobilized with respect to the binding surface), the analyte molecules may be exposed to at least a first type of binding ligand having an affinity for a first epitope on the analyte molecules and a second type of binding ligand having an affinity for the first or a second epitope on the analyte molecules. As described herein, more than two types of binding ligand may be employed. At least a portion of the plurality of the locations may be addressed, and the number of the locations addressed which contain at least a first type of binding ligand and a second type of binding ligand can be determined. Based on at least this determination, a measure of the concentration of analyte molecules may be determined. In some cases, the binding surface comprises the surface of a bead contained within one of a plurality of locations on a substrate. In such cases, the exposing step may be performed prior to or following the bead being contained within one of the plurality of locations on the substrate. Non-limiting examples of assay methods/systems are described in U.S. Patent Application Serial No. 12/731,130, entitled "Ultra-Sensitive Detection of Molecules or Particles using Beads or Other Capture Objects," by Duffy et al., filed March 24, 2010; International Patent Application No. (not yet determined), entitled "Ultra-Sensitive Detection of Molecules or Particles using Beads or Other Capture Objects," by Duffy et al., filed March 1, 2011 (Attorney Docket No. Q0052.70011WO00); U.S. Patent Application No. 20070259448, entitled "Methods

and arrays for target analyte detection and determination of target analyte concentration in solution," by Walt et al., filed Feb. 16, 2007; U.S. Patent Application No. 20070259385, entitled "Methods and arrays for detecting cells and cellular components in small defined volumes," by Walt et al., filed Feb. 16, 2007; U.S. Patent Application No. 20070259381, entitled "Methods and arrays for target analyte detection and determination of reaction components that affect a reaction," by Walt et al., filed Feb. 16, 2007; International Patent Application No. PCT/US07/019184, entitled "Methods for Determining the Concentration of an Analyte in Solution," by Walt et al., filed August 20, 2007; and International Patent Application No. PCT/US09/005428, entitled "Ultra-Sensitive Detection of Molecules or Enzymes," by Duffy et al., filed September 9, 2009, herein incorporated by reference.

Binding Ligands and Precursor Labeling Agents/Labeling Agent

Binding ligands may be selected from any suitable molecule, particle, or the like, as discussed more below, able to associate with an analyte molecule and/or to associate with another binding ligand. Certain binding ligands can comprise a component that is able to facilitate detection, either directly (e.g., via a detectable moiety) or indirectly. A component may facilitate indirect detection, for example, by converting a precursor labeling agent molecule into a labeling agent molecule (e.g., an agent that is detected in an assay). In some embodiments, the binding ligand may comprise an enzymatic component (e.g., horseradish peroxidase, beta-galactosidase, alkaline phosphatase, etc). A first type of binding ligand may be used in conjunction with additional binding ligands (e.g., second type, etc.), as discussed herein.

In some embodiments, and as described herein, more than one type of binding ligand may be used. In some embodiments, a first type of binding ligand and a second type of binding ligand may be provided. In some instances, at least two, at least three, at least four, at least five, at least eight, at least ten, or more, types of binding ligands may be provided. The binding ligands may be selected such that they interact with each other and/or an analyte molecule in a variety of different manners. Various examples of possible interactions are shown above in FIGS. 1, 3, and 4.

In some embodiments, each type of binding ligand which is used for detection may be distinguishable from each of the other type of binding ligands used for detection and employed in the assay method. That is, each type of binding ligand which is directly or indirectly detected produces a signal which is distinguishable from the signals

produced by the other types of binding ligand which are directly or indirectly detected. For example, the first type of binding ligand may comprise a first component (e.g., fluorescent entity) producing a first signal (e.g., a first fluorescence emission) and the second type of binding ligand may comprise a second component (e.g., fluorescent entity) producing a second signal (e.g., a second fluorescence emission), wherein the second signal is distinguishable from the first signal.

In some cases, however, each type of binding ligand which is directly or indirectly detected produces a signal which is the same as the signals produced by the other types of binding ligand which are directly or indirectly detected. In these embodiments, the detection of each type of binding ligand may be done sequentially so that the signals may be distinguished. For example, a first type of binding ligand may comprise a first enzymatic component and the second type of binding ligand may comprise a second type of enzymatic component. Both types of binding ligands may be immobilized with respect to an analyte molecule. The binding ligands may be exposed to a first type of precursor labeling agent, wherein the first type of precursor labeling agent is converted to a first type of labeling agent upon exposure to the first type of binding ligand. A first determination may be carried out to determine which reaction vessels contain a first type labeling agent. The binding ligands may be exposed to a second type of precursor labeling agent, wherein the second type of labeling agent is converted to a second type of labeling agent upon exposure to the second type of binding ligand. In some cases, the first type of labeling agent may be removed from the reaction vessels prior to exposure to a second type of precursor labeling agent (in some cases, a determination may be carried out to ensure that substantially all of the first type of labeling agent has been removed). The second type of labeling agent may produce a signal which is substantially the same as that produced by the first type of labeling agent. A second determination may be carried out to determine which reaction vessels contain the second type of labeling agent, and the first and second determination may be analyzed to determine which reaction vessels contained both the first type and the second type of labeling agent. As a specific example, the first type of binding ligand comprises first type of enzymatic component beta-galatosidase, and the first type of precursor labeling agent is resorufin-D-pyranogalactoside, whereas the second type of binding ligand comprises second type of enzymatic component, horseradish peroxidase, and the second type of precursor labeling agent is Amplex Red. Both precursor labeling agents

are converted to a labeling agent which comprises resorufin, the moiety which is detectable.

The binding ligands may be detected directly or indirectly. In the case of direct detection, the binding ligands may comprise a molecule or moiety that may be directly interrogated and/or detected. Those of ordinary skill in the art will be aware of components that can be directly detected. For example, the component may be a fluorescent entity (e.g., a fluorescent moiety, fluorescent bead, etc.), a metal nanoparticle or nanocluster (e.g., a gold nanocluster or nanoparticle, silver nanocluster or nanoparticle), a quantum dot (e.g., CdSe quantum dot, CdTe quantum dot, etc.), or a radioactive isotope.

In some embodiments, the binding ligands may be indirectly detected. The indirect approach can include, for example, exposing a binding ligand associated with an analyte molecule to a precursor labeling agent, wherein the precursor labeling agent is converted into a labeling agent upon exposure to the binding ligand associated with an analyte molecule. The labeling agent may comprise a molecule or moiety that can be directly interrogated and/or detected. The presence or absence of a binding ligand at a location may then be determined by determining the presence or absence of a labeling agent at/in the location.

A non-limiting example of indirect detection is shown in FIG. 5. Analyte molecule 200 is associated with binding ligand 202. Binding ligand comprises a component 204 which is capable of converting precursor labeling agent molecule 206 into labeling agent 208, as indicated by arrow 207. Labeling agent 208 may be directly detected and used to determine the presence of binding ligand 202 at the location. Another non-limiting example was described above in FIG. 4, step (D).

In some embodiments, a binding ligand may comprise an enzymatic component. In this instance, the precursor labeling agent may be an enzymatic label, for example, a chromogenic, fluorogenic, or chemiluminescent enzymatic precursor labeling agent, that upon contact with the enzymatic component, converts to a labeling agent. In some cases, the chromogenic, fluorogenic, or chemiluminescent enzymatic precursor labeling agent is provided in an amount sufficient to contact every analyte molecule which was partitioned across a plurality of locations. In some cases, the enzymatic component may comprise beta-galactosidase, alkaline phosphatase, or horseradish peroxidase.

Other non-limiting examples of systems or methods for detection include embodiments where nucleic acid precursors are replicated into multiple copies or

converted to a nucleic acid that can be detected readily, such as the polymerase chain reaction (PCR), rolling circle amplification (RCA), ligation, Loop-Mediated Isothermal Amplification (LAMP), etc. Such systems and methods will be known to those of ordinary skill in the art, for example, as described in "DNA Amplification: Current Technologies and Applications," Vadim Demidov et al., 2004.

As will be understood by those of ordinary skill in the art, a variety of appropriate chromogenic, fluorogenic, or chemiluminescent enzymatic precursor labeling agents may be selected for conversion by many different enzymes. Thus, any known chromogenic, fluorogenic, or chemiluminescent enzyme precursor labeling agent capable of producing a labeling agent in a reaction with a particular enzyme can potentially be used in the present invention as the precursor labeling agent in embodiments where the binding ligand comprises an enzymatic component. For example, many chromogenic, fluorogenic, or chemiluminescent precursor labeling agents suitable for use an enzymatic precursor labeling agent molecule are disclosed in *The Handbook -A Guide to Fluorescent Probes and Labeling Technologies,* Tenth Ed., Chapter 10.

In some embodiments, a first type of binding ligand may comprise a first type of enzymatic component and the second type of binding ligand may comprise a second type of enzymatic component which differs from the first type of enzymatic component. A capture object (e.g., a bead) comprising an analyte molecule, the first type of binding ligand, and the second type of binding ligand may be exposed to a first type of precursor labeling agent which is converted to a first type of labeling agent (e.g., comprising a first measurable property) upon exposure to the first type of enzymatic component and a second type of precursor labeling agent which is converted to a second type of labeling agent (e.g., comprising a measurable property which is distinguishable from the first measurable property) upon exposure to the second type of enzymatic component. Therefore, only locations which are determined to contain the first type of labeling agent and the second type of labeling agent are determined to contain a capture object carrying an analyte molecule. As described herein, however, in some cases, the first type of labeling agent and the second type of labeling agent may be the same or produce the same detectable signal, but be distinguished from each other via the time frame in which the signal is produced.

In some cases, the first type of binding ligand and the second type of binding ligand may each incorporate a component (e.g., such as a DNA label) and a third type of

binding ligand may comprise two complimentary components to the components of the first type and second type of binding ligands (e.g., two types of complimentary DNA labels), wherein the third type of binding ligand also comprises an molecule or moiety for direct or indirect detection (e.g., the presence of the third type of binding ligand in a reaction vessel is required to determine the presence or absence of an analyte molecule in a location). Both the first type of binding ligand and the second types of binding ligand are present in substantially close proximity to each other (e.g., via association with an analyte molecule) to allow for association of the third type of binding ligand, and therefore, for detection of the analyte molecule.

In some embodiments, techniques may be used to prevent or reduce dissociation of an analyte molecule from a capture component and/or capture object, and/or to prevent or reduce dissociation of a binding ligand from an analyte molecule and/or another binding ligand. As will be known to those of ordinary skill in the art, some reversible affinity interactions between selected analyte molecules, capture components, and/or binding ligands (e.g., between an antibody and an antigen) are governed by thermodynamics. Accordingly, at some point during certain assay methods, some dissociation may occur between an analyte molecule and a capture component and/or a binding ligand, and/or between a binding ligand and an analyte molecule and/or another binding ligand. This may result in a reduced number of analyte molecules (e.g., immunocomplexes) being detected than are actually present. The dissociation constant of a particular pair of components (e.g., antibody-antigen pair), washing and/or fluid exposure, time between exposure and interrogation, and/or other factors, may affect the degree to which a dissociation event alters determination of analyte molecules and/or particles. Accordingly, certain techniques may be used to reduce the effects of dissociation processes.

In a first embodiment, dissociation may be reduced or eliminated by removing fluids from the assay locations (e.g., wells) following spatial segregation of a plurality of analyte molecules (e.g., associated with a capture object via a capture component and/or associated with at least one binding ligand) into a plurality of such locations. That is, all or substantially all of the fluid surrounding or substantially contained in or at the locations may be removed. For example. the fluid may be removed by air and/or vacuum drying. Removal of the fluid may reduce or eliminate dissociation. Immediately prior to interrogation of the locations, a fluid may be added to the locations thereby rehydrating the complexes to facilitate interrogation using a detector.

In a specific example, a plurality of analyte molecules is associated (e.g., via a capture object) with beads (one embodiment of capture objects). The beads, and thus the analyte molecules, are optionally associated with at least one binding ligand and/or exposed to a material (e.g., a buffer) to keep the analyte molecules and/or binding ligands hydrated (e.g., immunocomplexes may be exposed to a buffer containing a certain carbohydrate). Following loading of the beads into a plurality of wells (e.g., locations), excess solution (e.g., buffers) is removed by air and/or vacuum drying. In this embodiment, by eliminating the contact of the beads with bulk solution, the dissociation of the analyte molecules and/or binding ligands is essentially eliminated or substantially reduced as compared to a similar system where the fluid is not removed. In this case, the wells comprising the beads may be stored (e.g., for 1 min, 2 min, 5 min, 10 min, 20 min, 30 min, 1 hour, 2 hours, 3 hours, 4 hours, 6 hours, 12 hours, or more) with none or substantially none (e.g., less than about 20%, less than about 15%, less than about 10%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, less than about 1%, less than about 0.5%, less than about 0.3%, less than about 0.1%, less than about 0.01%, or less) of the analyte molecules (and/or binding ligands) dissociating. Immediately prior to or close in time to the time when interrogations of the wells are performed (e.g., about 1 second, about 5 seconds, about 10 seconds, about 30 seconds, about 1 minute, about 2 minutes, or about 5 minutes prior), a solution may be provided to the wells, optionally followed by sealing (e.g., prior to interrogation). See, for example, Example 1.

In a second embodiment, dissociation may be reduced or eliminated by crosslinking an analyte molecule with a capture component, and/or crosslinking a binding ligand with an analyte molecule and/or a second binding ligand. For example, an analyte molecule comprising an antigen may be crosslinked with a binding ligand and/or capture component comprising an antibody. Crosslinking methods and techniques that may be employed are known to those of ordinary skill in the art. In some cases, following association of an analyte molecule with a capture component (e.g., associated with a capture object) and/or association of a binding ligand with the analyte molecule and/or a second binding ligand, the complex may be exposed to a crosslinking reagent (e.g., 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (i.e., EDC), glutaraldehyde, etc.), thereby promoting the desired crosslinking reaction. The crosslinking reagent may be selected according to the properties of the reagents used for assay, and various factors may be considered including the length of the crosslinking

reagent and/or the necessary functional groups when selecting a crosslinking agent, as would be understood by those skilled in the art. Excess crosslinking reagent may optionally be removed and/or unreacted crosslinking groups may be quenched prior to interrogation and/or prior exposure to a labeling agent precursor or other reagent. The assay may be then carried out according to the methods as described herein. See, for example, Example 2.

A non-limiting example of a crosslinking reaction is shown in FIG. 6A. In this figure, bead 502 comprising capture component 504 (e.g., capture antibodies) is associated with analyte molecule 506. Analyte molecule 506 is also associated with binding ligand 508. Complex 507 is exposed to crosslinking reagent 510 and a reaction occurs as indicated by arrow 511. First crosslinking reagent 512 forms a crosslink between analyte molecule 506 and capture component 504 and crosslinking reagent 514 forms a crosslink between analyte molecule 506 and binding ligand 508.

As another example, the binding ligand may comprise a crosslinking component capable of forming a crosslink between two components (e.g., an analyte molecule and a binding ligand, or a capture component and an analyte molecule, or a first binding ligand and a second binding ligand), as shown in FIG. 6B. In this figure, bead 522 comprising capture component 524 (e.g., capture antibody) is associated with analyte molecule 526. Analyte molecule 526 is also associated with binding ligand 528 comprising crosslinking component 530. Upon exposure to an external stimulus (e.g., UV-light, chemical stimulus, etc.), as indicated by arrow 531, crosslink 532 forms between analyte molecule 526 and binding ligand 524. Non-limiting examples of crosslinking components useful for this purpose include N-succinimidyl-6-[4'-azido-2'-nitrophenylamino]) hexanoate (i.e., SANPAH) and succinimidyl 6-(4,4'-azipentanamido)hexanoate (i.e., LC-SDA).

In certain embodiments, solubilized, or suspended precursor labeling agents may be employed, wherein the precursor labeling agents are converted to labeling agents which are insoluble in the liquid and/or which become immobilized within/near the location (e.g., within the reaction vessel in which the labeling agent is formed). Such precursor labeling agents and labeling agents and their use is described in commonly owned U.S. Patent Application Serial No. 12/236,484, entitled "High Sensitivity Determination of the Concentration of Analyte molecules in a Fluid Sample," by Duffy et al., filed September 23, 2008, incorporated herein by reference.

Methods and Systems for Segregating Analyte Molecules into Arrays of Locations

In certain embodiments, the assay methods of the present invention employ a step of spatially segregating a plurality of analyte molecules into a plurality of locations to facilitate detection/quantification, such that each location comprises/contains zero, one or more analyte molecules. Additionally, in some embodiments, the locations are provided in a manner such that each analyte molecule can be individually addressed. While numerous exemplary embodiments for spatially segregating a plurality of analyte molecules into a plurality of locations are described herein, numerous methods may be employed. Non-limiting examples of assay methods/systems are described in U.S. Patent Application Serial No. 12/731,130, entitled "Ultra-Sensitive Detection of Molecules or Particles using Beads or Other Capture Objects," by Duffy et al., filed March 24, 2010; International Patent Application No. (not yet determined), entitled "Ultra-Sensitive Detection of Molecules or Particles using Beads or Other Capture Objects," by Duffy et al., filed March 1, 2011 (Attorney Docket No. Q0052.70011WO00U.S. Patent Application No. 20070259448, entitled "Methods and arrays for target analyte detection and determination of target analyte concentration in solution," by Walt et al., filed Feb. 16, 2007; U.S. Patent Application No. 20070259385, entitled "Methods and arrays for detecting cells and cellular components in small defined volumes," by Walt et al., filed Feb. 16, 2007; U.S. Patent Application No. 20070259381, entitled "Methods and arrays for target analyte detection and determination of reaction components that affect a reaction," by Walt et al., filed Feb. 16, 2007; International Patent Application No. PCT/US07/019184, entitled "Methods for Determining The Concentration of an Analyte In Solution," by Walt et al., filed August 20, 2007; and International Patent Application No. PCT/US09/005428, entitled "Ultra-Sensitive Detection of Molecules or Enzymes," by Duffy et al., filed Sept. 9, 2009, herein incorporated by reference.

In some embodiments, the plurality of analyte molecules may be spatially segregated into a plurality of locations, wherein the locations comprise a plurality of reaction vessels. The analyte molecules may be partitioned across the plurality of reaction vessels such that at least some of the reaction vessels contain at least one analyte molecule and a statistically significant fraction of the reactions vessels contain no analyte molecules. A statistically significant fraction of reaction vessels that contain at least one analyte molecule (or no analyte molecules) will typically be able to be reproducibly detected and quantified using a particular system of detection and will typically be above

the background noise (e.g., non-specific binding) that is determined when carrying out the assay with a sample that does not contain any analyte molecules, divided by the total number of locations addressed. A "statistically significant fraction" as used herein for the present embodiments, may be calculated according to the Equation 1:

$$n > 3\sqrt{n} \qquad \text{(Eq. 1)}$$

wherein n is the number of determined events for a selected category of events. That is, a statistically significant fraction occurs when the number of events n is greater than three times square root of the number of events. For example, to determine a statistically significant fraction of the reaction vessels which contain an analyte molecule or particle, n is the number of reaction vessels which contain an analyte molecule. As another example, to determine a statistically significant fraction of capture objects associated with a single analyte molecule, n is the number of capture objects associated with a single analyte molecule.

In some embodiments, the statistically significant fraction of locations that contain at least one analyte molecule (or a single analyte molecule in some cases where the ratio of locations to analyte molecules would lead, statistically, to substantially only zero or one analyte molecule contained in each location) to the total number of locations containing sample is less than about 1:2, less than about 1:3, less than about 1:4, less than about 2:5, less than about 1:5, less than about 1:10, less than about 1:20, less than about 1:100, less than about 1:200, or less than about 1:500. In such embodiments, the fraction of locations not containing any analyte molecules to the total number of locations may be at least about 1:100, about 1:50, about 1:20, about 1:10, about 1:5, about 1:4, about 1:3, about 1:2, about 1:1, about 2:1, about 3:1, about 4:1, about 5:1, about 10:1, about 20:1, about 50:1, about 100:1, or more.

In some embodiments, the percentage of locations which contain at least one analyte molecule is less than about 50%, less than about 40%, less than about 30%, less than about 20%, less than about 10%, less than about 5%, less than about 2%, less than about 1%, less than about 0.5%, less than about 0.01%, or less, the total number of locations. In some embodiments, the percentage of locations which do not contain any analyte molecule is at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 98%, or greater, the total number of locations.

In some embodiments, the assay methods may involve the use of a plurality of capture objects. The plurality of capture objects (e.g., beads) may be configured to

capture an analyte molecule or particle. In some cases, the plurality of capture objects comprises a plurality of beads. The beads may or may not be magnetic. At least a portion of the capture objects may be spatially segregated into a plurality of locations (e.g., reaction vessels/wells). The plurality of analyte molecules may be exposed to a plurality of types of binding ligands prior to or following association of the plurality of analyte molecules with respect to the capture components. Various other aspects of assay methods using such capture components are described in commonly owned U.S. Patent Application Serial No. 12/731,130, entitled "Ultra-Sensitive Detection of Molecules or Particles using Beads or Other Capture Objects" by Duffy et al., filed March 24, 2010; and International Patent Application No. (not yet determined), entitled "Ultra-Sensitive Detection of Molecules or Particles using Beads or Other Capture Objects," by Duffy et al., filed March 1, 2011 (Attorney Docket No. Q0052.70011WO00), incorporated by reference. Specifically, the methods and systems described herein may be used in combination with and in context with the single molecules methods and systems described in the above-referenced applications.

In some embodiments, a plurality of locations may be addressed and/or a plurality of capture objects and/or species/molecules/particles of interest may be detected substantially simultaneously. "Substantially simultaneously" when used in this context, refers to addressing/detection of the locations/capture objects/species/molecules/particles of interest at approximately the same time such that the time periods during which at least two locations/capture objects/species/molecules/particles of interest are addressed/detected overlap, as opposed to being sequentially addressed/detected, where they would not. Simultaneous addressing/detection can be accomplished by using various techniques, including optical techniques (e.g., CCD detector). Spatially segregating capture objects/species/molecules/particles into a plurality of discrete, resolvable locations, according to some embodiments facilitates substantially simultaneous detection by allowing multiple locations to be addressed substantially simultaneously. For example, for embodiments where individual species/molecules/particles are associated with capture objects that are spatially segregated with respect to the other capture objects into a plurality of discrete, separately resolvable locations during detection, substantially simultaneously addressing the plurality of discrete, separately resolvable locations permits individual capture objects, and thus individual species/molecules/particles (e.g., analyte molecules) to be resolved. For example, in certain embodiments, individual molecules/particles of a plurality of

molecules/particles are partitioned across a plurality of reaction vessels such that each reaction vessel contains zero or only one species/molecule/particle. In some cases, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, at least about 99.5% of all species/molecules/particles are spatially separated with respect to other species/molecules/particles during detection. A plurality of species/molecules/particles may be detected substantially simultaneously within a time period of less than about 1 second, less than about 500 milliseconds, less than about 100 milliseconds, less than about 50 milliseconds, less than about 10 milliseconds, less than about 1 millisecond, less than about 500 microseconds, less than about 100 microseconds, less than about 50 microseconds, less than about 10 microseconds, less than about 1 microsecond, less than about 0.5 microseconds, less than about 0.1 microseconds, or less than about 0.01 microseconds, less than about 0.001 microseconds, or less. In some embodiments, the plurality of species/molecules/particles may be detected substantially simultaneously within a time period of between about 100 microseconds and about 0.001 microseconds, between about 10 microseconds and about 0.01 microseconds, or less.

During the addressing step of the method where the locations into which the capture objects/analyte molecules have been segregated are addressed, any of a variety of parameters may be determined. In some embodiments, the number of locations which contain both a first type of binding ligand and a second type of binding ligand is determined. The number of locations which do not contain both a first type of binding ligand and a second type of binding ligand may also be determined. A single interrogation or multiple interrogations of any subset or all of the locations ultimately addressed may be made at any given time to facilitate one or all of the above described determinations. The portion of locations addressed may be at least about 5%, or at least about 10%, or at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90%, or more of the total number of locations

In some cases, the locations which contain the specified types of binding ligands may be determined by a single interrogation of the locations. In such embodiments, the detection system is configured such that it is able to detect and distinguish between each type of binding ligand. In some cases, however, the locations which contain each type of binding ligand may be determined by multiple interrogations of the locations. The multiple interrogations of each location may be combined/compared to determine which

locations contain both binding ligands. For example, in some cases, a first interrogation of the locations may be conducted using light of a first range of wavelengths (e.g., a wavelength range where the first type of binding ligand produces a detectable fluorescence) to detect which locations contain a first type of binding ligand whereas a second interrogation is conducted using light of a second, differing range of wavelengths (e.g., a wavelength range where the second type of binding ligand produces a detectable fluorescence) to detect which locations contain a second type of binding ligand. The locations which contain both a first type of binding ligand and a second type of binding ligand may be determined by comparing/combining the results from the two interrogations for each location. It should be understood, however, that in some cases, a single type of binding ligand (e.g., a third type of binding ligand) may be detected to determine the presence of both a first type of binding ligand and a second type of binding ligand contained in a single location (e.g., in embodiments where a third type of binding ligand associates with an analyte molecule via association with both a first type of binding ligand and a second type of binding ligand, both immobilized with respect to an analyte molecule, and as outlined in FIG. 3).

In a specific embodiment of the present invention, the locations are optically interrogated. The locations exhibiting changes in their optical signature may be identified by a conventional optical train and optical detection system. Depending on the detected species (e.g., type of fluorescence entity, etc.) and the operative wavelengths, optical filters designed for a particular wavelength may be employed for optical interrogation of the locations. In embodiments where optical interrogation is used, the system may comprise more than one light source and/or a plurality of filters to adjust the wavelength and/or intensity of the light source. In some embodiments, the optical signal from a plurality of locations is determined using a CCD camera.

Other non-limiting examples of camera imaging types that can be used to capture images include charge injection devices (CIDs), complimentary metal oxide semiconductors (CMOSs) devices, scientific CMOS (sCMOS) devices, and time delay integration (TDI) devices, as will be known to those of ordinary skill in the art. The camera may be obtained from a commercial source. CIDs are solid state, two dimensional multi pixel imaging devices similar to CCDS, but differ in how the image is captured and read. For examples of CCDs, see U.S. Patent No. 3,521,244 and U.S. Patent No. 4,016,550. CMOS devices are also two dimensional, solid state imaging devices but differ from standard CCD arrays in how the charge is collected and read out.

The pixels are built into a semiconductor technology platform that manufactures CMOS transistors thus allowing a significant gain in signal from substantial readout electronics and significant correction electronics built onto the device. For example, see U.S. Patent No. 5,883,83). sCMOS devices comprise CMOS imaging technology with certain technological improvements that allows excellent sensitivity and dynamic range. TDI devices employ a CCD device which allows columns of pixels to be shifted into and adjacent column and allowed to continue gathering light. This type of device is typically used in such a manner that the shifting of the column of pixels is synchronous with the motion of the image being gathered such that a moving image can be integrated for a significant amount of time and is not blurred by the relative motion of the image on the camera. In some embodiments, a scanning mirror system coupled with a photodiode or photomultiplier tube (PMT) could be used to for imaging.

The plurality of locations may be formed using any suitable technique. In some embodiments, the plurality of locations comprises a plurality of reaction vessels/wells on a substrate. The reactions vessels, in certain embodiments, may be configured to receive and contain only a single capture object.

In some embodiments of the present invention, the plurality of reaction vessels may be sealed (e.g., after the introduction of the analyte molecules, binding ligands, and/or precursor labeling agent), for example, through the mating of the second substrate and a sealing component. The sealing of the reaction vessels may be such that the contents of each reaction vessel cannot escape the reaction vessel during the remainder of the assay. In some cases, the reaction vessels may be sealed after the addition of the analyte molecules and, optionally, at least one type of precursor labeling agent to facilitate detection of the analyte molecules. For embodiments employing precursor labeling agents, by sealing the contents in some or each reaction vessel, a reaction to produce the detectable labeling agents can proceed within the sealed reaction vessels, thereby producing a detectable amount of labeling agents that is retained in the reaction vessel for detection purposes.

The plurality of locations comprising a plurality of reaction vessels may be formed using a variety of methods and/or materials. In some cases, the plurality of reaction vessels is formed as an array of depressions on a first surface. In other cases, however, the plurality of reaction vessels may be formed by mating a sealing component comprising a plurality of depressions with a substrate that may either have a featureless surface or include depressions aligned with those on the sealing component. Any of the

device components, for example, the substrate or sealing component, may be fabricated from a compliant material, e.g., an elastomeric polymer material, to aid in sealing. The surfaces may be or made to be hydrophobic or contain hydrophobic regions to minimize leakage of aqueous samples from the microwells.

In some cases, the sealing component may be capable of contacting the exterior surface of an array of microwells (e.g., the cladding of a fiber optic bundle as described in more detail below) such that each reaction vessel becomes sealed or isolated such that the contents of each reaction vessel cannot escape the reaction vessel. According to one embodiment, the sealing component may be a silicone elastomer gasket that may be placed against an array of microwells with application of substantially uniform pressure across the entire substrate. In some cases, the reaction vessels may be sealed after the addition of the plurality of capture objects used for analyte capture and, optionally, any precursor labeling agent molecule that may be used to facilitate detection of the analyte molecule.

A non-limiting example of the formation of a plurality of reaction vessels containing assay solution on/in a substrate is depicted in FIG. 7. FIG. 7, panel (A) shows a surface comprising a plurality of microwells 139, which have been exposed to an assay solution 141 (e.g., a solution containing the analyte molecules), and a sealing component 143. Sealing component 143 in this example comprises a substantially planar bottom surface. Mating of substrate 139 with sealing component 143 forms a plurality of sealed reaction vessels 145. The areas between the reaction vessels 148 may be modified to aid in the formation of a tight seal between the reaction vessels.

A second embodiment is shown in FIG. 7, panel (B), in which sealing component 162 comprising a plurality of microwells 163 is mated with a substantially planar surface 158 which has been exposed to assay solution 162, thereby forming a plurality of reaction vessels 164.

In a third embodiment, as shown in FIG. 7, panel (C), substrate surface 166 comprising a plurality of microwells 167 is mated with sealing component 170 also comprising a plurality of microwells 171. In this embodiment, the microwells in the substrate and the microwells in the sealing components are substantially aligned so each reaction vessel 172 formed comprises a portion of the microwell from the sealing component and a portion of a microwell from the substrate. In FIG. 7, panel (D), the microwells are not aligned such that each reaction vessel comprises either a microwell from the sealing component 173 or a microwell from the substrate 175.

The sealing component may be essentially the same size as the substrate or may be different in size. In some cases, the sealing component is approximately the same size as the substrate and mates with substantially the entire surface of the substrate. In other cases, as depicted in FIG. 7, panel (E), the sealing component 176 is smaller than the substrate 174 and the sealing component only mates with a portion 178 of the substrate. In yet another embodiment, as depicted in FIG. 7, panel (F), the sealing component 182 is larger than the substrate 180, and only a portion 184 of the sealing component mates with the substrate 180.

In some embodiments, the reaction vessels may all have approximately the same volume. In other embodiments, the reaction vessels may have differing volumes. The volume of each individual reaction vessel may be selected to be appropriate to facilitate any particular assay protocol. For example, in one set of embodiments where it is desirable to limit the number of capture objects used for analyte capture contained in each vessel to a small number, the volume of the reaction vessels may range from attoliters or smaller to nanoliters or larger depending upon the nature of the capture objects, the detection technique and equipment employed, the number and density of the wells on the substrate and the expected concentration of capture objects in the fluid applied to the substrate containing the wells. In one embodiment, the size of the reaction vessel may be selected such only a single capture object used for analyte capture can be fully contained within the reaction vessel.

In accordance with one embodiment of the present invention, the reaction vessels may have a volume between about the reaction vessels may have a volume between about 1 femtoliter and about 1 picoliter, between about 1 femtoliters and about 100 femtoliters, between about 10 attoliters and about 100 picoliters, between about 1 picoliter and about 100 picoliters, between about 1 femtoliter and about 1 picoliter, or between about 30 femtoliters and about 60 femtoliters. In some cases, the reaction vessels have a volume of less than about 1 picoliter, less than about 500 femtoliters, less than about 100 femtoliters, less than about 50 femtoliters, or less than about 1 femtoliter. In some cases, the reaction vessels have a volume of about 10 femtoliters, about 20 femtoliters, about 30 femtoliters, about 40 femtoliters, about 50 femtoliters, about 60 femtoliters, about 70 femtoliters, about 80 femtoliters, about 90 femtoliters, or about 100 femtoliters.

The total number of locations and/or density of the locations employed in an assay (e.g., the number/density of reaction vessels in an array) can depend on the

composition and end use of the array. For example, the number of reaction vessels employed may depend on the number of types of analyte molecule and/or binding ligand employed, the suspected concentration range of the assay, the method of detection, the size of the capture objects, the type of detection entity (e.g., free labeling agent in solution, precipitating labeling agent, etc.). Arrays containing from about 2 to many billions of reaction vessels (or total number of reaction vessels) can be made by utilizing a variety of techniques and materials. Increasing the number of reaction vessels in the array can be used to increase the dynamic range of an assay or to allow multiple samples or multiple types of analyte molecules to be assayed in parallel. The array may comprise between one thousand and one million reaction vessels per sample to be analyzed. In some cases, the array comprises greater than one million reaction vessels. In some embodiments, the array comprises between about 1,000 and about 50,000, between about 1,000 and about 1,000,000, between about 1,000 and about 10,000, between about 10,000 and about 100,000, between about 100,000 and about 1,000,000, between about 100,000 and about 500,000, between about 1,000 and about 100,000, between about 50,000 and about 100,000, between about 20,000 and about 80,000, between about 30,000 and about 70,000, between about 40,000 and about 60,000, reaction vessels. In some embodiments, the array comprises about 10,000, about 20,000, about 50,000, about 100,000, about 150,000, about 200,000, about 300,000, about 500,000, about 1,000,000, or more, reaction vessels.

The array of reaction vessels may be arranged on a substantially planar surface or in a non-planar three-dimensional arrangement. The reaction vessels may be arrayed in a regular pattern or may be randomly distributed. In a specific embodiment, the array is a regular pattern of sites on a substantially planar surface permitting the sites to be addressed in the X-Y coordinate plane.

In some embodiments, the reaction vessels are formed in a solid material. As will be appreciated by those in the art, the number of potentially suitable materials in which the reaction vessels can be formed is very large, and includes, but is not limited to, glass (including modified and/or functionalized glass), plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, cyclic olefin copolymer (COC), cyclic olefin polymer (COP), Teflon®, polysaccharides, nylon or nitrocellulose, etc.), elastomers (such as poly(dimethyl siloxane) and poly urethanes), composite materials, ceramics, silica or silica-based materials (including silicon and modified silicon), carbon, metals, optical

fiber bundles, or the like. In general, the substrate material may be selected to allow for optical detection without appreciable autofluorescence. In certain embodiments, the reaction vessels may be formed in a flexible material.

A reaction vessel in a surface (e.g., substrate or sealing component) may be formed using a variety of techniques known in the art, including, but not limited to, photolithography, stamping techniques, molding techniques, etching techniques, or the like. As will be appreciated by those of the ordinary skill in the art, the technique used can depend on the composition and shape of the supporting material and the size and number of reaction vessels.

In a particular embodiment, an array of reaction vessels is formed by creating microwells in an end of a fiber optic bundle and utilizing a planar compliant surface as a sealing component. In certain such embodiments, an array of reaction vessels in the end of a fiber optic bundle may be formed as follows. First, an array of microwells is etched into the end of a polished fiber optic bundle. Techniques and materials for forming and etching a fiber optic bundle are known to those of ordinary skill in the art. For example, the diameter of the optical fibers, the presence, size and composition of core and cladding regions of the fiber, and the depth and specificity of the etch may be varied by the etching technique chosen so that microwells of the desired volume may be formed. In certain embodiments, the etching process creates microwells by preferentially etching the core material of the individual glass fibers in the bundle such that each well is approximately aligned with a single fiber and isolated from adjacent wells by the cladding material. Potential advantages of the fiber optic array format is that it can produce thousands to millions of reaction vessels without complicated microfabrication procedures and that it can provide the ability to observe and optically address many reaction vessels simultaneously.

Each microwell may be aligned with an optical fiber in the bundle so that the fiber optic bundle can carry both excitation and emission light to and from the wells, enabling remote interrogation of the well contents. Further, an array of optical fibers may provide the capability for simultaneous or non-simultaneous excitation of molecules in adjacent vessels, without signal "cross-talk" between fibers. That is, excitation light transmitted in one fiber does not escape to a neighboring fiber.

Alternatively, the equivalent structures of a plurality of reaction vessels may be fabricated using other methods and materials that do not utilize the ends of an optical fiber bundle as a substrate. For example, the array may be a spotted, printed or

photolithographically fabricated substrate produced by techniques known in the art; see for example WO95/25116; WO95/35505; PCT US98/09163; U.S. Patent Nos. 5,700,637, 5,807,522, 5,445,934, 6,406,845, and 6,482,593. In some cases, the array may be produced using molding, embossing, and/or etching techniques as will be known to those of ordinary skill in the art.

In certain embodiments, the present invention provides a system equipped with a mechanical platform that applies a sealing component to a substrate. The platform may be positioned beneath a stage on the system. After the chosen reaction components have been added to an array of reaction vessels, the sealing component may be mated with the array. For example, the sealing component may be sandwiched between a flat surface (such as, for example, a microscope slide) and the array of reaction vessels using uniform pressure applied by the mechanical platform.

A non-limiting embodiment is illustrated in FIG. 8A. A sealing component 300 is placed on top of mechanical platform 302. The assay solution 304 is placed on top of the sealing component 300. The mechanical platform is moved upwards towards the array 306 (e.g., fiber optic array) such that uniform pressure is applied. As shown in FIG. 8B, the sealing component 300 forms a tight seal with the array 306. In other instances, varying pressure may be applied to the sealing component to form a tight seal between the sealing component and the array. The system may also comprise additional components 312 that may be utilized to analyze the array (e.g., microscope, computer, etc.) as discussed more herein.

In some embodiments, the plurality of locations may not comprise a plurality of reaction vessels/wells. For example, in embodiments where capture objects are employed, a patterned substantially planar surface may be employed and the patterned areas form a plurality of locations. In some cases, the patterned areas may comprise substantially hydrophilic surfaces which are substantially surrounded by substantially hydrophobic surfaces. In certain embodiments, a plurality of capture objects (e.g., beads) may be substantially surrounded by a substantially hydrophilic medium (e.g., comprising water), and the beads may be exposed to the patterned surface such that the beads associate in the patterned areas (e.g., the hydrophilic locations on the surface), thereby spatially segregating the plurality of beads. For example, in one such embodiment, a substrate may be or include a gel or other material able to provide a sufficient barrier to mass transport (e.g., convective and/or diffusional barrier) to prevent capture objects used for analyte capture and/or precursor labeling agent and/or labeling

agent from moving from one location on or in the material to another location so as to cause interference or cross-talk between spatial locations containing different capture objects during the time frame required to address the locations and complete the assay. For example, in one embodiment, a plurality of capture objects is spatially separated by dispersing the capture objects on and/or in a hydrogel material. In some cases, a precursor labeling agent may be already present in the hydrogel, thereby facilitating development of a local concentration of the labeling agent (e.g., upon exposure to a binding ligand or analyte molecule carrying an enzymatic component). As still yet another embodiment, the capture objects may be confined in one or more capillaries. In some cases, the plurality of capture objects may be absorbed or localized on a porous or fibrous substrate, for example, filter paper. In some embodiments, the capture objects may be spatially segregated on a uniform surface (e.g., a planar surface), and the capture objects may be detected using precursor labeling agents which are converted to substantially insoluble or precipitating labeling agents that remain localized at or near the location of where the corresponding capture object is localized. The use of such substantially insoluble or precipitating labeling agents is described herein. In some cases, single analyte molecules may be spatially segregated into a plurality of droplets. That is, single analyte molecules may be substantially contained in a droplet containing a first fluid. The droplet may be substantially surrounded by a second fluid, wherein the second fluid is substantially immiscible with the first fluid.

Quantification

According to some embodiments of the present invention, the methods, systems, and/or devices are used to determine the presence and/or a measure of the concentration of a plurality of analyte molecules (or particles) in a fluid sample based at least in part on detecting and/or quantifying at least some of a plurality of locations comprising at least a first type of binding ligand and a second type of binding ligand. In some cases, the concentration of the analyte molecules in a fluid sample may be linearly proportional to the number of locations determined to contain at least a first type of binding ligand and a second type of binding ligand. In other cases, the measure of concentration of the analyte molecules in a fluid sample may be related to the determination and/or quantification of the number of locations containing at least a first type of binding ligand and a second type of binding ligand by a non-linear relationship. In some embodiments, a measure of the concentration of analyte molecules in a fluid sample may be determined

at least in part using a calibration curve. Methods to determine a measure of the concentration of analyte molecules in a fluid sample are discussed more below. In some cases, methods for determining a measure of the concentration of analyte molecules in a fluid same may be carried out according to the methods described in U.S. Patent Application Serial No. (not yet determined), entitled "Ultra-Sensitive Detection of Molecules or Particles using Beads or Other Capture Objects," by Duffy et al., filed March 23, 2010 (Attorney Docket No. Q0052.70011US01).

In some embodiments, additional determinations may be carried out. In some cases, the number of locations which do not comprise at least a first type of binding ligand and a second type of binding ligand (e.g., locations which contain only a first type of binding ligand or a second type of binding ligand, or no binding ligands). In these cases, a measure of the concentration of analyte molecules in a fluid sample may be based on the ratio of locations comprising at least a first type of binding ligand and a second type of binding ligand, to the number of locations comprising which do not contain at least a first type of binding ligand and a second type of binding ligand.

In certain embodiments, the fraction of locations (e.g., the statistically significant fraction) which comprises a single analyte molecule (e.g., associated with at least a first type of binding ligand and a second type of binding ligand) is less than about 50%, less than about 40%, less than about 25%, less than about 10%, less than about 5%, less than about 1%, less than about 0.5%, or less than about 0.1% of the total number of locations (e.g., in some cases, containing a capture object). In some cases, the percentage of locations which do not contain at least a first type of binding ligand and a second type of binding ligand is at least about 20%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 90%, or at least about 95%, at least about 99%, at least about 99.5%, at least about 99.9%, or greater, of the total number of locations.

According to one embodiment, the quantification method of the present invention can be performed as follows. A fluid sample containing or suspected of containing an analyte molecule of interest is contacted with one or more types of binding ligands (and optionally, a plurality of capture objects) and the analyte molecules (or capture objects) are partitioned across an array of locations, such as reaction vessels/wells (as described previously). In some embodiments, where a binary read-out method is desired to be used for determination, in the step of contacting the fluid sample with the locations, the relative amounts/concentrations of fluid sample and number of locations may be selected

(e.g., based on a known or estimated/suspected approximate concentration range of analyte molecules in the sample) so that the ratio of analyte molecules in the fluid sample to total number of locations is less than about 1:5, less than about 1:10, less than about 1:12, less than about 1:15, less than about 1:20, less than about 1:50, less than about 1:100, or less. With such ratios, at least some of the locations statistically will be expected to contain a single analyte molecule and the majority of the remainder of the locations will not contain any analyte molecules. The number of locations containing multiple analyte molecules under such conditions may be low enough to be neglected, such that locations determined to comprise an analyte molecule can be assumed to comprise a single analyte molecule. Under such conditions, an analysis system configured to perform a binary read out quantification may be used to determine the number of locations which comprise an analyte molecule by any detection method as described herein (e.g., by detecting the presence of more than one type of binding ligand in the reaction vessel). The number of locations which comprise an analyte molecule capture object associated with an analyte molecule is then counted. Utilization of a zero (no analyte molecule detected) or one (an analyte molecule detected) response, in conjunction with using an array with a large number of locations can permit a determination of bulk concentrations of analyte molecules in the sample by counting the actual number of molecules contained in the volume of sample partitioned across and contained in the locations.

In some assays, a Poisson distribution adjustment may be applied to numbers and/or ratios determined by a binary read-out method to facilitate and/or improve accuracy of determining a concentration of analyte molecules in a fluid sample. For example, in embodiments where the ratio of analyte molecules in the fluid sample to the total number of capture objects contacted with the fluid sample is greater than about 1:10, greater than about 1:5, greater than about 1:4, greater than about 1:3, or greater than about 1:2, or between about 1:10 and about 1:2, between about 1:5 and about 1:2, the number of analyte molecules immobilized per capture may be zero or one, with a greater proportion containing more than one than for the regime described in the paragraph above. In some such cases, performance and accuracy of the concentration determinations may be improved over use of an assumption that all positive locations contain only a single analyte molecule (as described in the paragraph above) by employing a Poisson distribution adjustment to predict the number of locations expected to contain 0, 1, 2, 3, 4, etc., analyte molecules (e.g., per capture object). Poisson

distribution adjustment will be known to those of ordinary skill in the art. For example, see U.S. Patent Application Serial No. (not yet determined), entitled "Ultra-Sensitive Detection of Molecules or Particles using Beads or Other Capture Objects" by Duffy et al., filed March 24, 2010 (Attorney Docket No. Q0052.70011US01)

In some embodiments, a measure of the concentration of analyte molecules or particles in the fluid sample may be determined at least in part by comparison of a measured parameter to a calibration standard. For example, the number and/or fraction of locations that comprise an analyte molecule associated with at least a first type of binding ligand and a second type of binding ligand (e.g., based on a binary read-out) may be calibrated against a calibration curve to determine a measure of the concentration of the analyte molecule in the fluid sample. The calibration curve may be produced by completing the assay with a plurality of standardized samples of known concentration under substantially similar conditions as those used to analyze a test sample with unknown concentration. A reading may be taken for the signal related to the detection/quantification of the analyte molecule associated with at least a first type of binding ligand and a second type of binding ligand for each standardized sample, therefore allowing for the formation of a calibration curve relating the detection of the analyte molecules associated with a first type of binding ligand and a second type of binding ligand with a known concentration of the analyte molecule. The assay may then be completed on a sample containing the analyte molecule in an unknown concentration, and the detection of the analyte molecule associated with at least a first type of binding ligand and a second type of binding ligand from this assay may be compared to the calibration curve to determine a measure of the concentration of the analyte molecule in the fluid sample.

In some embodiments, the concentration of analyte molecules in the fluid sample may be determined through use of a calibration curve using an assay system employing a computer. The computer may execute software that may use the data collected to produce the calibration curve and/or to determine a measure of the concentration of analyte molecules in a test fluid sample from such calibration curve. For example, a fluorescence image of an array comprising the plurality of capture objects partitioned across the array may be collected and analyzed using image analysis software (e.g., IP Lab, BD Biosciences). The analysis software may automatically calculate the number of locations that have fluorescence intensity over the background intensity (e.g., a number that correlates to the number of locations which comprise both a first type of binding

ligand and a second type of binding ligand). The number of locations which comprise fluorescence intensity over the background intensity may be divided by the total number of locations addressed, for example, to determine the fraction of locations which comprise an analyte molecule. The active location fraction may be compared to a calibration curve to determine a measure of the concentration of analyte molecules in the fluid sample.

In certain embodiments, it may be possible to increase both the dynamic range and the sensitivity of the assay by expanding the number of locations into which the capture objects are partitioned and/or by adjusting the ratio of capture objects (e.g. beads) to analyte molecules in the initial capture step. In certain cases, decreasing or increasing the analyte-to bead ratio may result in more dynamic range. In some cases, as the volume of a sample increases, detecting small numbers of analyte molecule with accuracy, may, in some cases, become more challenging for example, due to limitations of equipment, time constraints, etc. For example, to achieve the same efficiencies in larger volume sample (e.g., 1 mL, 10 mL) as achieved with a smaller volume sample (e.g., 100 μL), more beads (e.g., 10 and 100 times more beads, respectively) may be necessary, and thus, the beads may need to be spatially segregated into larger number of locations, wherein the larger number of locations may require an increased imaging area.

For the capture step, the choice of bead concentration may depend on several competing factors. For example, it can be advantageous if sufficient beads are present to capture most of the target analyte from thermodynamic and kinetic perspectives. As an exemplary illustration, thermodynamically, 200,000 beads in 100 μL that each have about 80,000 capture components (e.g. antibodies) bound to correlates to an antibody concentration of about 0.3 nM, and the antibody-protein equilibrium at that concentration may give rise to a relatively high capture efficiency of target analyte molecules in certain cases (e.g. >70%). Kinetically, for 200,000 beads dispersed in 100 μL, the average distance between beads can be estimated to be about 80 μm. Proteins the size of TNF-α and PSA (17.3 and 30 kDa, respectively), as exemplary analyte molecules, for example, will typically tend to diffuse 80 μm in less than 1 min, such that, over a 2 hour incubation, capture of such analyte molecules will tend not to be limited kinetically. In addition, it can also be advantageous to provide sufficient beads loaded onto the arrays to limit Poisson noise to a desired or acceptable amount. Considering as an example a situation where 200,000 beads in a in 10 μL volume are loaded onto an array, typically about 20,000–30,000 beads may become trapped in femtoliter sized wells of the array.

For a typical background signal (e.g. due to non specific binding, etc.) of 1% active beads, this loading would be expected to result in a background signal of 200–300 active beads detected, corresponding to a coefficient of variation (CV) from Poisson noise of 6–7%, which may be acceptable in typical embodiments. However, bead concentrations above certain concentrations may be undesirable in certain cases in that they may lead to: a) increases in non-specific binding that may reduce signal-to-background; and/or b) undesirably low ratios of analyte-to-bead such that the fraction of active beads is too low, resulting in high CVs from Poisson noise. In certain embodiments, considering a balance of factors such as those discussed above, providing about 200,000 to 1,000,000 beads per 100 µL of test sample may be desirable or, in certain cases optimal, for performing certain assays of the invention.

For embodiments of the inventive assay employing one or more binding ligand(s) to label the captured analyte molecules, it may be advantageous to, in certain instances, adjust the concentrations used to yield desirable or optimal performance. For example, considering an embodiment involving an analyte molecule that is a protein (captured protein) and employing a first binding ligand comprising a detection antibody and a second binding ligand comprising an enzyme conjugate (e.g. SβG), the concentrations of detection antibody and enzyme conjugate (SβG) used to label the captured protein may in some cases be limited or minimized to yield an acceptable background signal (e.g. 1% or less) and Poisson noise. The choice of the concentrations of detection antibody and enzyme conjugate (SβG) used to label the captured protein can be factors in improving the performance of or optimizing certain of the inventive assay methods. In certain cases, it may be desirable for only a fraction of the capture proteins to be labeled so as to avoid saturating signals produced by the assay. For example, for a particular assay where background levels observed are equivalent to ~1–2 fM of target protein, such that the ratio of analyte to bead may be about 0.3–0.6, the number of active beads may be in the range of about 25–40% if every protein was labeled with an enzyme, which may be higher than desirable in some cases. To produce background signals that may be closer to a lower end of the dynamic range for a digital detection assay— considering e.g. that in certain cases 1% active beads may provide a reasonable noise floor for background in digital detection assays of the invention—appropriate labeling of the captured protein can potentially be achieved by kinetic control of the labeling steps, either by limiting or minimizing the concentrations of both labeling reagents or by using shorter incubation times. For example, in an embodiment where label concentrations are minimized, use of

a standard ELISA incubation time may provide acceptable results; e.g. using a total assay time of ~6 h. This length of time may be acceptable for testing that tolerates a daily turnaround time for samples. For shorter turnaround times of, for example, <1 hour (e.g., for point-of-care applications), the assay could be performed with shorter incubations with higher concentrations of labels.

In some embodiments, the concentration of analyte molecules or particles in the fluid sample that may be substantially accurately determined is less than about 5000 fM, less than about 3000 fM, less than about 2000 fM, less than about 1000 fM, less than about 500 fM, less than about 300 fM, less than about 200 fM, less than about 100 fM, less than about 50 fM, less than about 25 fM, less than about 10 fM, less than about 5 fM, less than about 2 fM, less than about 1 fM, less than about 500 aM (attomolar), less than about 100 aM, less than about 10 aM, less than about 5 aM, less than about 1 aM, less than about 0.1 aM, less than about 500 zM (zeptomolar), less than about 100 zM, less than about 10 zM, less than about 5 zM, less than about 1 zM, less than about 0.1 zM, or less. In some cases, the limit of detection (e.g., the lowest concentration of an analyte molecule which may be determined in solution substantially accurately) is about 100 fM, about 50 fM, about 25 fM, about 10 fM, about 5 fM, about 2 fM, about 1 fM, about 500 aM (attomolar), about 100 aM, about 50 aM, about 10 aM, about 5 aM, about 1 aM, about 0.1 aM, about 500 zM (zeptomolar), about 100 zM, about 50 zM, about 10 zM, about 5 zM, about 1 zM, about 0.1 zM, or less. In some embodiments, the concentration of analyte molecules or particles in the fluid sample that may be substantially accurately determined is between about 5000 fM and about 0.1 fM, between about 3000 fM and about 0.1 fM, between about 1000 fM and about 0.1 fM, between about 1000 fM and about 0.1 zM, between about 100 fM and about 1 zM, between about 100 aM and about 0.1 zM.

The concentration of analyte molecules or particles in a fluid sample may be considered to be substantially accurately determined if the measured concentration of the analyte molecules or particles in the fluid sample is within about 10% of the actual (e.g., true) concentration of the analyte molecules or particles in the fluid sample. In certain embodiments, the measured concentration of the analyte molecules or particles in the fluid sample may be within about 5%, within about 4%, within about 3%, within about 2%, within about 1%, within about 0.5%, within about 0.4%, within about 0.3%, within about 0.2% or within about 0.1%, of the actual concentration of the analyte molecules or particles in the fluid sample. In some cases, the measure of the concentration determined

differs from the true (e.g., actual) concentration by no greater than about 20%, no greater than about 15%, no greater than 10%, no greater than 5%, no greater than 4%, no greater than 3%, no greater than 2%, no greater than 1%, or no greater than 0.5%. The accuracy of the assay method may be determined, in some embodiments, by determining the concentration of analyte molecules in a fluid sample of a known concentration using the selected assay method.

Exemplary Target Analytes

As will be appreciated by those in the art, a large number of analyte molecules and particles may be detected and, optionally, quantified using methods and systems of the present invention; basically, any analyte molecule that is able to be made to become immobilized with respect to a binding ligand can be potentially investigated using the invention. Certain more specific targets of potential interest that may comprise an analyte molecule are mentioned below. The list below is exemplary and non-limiting.

In some embodiments, the analyte molecule may be a biomolecule. Non-limiting examples of biomolecules include hormones, antibodies, cytokines, proteins, nucleic acids, lipids, carbohydrates, lipids cellular membrane antigens and receptors (neural, hormonal, nutrient, and cell surface receptors) or their ligands, or combinations thereof. Non-limiting embodiments of proteins include peptides, polypeptides, protein fragments, protein complexes, fusion proteins, recombinant proteins, phosphoproteins, glycoproteins, lipoproteins, or the like. As will be appreciated by those in the art, there are a large number of possible proteinaceous analyte molecules that may be detected or evaluated for binding partners using the present invention. In addition to enzymes as discussed above, suitable protein analyte molecules include, but are not limited to, immunoglobulins, hormones, growth factors, cytokines (many of which serve as ligands for cellular receptors), cancer markers, etc. Non-limiting examples of biomolecules include PSA and TNF-alpha.

In certain embodiments, the analyte molecule may be a host-translationally modified protein (e.g., phosphorylation, methylation, glycosylation) and the capture component may be an antibody specific to a post-translational modification. Modified proteins may be captured with capture components comprising a multiplicity of specific antibodies and then the captured proteins may be further bound to a binding ligand comprising a secondary antibody with specificity to a post-translational modification. Alternatively, modified proteins may be captured with capture components comprising

an antibody specific for a post-translational modification and then the captured proteins may be further bound to binding ligands comprising antibodies specific to each modified protein.

In another embodiment, the analyte molecule is a nucleic acid. A nucleic acid may be captured with a complementary nucleic acid fragment (e.g., an oligonucleotide) and then optionally subsequently labeled with a binding ligand comprising a different complementary oligonucleotide.

Suitable analyte molecules and particles include, but are not limited to small molecules (including organic compounds and inorganic compounds), environmental pollutants (including pesticides, insecticides, toxins, etc.), therapeutic molecules (including therapeutic and abused drugs, antibiotics, etc.), biomolecules (including hormones, cytokines, proteins, nucleic acids, lipids, carbohydrates, cellular membrane antigens and receptors (neural, hormonal, nutrient, and cell surface receptors) or their ligands, etc), whole cells (including prokaryotic (such as pathogenic bacteria) and eukaryotic cells, including mammalian tumor cells), viruses (including retroviruses, herpesviruses, adenoviruses, lentiviruses, etc.), spores, etc.

In some embodiments, the analyte molecule may be an enzyme. Non-limiting examples of enzymes include an oxidoreductase, transferase, kinase, hydrolase, lyase, isomerase, ligase, and the like. Additional examples of enzymes include, but are not limited to, polymerases, cathepsins, calpains, amino-transferases such as, for example, AST and ALT, proteases such as, for example, caspases, nucleotide cyclases, transferases, lipases, enzymes associated with heart attacks, and the like. When a system/method of the present invention is used to detect the presence of viral or bacterial agents, appropriate target enzymes include viral or bacterial polymerases and other such enzymes, including viral or bacterial proteases, or the like.

In other embodiments, the analyte molecule may comprise an enzymatic component. For example, the analyte particle can be a cell having an enzyme or enzymatic component present on its extracellular surface. Alternatively, the analyte particle is a cell having no enzymatic component on its surface. Such a cell is typically identified using an indirect assaying method described below. Non-limiting example of enzymatic components are horseradish peroxidase, beta-galactosidase, and alkaline phosphatase.

The fluid sample containing or suspected of containing an analyte molecule may be derived from any suitable source. In some cases, the sample may comprise a liquid,

fluent particulate solid, fluid suspension of solid particles, supercritical fluid, and/or gas. In some cases, the analyte molecule may be separated or purified from its source prior to determination; however, in certain embodiments, an untreated sample containing the analyte molecule may be tested directly. The source of the analyte molecule may be synthetic (e.g., produced in a laboratory), the environment (e.g., air, soil, etc.), a mammal, an animal, a plant, or any combination thereof. In a particular example, the source of an analyte molecule is a human bodily substance (e.g., blood, serum, plasma, urine, saliva, tissue, organ, or the like). The volume of the fluid sample analyzed may potentially be any amount within a wide range of volumes, depending on a number of factors such as, for example, the number of capture objects used/available, the number of locations us/available, etc. In a few particular exemplary embodiments, the sample volume may be about 0.01 ul, about 0.1 uL, about 1 uL, about 5 uL, about 10 uL, about 100 uL, about 1 mL, about 5 mL, about 10 mL, or the like. In some cases, the volume of the fluid sample is between about 0.01 uL and about 10 mL, between about 0.01 uL and about 1 mL, between about 0.01 uL and about 100 uL, or between about 0.1 uL and about 10 uL.

In some cases, the fluid sample may be diluted prior to use in an assay. For example, in embodiments where the source of an analyte molecule is a human body fluid (e.g., blood, serum), the fluid may be diluted with an appropriate solvent (e.g., a buffer such as PBS buffer). A fluid sample may be diluted about 1-fold, about 2-fold, about 3-fold, about 4-fold, about 5-fold, about 6-fold, about 10-fold, or greater, prior to use. The sample may be added to a solution comprising the plurality of capture objects, or the plurality of capture objects may be added directly to or as a solution to the sample.

Capture Components

In some embodiments of the present invention, the analyte molecules may immobilized with respect to a surface (e.g., the surface of a capture object, the surface of a location (e.g., reaction vessel), or the like). The analyte molecules may be immobilized with respect to a surface prior to or following exposure to a plurality of types of binding ligands. In some embodiments, immobilization of the analyte molecules with respect to a surface may aid in removal of any excess binding ligands from the solution without concern of dislodging the analyte molecule from the surface (e.g., from the reaction vessel). Generally, a capture component allows the attachment of a molecule, particle, or complex to a solid support (e.g., capture object, location, etc.) for the purposes of

immobilization, detection, quantification, and/or other analysis of the molecule, particle, or complex.

As will be appreciated by those in the art, the composition of the capture component will depend on the composition of the analyte molecule. Capture components for a wide variety of target molecules are known or can be readily found or developed using known techniques. For example, when the target molecule is a protein, the capture components may comprise proteins, particularly antibodies or fragments thereof (e.g., antigen-binding fragments (Fabs), Fab' fragments, pepsin fragments, $F(ab')_2$ fragments, full-length polyclonal or monoclonal antibodies, antibody-like fragments, etc.), other proteins, such as receptor proteins, Protein A, Protein C, etc., or small molecules. In some cases, capture components for proteins comprise peptides. For example, when the target molecule is an enzyme, suitable capture components may include enzyme substrates and/or enzyme inhibitors. In some cases, when the target analyte is a phosphorylated species, the capture component may comprise a phosphate-binding agent. For example, the phosphate-binding agent may comprise metal-ion affinity media such as those describe in U.S. Patent No. 7,070,921 and U.S. Patent Application No. 20060121544. In addition, when the target molecule is a single-stranded nucleic acid, the capture component may be a complementary nucleic acid. Similarly, the target molecule may be a nucleic acid binding protein and the capture component may be a single-stranded or double-stranded nucleic acid; alternatively, the capture component may be a nucleic acid-binding protein when the target molecule is a single or double stranded nucleic acid. Alternatively, as is generally described in U.S. Patents 5,270,163, 5,475,096, 5,567,588, 5,595,877, 5,637,459, 5,683,867, 5,705,337, and related patents, nucleic acid "aptamers" may be developed for capturing virtually any target molecule. Also, for example, when the target molecule is a carbohydrate, potentially suitable capture components include, for example, antibodies, lectins, and selectins. As will be appreciated by those of ordinary skill in the art, any molecule that can specifically associate with a target molecule of interest may potentially be used as a capture component.

For certain embodiments, suitable target analyte molecule/capture component pairs can include, but are not limited to, antibodies/antigens, receptors/ligands, proteins/nucleic acid, nucleic acids/nucleic acids, enzymes/substrates and/or inhibitors, carbohydrates (including glycoproteins and glycolipids)/lectins and/or selectins, proteins/proteins, proteins/small molecules; small molecules/small molecules, etc.

According to one embodiment, the capture components are portions (particularly the extracellular portions) of cell surface receptors that are known to multimerize, such as the growth hormone receptor, glucose transporters (particularly GLUT 4 receptor), and T-cell receptors and the target analytes are one or more receptor target ligands.

In a particular embodiment, the capture component may be attached to the surface via a linkage, which may comprise any moiety, functionalization, or modification of the binding surface and/or capture component that facilitates the attachment of the capture component to the surface. The linkage between the capture component and the surface may comprise one or more chemical or physical (e.g., non-specific attachment via van der Waals forces, hydrogen bonding, electrostatic interactions, hydrophobic/hydrophilic interactions; etc.) bonds and/or chemical linkers providing such bond(s). In certain embodiments, the capture component comprises a capture extender component. In such embodiments, the capture component comprises a first portion that binds the analyte molecule and a second portion that can be used for attachment to the binding surface.

In certain embodiments, a surface may also comprise a protective or passivating layer that can reduce or minimize non-specific attachment of non-capture components (e.g., analyte molecules, binding ligands) to the binding surface during the assay which may lead to false positive signals during detection or to loss of signal. Examples of materials that may be utilized in certain embodiments to form passivating layers include, but are not limited to: polymers, such as poly(ethylene glycol), that repel the non-specific binding of proteins; naturally occurring proteins with this property, such as serum albumin and casein; surfactants, e.g., zwitterionic surfactants, such as sulfobetaines; naturally occurring long-chain lipids; and nucleic acids, such as salmon sperm DNA.

One embodiment utilizes proteinaceous capture components. As is known in the art, any number of techniques may be used to attach a proteinaceous capture component to a wide variety of solid surfaces. "Protein" or "proteinaceous" in this context includes proteins, polypeptides, peptides, including, for example, enzymes, and antibodies. A wide variety of techniques are known to add reactive moieties to proteins, for example, the method outlined in U.S. Patent No. 5,620,850. The attachment of proteins to surfaces is known, for example, see Heller, Acc. Chem. Res. 23:128 (1990), and many other similar references.

In some embodiments, the capture component (or binding ligand) may comprise Fab' fragments. The use of Fab' fragments as opposed to whole antibodies may help

reduce non-specific binding between the capture component and the binding ligand. In some cases, the Fc region of a capture component (or binding ligand) may be removed (e.g., proteolytically). In some cases, an enzyme may be used to remove the Fc region (e.g., pepsin, which may produce $F(ab')_2$ fragments and papain, which may produce Fab fragments). In some instances, the capture component may be attached to a binding surface using amines or may be modified with biotin (e.g., using NHS-biotin) to facilitate binding to an avidin or streptavidin coated capture object surface. $F(ab')_2$ fragments may be subjected to a chemical reduction treatment (e.g., by exposure to 2-mercaptoethylamine) to, in some cases, form two thiol-bearing Fab' fragments. These thiol-bearing fragments can then be attached via reaction with a Michael acceptor such as maleimide. For example, the Fab' fragments may then be treated with a reagent (e.g., maleimide-biotin) to attach at least one biotin moiety (i.e., biotinylated) to facilitate attachment to streptavidin-coated surfaces as described above.

Certain embodiments utilize nucleic acids as the capture component, for example for when the analyte molecule is a nucleic acid or a nucleic acid binding protein, or when the it is desired that the capture component serve as an aptamer for binding a protein, as is well known in the art.

According to one embodiment, each binding surface comprises a plurality of capture components. The plurality of capture components, in some cases, may be distributed randomly on the binding surface like a "lawn." Alternatively, the capture components may be spatially segregated into distinct region(s) and distributed in any desired fashion.

Binding between the capture component and the analyte molecule, in certain embodiments, is specific, e.g., as when the capture component and the analyte molecule are complementary parts of a binding pair. In certain such embodiments, the capture component binds both specifically and directly to the analyte molecule. By "specifically bind" or "binding specificity," it is meant that the capture component binds the analyte molecule with specificity sufficient to differentiate between the analyte molecule and other components or contaminants of the test sample. For example, the capture component, according to one embodiment, may be an antibody that binds specifically to some portion of an analyte molecule (e.g., an antigen). The antibody, according to one embodiment, can be any antibody capable of binding specifically to an analyte molecule of interest. For example, appropriate antibodies include, but are not limited to, monoclonal antibodies, bispecific antibodies, minibodies, domain antibodies, synthetic

antibodies (sometimes referred to as antibody mimetics), chimeric antibodies, humanized antibodies, antibody fusions (sometimes referred to as "antibody conjugates"), and fragments of each, respectively. As another example, the analyte molecule may be an antibody and the capture component may be an antigen.

According to one embodiment in which an analyte particle is a biological cell (e.g., mammalian, avian, reptilian, other vertebrate, insect, yeast, bacterial, cell, etc.), the capture component may be a ligand having specific affinity for a cell surface antigen (e.g., a cell surface receptor). In one embodiment, the capture component is an adhesion molecule receptor or portion thereof, which has binding specificity for a cell adhesion molecule expressed on the surface of a target cell type. In use, the adhesion molecule receptor binds with an adhesion molecule on the extracellular surface of the target cell, thereby immobilizing or capturing the cell. In one embodiment in which the analyte particle is a cell, the capture component is fibronectin, which has specificity for, for example, analyte particles comprising neural cells.

In some embodiments, as will be appreciated by those of ordinary skill in the art, it is possible to detect analyte molecules using capture components for which binding to analyte molecules that is not highly specific. For example, such systems/methods may use different capture components such as, for example, a panel of different binding ligands, and detection of any particular analyte molecule is determined via a "signature" of binding to this panel of binding ligands, similar to the manner in which "electronic noses" work. This may find particular utility in the detection of certain small molecule analytes. In some embodiments, the binding affinity between analyte molecules and capture components should be sufficient to remain bound under the conditions of the assay, including wash steps to remove molecules or particles that are non-specifically bound. In some cases, for example in the detection of certain biomolecules, the binding constant of the analyte molecule to its complementary capture component may be between at least about 10^4 and about 10^6 M^{-1}, at least about 10^5 and about 10^9 M^{-1}, at least about 10^7 and about 10^9 M^{-1}, greater than about 10^9 M^{-1}, or greater. For example, typical affinities for IgG antibodies for their antigens are in the range 10^5-10^{10} M^{-1}. The affinity of biotin for streptavidin is 10^{15} M^{-1}.

The following examples are included to demonstrate various features of the invention. Those of ordinary skill in the art should, in light of the present disclosure, will appreciate that many changes can be made in the specific embodiments which are disclosed while still obtaining a like or similar result without departing from the scope of

the invention as defined by the appended claims. Accordingly, the following examples are intended only to illustrate certain features of the present invention, but do not necessarily exemplify the full scope of the invention.

Example 1

In the certain embodiments of assay methods of the present invention, there may be a variation in the elapsed time between addition of detector antibody and interrogation of certain arrays or regions of arrays versus interrogation of other arrays or regions. In certain cases, dissociation that may occur over such time intervals may result in inaccuracies in later made versus earlier made determinations of analyte molecule concentrations. This example investigated the effect of measures to reduce dissociation on the time variant accuracy of assay determinations. In this example, the assay comprised the following steps:

1. Immunocomplexes of enzyme-labeled PSA molecules (capture anti-PSA, PSA in serum, biotinylated detection anti-PSA, and SβG) were formed on magnetic beads and washed using known methods. For example, test solutions containing PSA were incubated with suspensions of magnetic beads functionalized with capture antibody (e.g., capture anti-PSA) for 1 h at 37 °C. The beads were then separated and washed three times in PBS. The beads were resuspended and incubated with solutions containing detection antibodies (e.g., biotinylated detection anti-PSA) for 30 min at 37 °C. The beads were then separated and washed three times in PBS. The beads were incubated with solutions containing SβG for 30 min at 37 °C, separated, and washed six times in PBS and 0.1% Tween-20. The beads were then resuspended in 10 uL of PBS in order to load into the wells of the fiber bundle arrays. Magnetic beads presenting immunocomplexes were exchanged into buffer containing a certain carbohydrate (e.g., sucrose, tehalose, etc.), or any other molecular species that can help maintain hydration state of proteins.
2. After loading beads into the microwells of fiber bundle arrays, excess of buffer was removed by air/vacuum drying. In some cases, the beads were loaded in the wells as follows: to apply the solution of beads to the etched wells in a fiber bundle, clear PVC tubing (1/16" I.D. 1/8" O.D.) and clear heat shrink (3/16" ID) were cut into approximately 1 cm long. A piece of PVC tubing was first put onto the etched and functionalized end of a fiber bundle to create a reservoir to hold

the bead solution, followed by the application of heat shrink around the interface between the PVC tubing and fiber bundle to provide a tight seal. 10 uL of the concentrated bead solution was pipetted into the reservoir created by the PVC tubing. The fiber bundle was then centrifuged at 3000 rpm (~1333 g) for 10 minutes to force the beads into the etched wells. The PVC tubing/heat shrink assembly was removed after centrifugation. The distal end of the fiber bundle was dipped in PBS solution to wash off excess bead solution, followed by swabbing the surface with deionized water.

3. The fiber bundle with beads loaded were stored for various periods of time. Right before signal generating/array read out, the dried fiber bundles were dipped in PBS buffer, wiped with a swab, dipped in PBS buffer again, and then dipped in a solution containing 100 μM RGP. The arrays were then sealed and read out. For example, in some cases, the system employed a custom-built imaging system containing a mercury light source, filter cubes, objectives, and a CCD camera was used for acquiring fluorescence images. Fiber bundle arrays were mounted on the microscope stage using a custom fixture. A droplet of β-galactosidase substrate (100 μM RPG) was placed on the silicone gasket material, and put into contact with the distal end of the fiber array. The precision mechanical platform moved the silicone sheet into contact with the distal end of the etched optical fiber array, creating an array of isolated femtoliter reaction vessels. Fluorescence images were acquired at 577 nm with an exposure time 1011 ms. Five frames (at 30 seconds per frame) were taken for each fiber bundle array. The fluorescent images were analyzed using image analysis software to determine the presence or absence of enzymatic activity within each well of the microwell array. The data was analyzed using a developed image processing software using MathWorks MATLAB and MathWorks Image Processing toolbox. The software aligned acquired image frames, identified reaction vessel positions, located reaction vessels with beads and measured the change in reaction vessel intensity over a predefined time period. Reaction vessels containing beads with sufficient intensity growth over all data frames were counted and the final number of active reaction vessels was reported as a percentage of all identified reaction vessels.

As well as fluorescence, the arrays were imaged with white light to identify those wells that contain beads. After acquiring the fluorescence images, the distal (sealed) end of the fiber bundle arrays were illuminated with white light

and imaged on the CCD camera. Due to scattering of light by the beads, those wells that contained a bead appeared brighter in the image than wells without beads. Beaded wells were identified using this method by software.

Dissociation of the biotinylated detection antibody from the captured analyte molecule, in particular, can cause a continual signal drop over time. In certain cases, dissociation can account for more than a 50% signal drop over the course of interrogation of an array or series of arrays during conduct of an assay (e.g., see FIG. 9A). FIG. 9A shows data from the similar experiments carried out over four different days. In these experiments, immunocomplexes of enzyme-labeled PSA were prepared simultaneously on beads in 64 wells of a 96-well plate that were arranged in 8-well columns. The beads from each column were then tested on a strip of 8 arrays using single molecule detection (Example 10) at increasing times (shown on the x-axis). Each data point is the average concentration of PSA determined from a column of 8 wells by comparison to a calibration curve that was obtained before the first column was tested. . This signal drop over time (which is manifested as a drop in the concentration of PSA that is determined) may be more pronounced in assays using proteins as reagents/targets than in assays involving nucleic acids (e.g. DNA). The decrease of signal fits to a dissociation model in certain cases. By carrying out drying of the wells in the present Example, the dissociation effects was eliminated and/or reduced by up to 90% (e.g., see FIG. 9B).

Those of ordinary skill in the art will be aware that while traditional immunoassays (e.g., ELISA) can suffer from signal loss due to dissociation of detection entities, in general, they do not require special treatment for the dissociation of immunocomplex because the plates are read out very quickly. For example, within a minute or so, a microplate reader can generate signal for a full 96-well plate. The difference in dissociation between well 1 and well 96 within this time frame is negligible. However, for assays like the methods described herein wherein interrogation of the assay sites may in certain cases take a longer time period, dissociation may affect the precision and/or accuracy of the methods. Accordingly, the process of drying the assay sites can prevent loss of an analyte molecule and/or binding ligand and reduce effects of dissociation.

In some embodiments, additional components may be provided which maintain the native configuration and/or biological activity of analyte molecules (e.g., proteins) in the microwells and which can protect the immunocomplexes and/or labeling agent(s)

(e.g. enzyme(s)) during the drying/rehydration process. For example, routine screening tests may be used to identify agents that can keep an antibody/antigen/enzyme complex intact and/or maintain enzyme activity after drying/rehydration. Without such agents, drying may denature and/or deactivate enzymes or other components of the assay. For example, screening of known nonionic kosmotropes revealed that carbohydrates help keep 90-100% of the immunocomplex signal after drying/rehydration treatment. Carbohydrates and other molecules may be used to maintain antibody active during dehydration in protein array assay and/or may be used to keep protein activity during dehydration/lyophilization.

Example 2

The following example describes the crosslinking of a binding ligand to an analyte molecule to aid in the prevention of disassociation of the binding ligand from the analyte molecule. In this assay, enzyme labeled PSA immunocomplexes were formed on beads using methods similar to those described in Example 1, using concentrations of PSA of 0, 0.1, and 1 pg/mL spiked into serum. One set of beads were reacted with 10 mg/mL of EDC for 10 minutes to cross-link the detection antibody to other proteins associated with the beads, e.g., PSA, capture antibody and blocking proteins. Control beads were not reacted with EDC. Control bead were then read using methods similar to those described above in Example 10 at t = 0 and then 2 hours later. The cross-linked beads were also read using methods similar to as described in Example 10 at about 2 hours after crosslinking The result showed that after 2 hours the average number of enzymes per bead on the control beads was reduced because of dissociation of the detection antibody. The crosslinked beads, however, maintained the high AEB even after 2 hours. These data suggest that crosslinking the detection antibody to proteins irreversibly associated with the beads substantially eliminated the dissociation effects. In addition, crosslinking boosted the signal, also as shown in FIG. 10, (EDAC-treated; (ii)) relative to the 0 hour wait (i) presumably because of the extra dissociation time the control beads experienced before being read.

While several embodiments of the present invention have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the functions and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or

modifications is deemed to be within the scope of the present invention. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the teachings of the present invention is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, the invention may be practiced otherwise than as specifically described and claimed. The present invention is directed to each individual feature, system, article, material, kit, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, kits, and/or methods, if such features, systems, articles, materials, kits, and/or methods are not mutually inconsistent, is included within the scope of the present invention.

The indefinite articles "a" and "an," as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean "at least one."

In the claims, as well as in the specification above, all transitional phrases such as "comprising," "including," "carrying," "having," "containing," "involving," "holding," and the like are to be understood to be open-ended, i.e., to mean including but not limited to. Only the transitional phrases "consisting of" and "consisting essentially of" shall be closed or semi-closed transitional phrases, respectively.

What is claimed:

Claims

1. A method for determining a measure of the concentration of analyte molecules or particles in a fluid sample, comprising:

providing the analyte molecules or particles immobilized with respect to a binding surface having affinity for at least one type of analyte molecule or particle, the binding surface forming or contained within one of a plurality of locations on a substrate, such that at least some of the plurality of locations contain at least one analyte molecule or particle and a statistically significant fraction of the plurality of locations do not contain any analyte molecules or particles;

exposing the analyte molecules or particles to a first type of binding ligand having an affinity for a first epitope on the analyte molecules or particles and a second type of binding ligand having an affinity for a second epitope on the analyte molecules or particles;

addressing at least a portion of the plurality of the locations and determining the number of said locations containing a first type of binding ligand and a second type of binding ligand; and

determining a measure of the concentration of analyte molecules or particles in the fluid sample based at least in part on the number of locations containing both a first type of binding ligand and a second type of binding ligand.

2. The method of claim 1, wherein the exposing step is performed prior to the analyte molecules or particles being immobilized with respect to the binding surface.

3. The method of claim 1, wherein the exposing step is performed after the analyte molecules or particles have been immobilized with respect to the binding surface.

4. The method of claim 3, wherein the binding surface comprises the surface of a bead contained within one of a plurality of locations on a substrate, and wherein the exposing step is performed prior to the bead being contained within one of the plurality of locations on the substrate.

5. The method of claim 3, wherein the binding surface comprises the surface of a bead contained within one of a plurality of locations on a substrate, and wherein the

exposing step is performed after the bead is contained within one of the plurality of locations on the substrate.

6. A method for determining a measure of the concentration of analyte molecules or particles in a fluid sample, comprising:

providing a fluid sample containing or suspected of containing a plurality of analyte molecules or particles;

exposing the plurality of analyte molecules or particles to a first type of binding ligand having an affinity for a first epitope on the analyte molecules or particles and a second type of binding ligand having an affinity for a second epitope on the analyte molecules or particles;

spatially separating at least a portion of the analyte molecules or particles into a plurality of locations, such that at least some of the plurality of locations contain at least one analyte molecule or particle and a statistically significant fraction of the plurality of locations do not contain any analyte molecules or particles;

addressing at least a portion of the plurality of locations and determining the number of said locations containing both a first type binding ligand and a second type of binding ligand; and

determining a measure of the concentration of analyte molecules or particles in the fluid sample based at least in part on the number of locations containing both a first type of binding ligand and a second type of binding ligand.

7. The method of claim 6, wherein the exposing step occurs prior to the spatially separating step.

8. The method of claim 6, wherein the spatially separating steps occurs prior to the exposing step.

9. The method of claim 6, wherein the plurality of location each comprise a binding surface having an affinity for the plurality of analyte molecules or particles.

10. The method of claim9, further comprising immobilizing the portion of analyte molecules which were spatially separating into the plurality of locations with respect to the binding surface of the locations.

11. The method of claim 6, further comprising immobilizing the plurality of analyte molecules or particles to a plurality of discrete objects such that a statistically significant fraction of the discrete objects are immobilized with respect to only a single analyte molecule or particle and a statistically significant fraction of the discrete objects are free of any analyte molecules or particles, prior to the exposing and spatially separating steps.

12. The method of claim 1 or 6, wherein the binding surfaces are presented on a plurality of discrete objects.

13. The method of claim 12, wherein the exposing step is conducted prior to containing the plurality of binding surfaces within a plurality of locations.

14. The method of claim 12, wherein the exposing step is conducted following containing the plurality of binding surfaces within a plurality of locations.

15. The method of claims 12, wherein the discrete objects comprise beads.

16. The method of claim 15, wherein the beads are magnetic.

17. The method of claim 1 or 6, wherein the first type of binding ligand and the second type of binding ligand are directly detected.

18. The method of claim 1 or 6, wherein the first type of binding ligand and the second type of binding ligand are indirectly detected.

19. The method of claim 1 or 6, wherein the first type of binding ligand comprises a first type of enzymatic component and the second type of binding ligand comprises a second type of enzymatic component.

20. The method of any claim 1 or 6, further comprising exposing the plurality of locations to a plurality of a first type of precursor labeling agent and a second type of precursor labeling agent.

21. The method of claim 20, wherein the first type of precursor labeling agent is converted to a first type of labeling agent upon exposure to the first type of binding ligand and the second precursor labeling agent is converted to a second type of labeling agent upon exposure to the second type of binding ligand, wherein the first type of labeling agent is distinguishable from the second type of labeling agent.

22. The method of claim 21, wherein the presence of a first type of binding ligand in a location is determined by determining the presence of the first type of labeling agent in that location and the presence of a second type of binding ligand in a location is determined by determining the presence of the second type of labeling agent in that location.

23. The method of claims 21, wherein the first type of labeling agent and the second type of labeling agent are chromogenic, fluorescent, or chemiluminescent.

24. The method of claim 1 or 6, further comprising exposing the plurality of analyte molecules to a third type of binding ligand.

25. The method of claim 24, wherein the third type of binding ligand associates with a first type of binding ligand and a second type of ligand, wherein said first type of binding ligand and said second type of binding ligand are immobilized with respect to a single analyte molecule.

26. The method of claim 24, wherein the presence of a first type of binding ligand and a second type of binding ligand in a location is determined by determining the presence of a third type of binding ligand in the location.

27. The method of claim 24, wherein a measure of the concentration of analyte molecules or particles in the fluid sample is based at least a part on the number of locations containing a third type of binding ligand.

28. The method of claim 24, further comprising exposing the plurality of analyte molecules or particles to a fourth type of binding ligand.

29. The method of claim 28, wherein the third type of binding ligand associates with the first type of binding ligand and the fourth type of binding ligand associates with the second type of binding ligand.

30. The method of claim 29, wherein the presence of a first type of binding ligand in a location is determined by determining the presence of the third type of binding ligand in that location and the presence of a second type of binding ligand in a location is determined by determining the presence of the fourth type of binding ligand in that location.

31. The method of claim 29, wherein the third type of binding ligand and the fourth type of binding ligand is directly detected.

32. The method of claim 29, wherein the third type of binding ligand and fourth type of binding ligand are indirectly detected.

33. The method of claim 1 or 6, wherein the first epitope and the second epitope are the same.

34. The method of claim 1 or 6, wherein the first epitope and the second epitope are different.

35. The method of claim 1 or 6, wherein the number of said locations containing a first type of binding ligand and a second type of binding ligand are determined using optical techniques.

36. The method of claim 1 or 6, wherein the number of said locations containing a first type of binding ligand and a second type of binding ligand are determined using fluorescence.

37. The method of claim 35, wherein the optical techniques comprises the use of a CCD detector.

38. The method of claim 1 or 6, wherein the plurality of locations comprises a plurality of reaction vessels.

39. The method of claim 38, wherein the plurality of reaction vessels is formed on the end of a fiber optic bundle.

40. The method of claim 38, further comprising sealing the plurality of reaction vessels.

41. The method of claim 38, wherein the average volume of the plurality of reaction vessels is between about 10 attoliters and about 100 picoliters, or between about 1 femtoliter and about 100 femtoliters, or between about 1 femtoliter and about 1 picoliter, or between about 1 micrometer and about 10 micrometers

42. The method of claim 1 or 6, wherein the concentration of analyte molecules or particles in the fluid sample is less than about 50×10^{-15} M, or less than about 40×10^{-15} M, or less than about 30×10^{-15} M, or less than about 20×10^{-15} M, or less than about 10×10^{-15} M, or less than about, or less than about 1×10^{-15} M.

43. The method of claim 1 or 6, wherein the measure of the concentration of analyte molecules or particles in the fluid sample is determined at least in part by comparison of a measured parameter to a calibration standard.

44. The method of claim 1 or 6, wherein the number of locations addressed in the addressing step is at least about 5%, or at least about 10%, or at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% of the total number of locations.

45. The method of claim 1 or 6, wherein the analyte molecules or particles are proteins or nucleic acids.

46. The method of claim 1 or 6, further comprising performing at least one wash step.

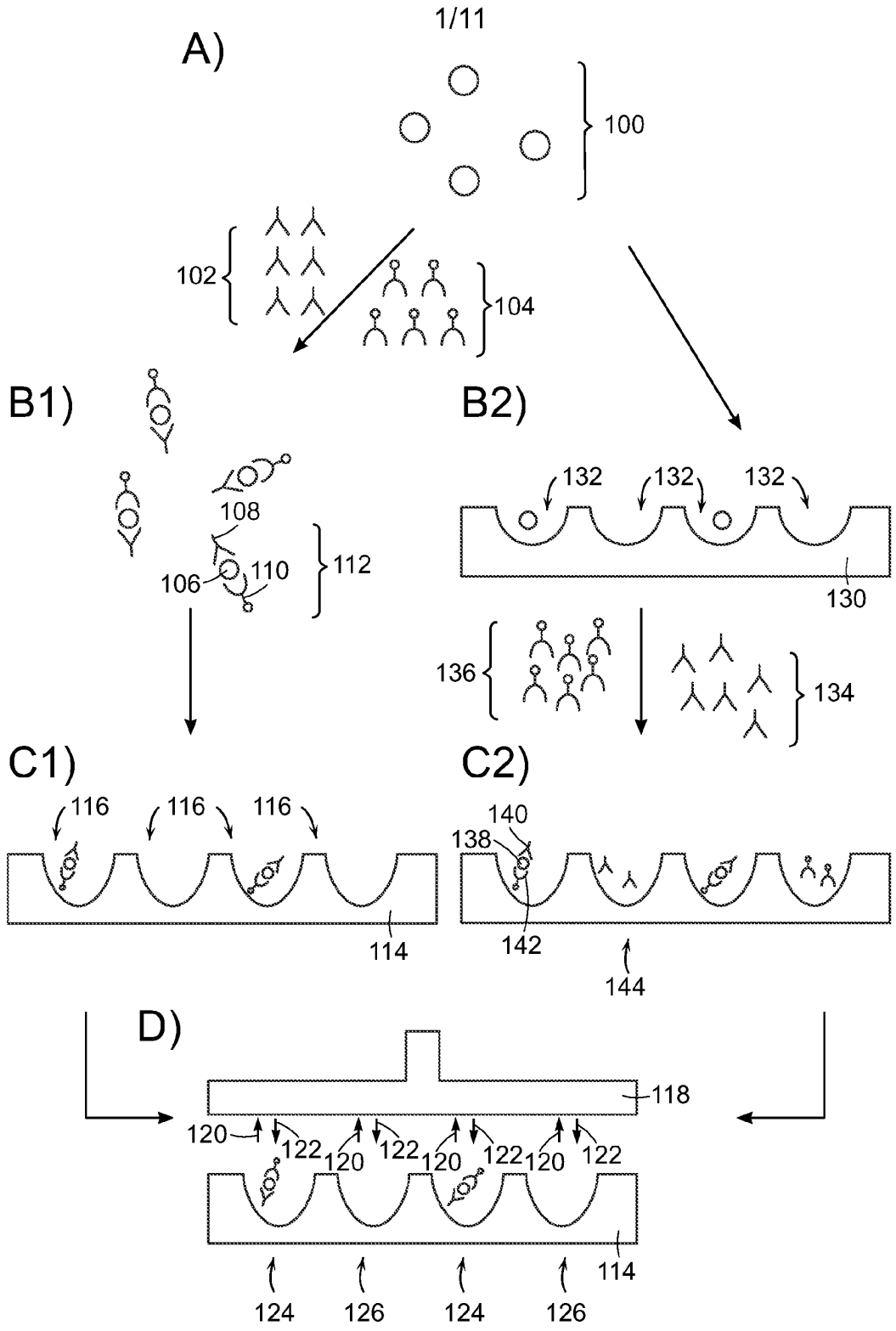


FIG. 1

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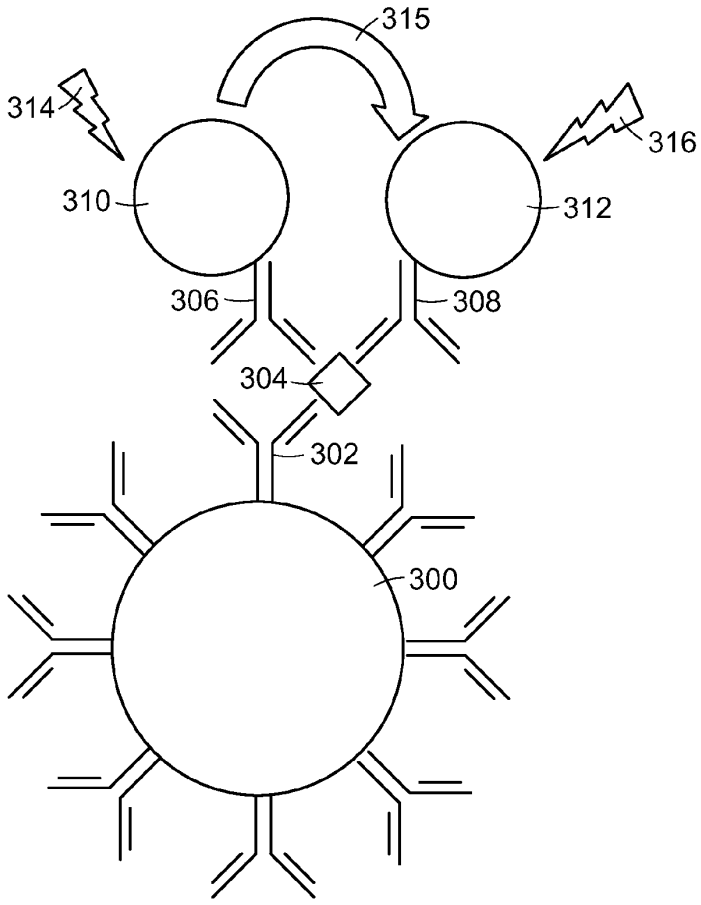


FIG. 2

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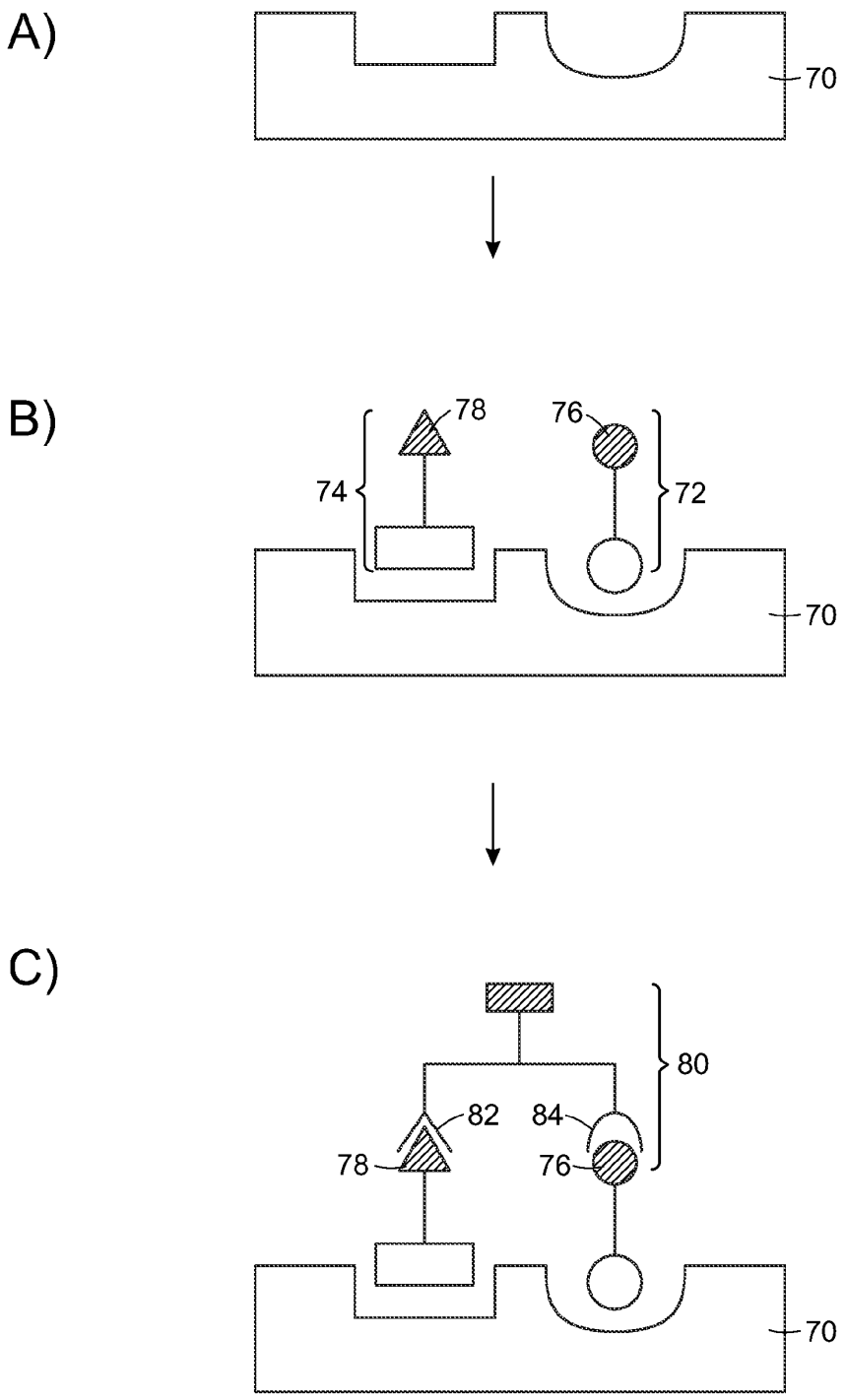


FIG. 3

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
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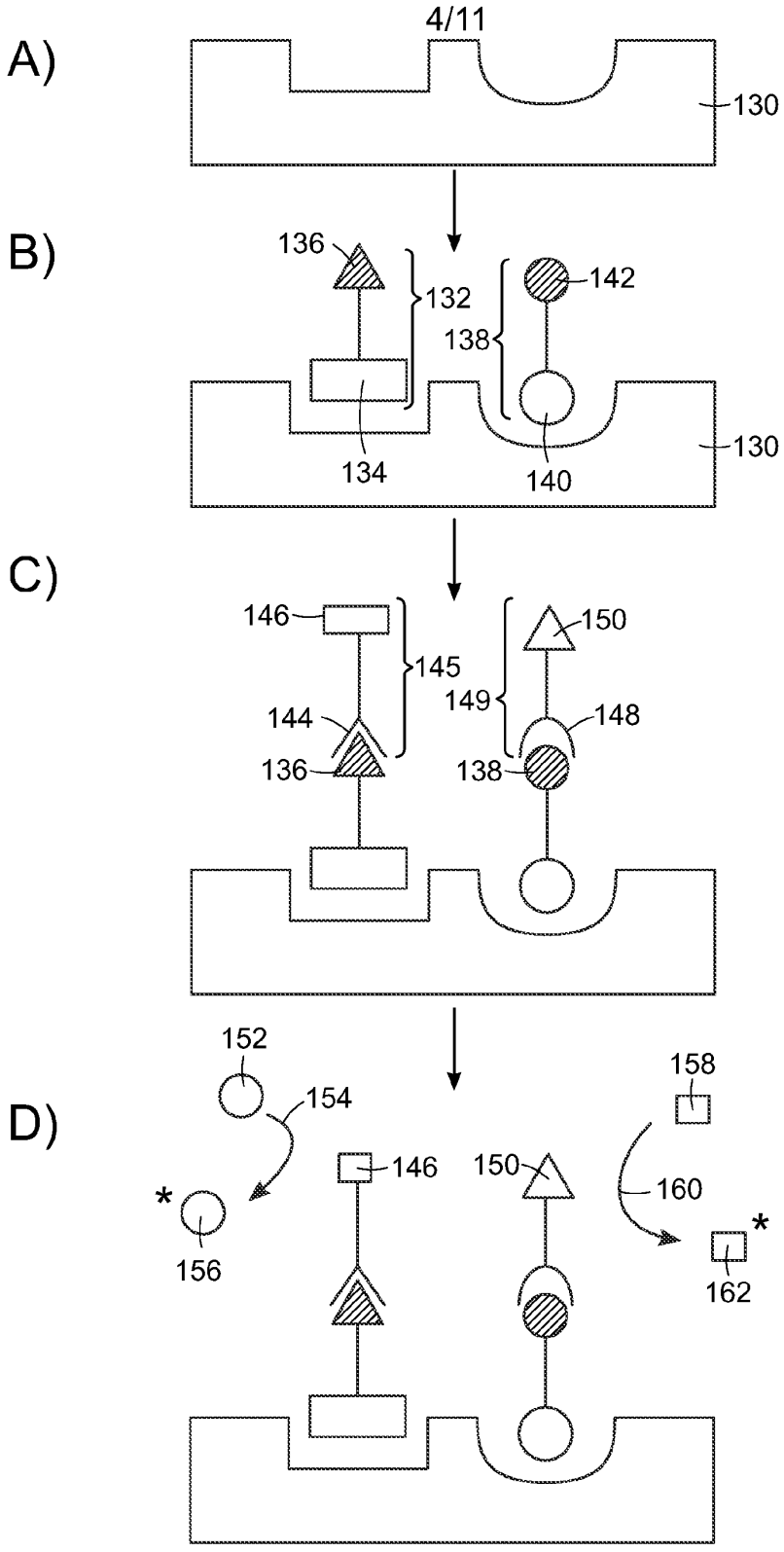


FIG. 4

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
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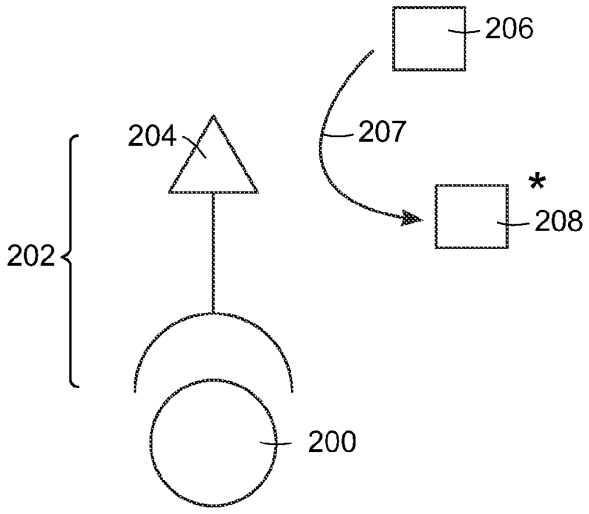


FIG. 5

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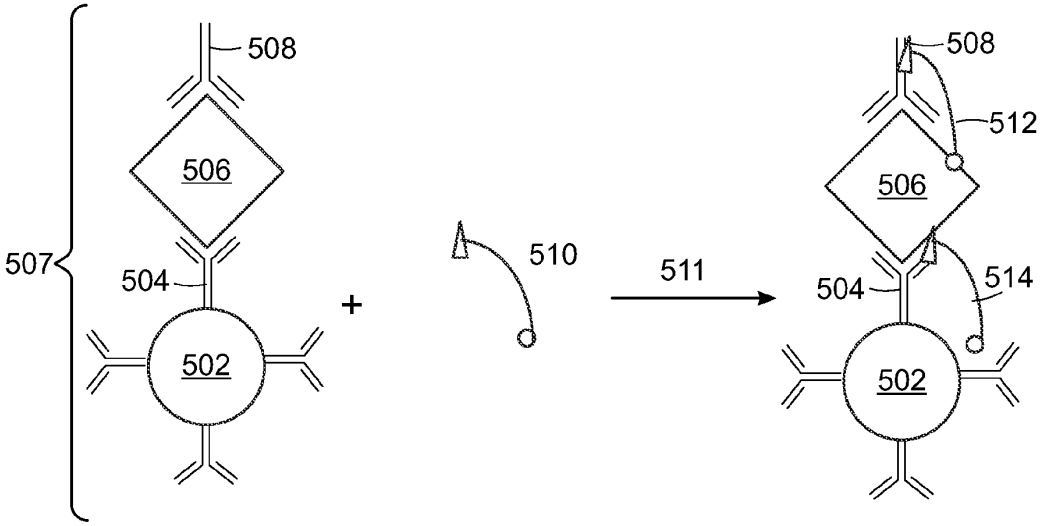


FIG. 6A

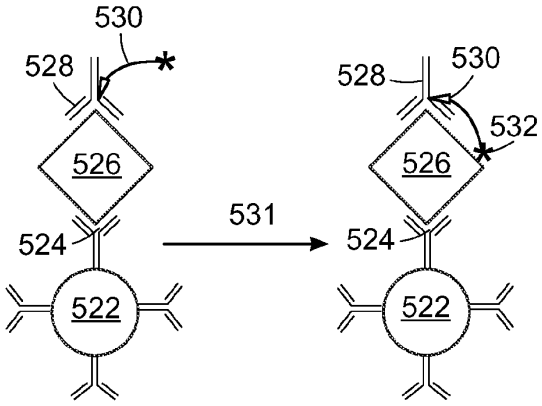


FIG. 6B

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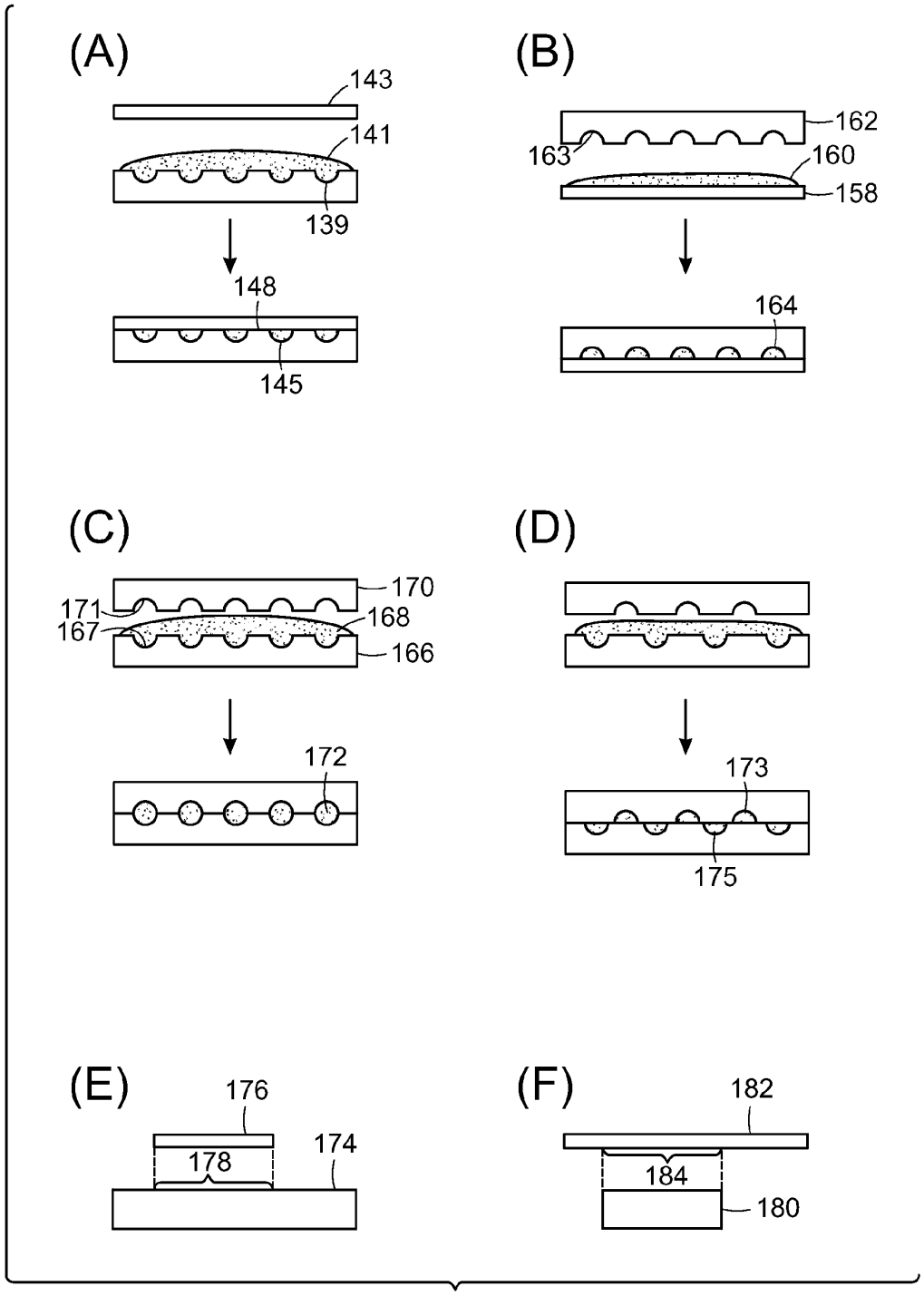


FIG. 7

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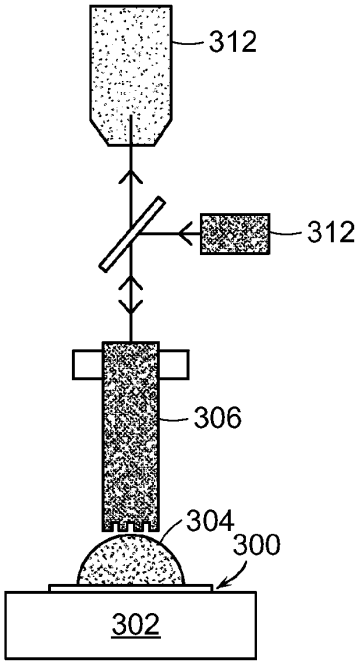


FIG. 8A

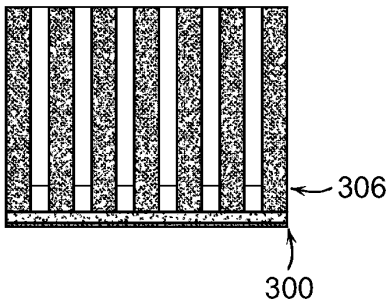


FIG. 8B

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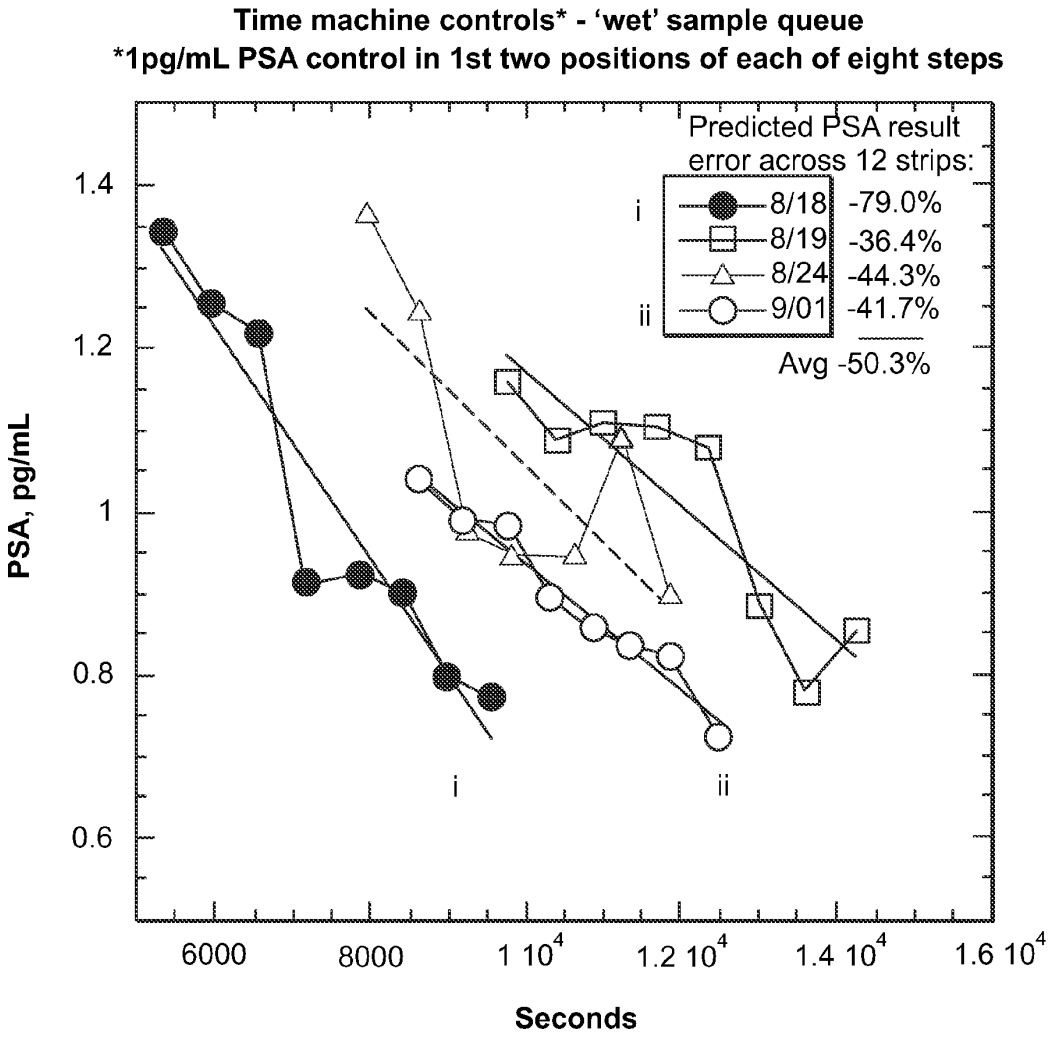


FIG. 9A

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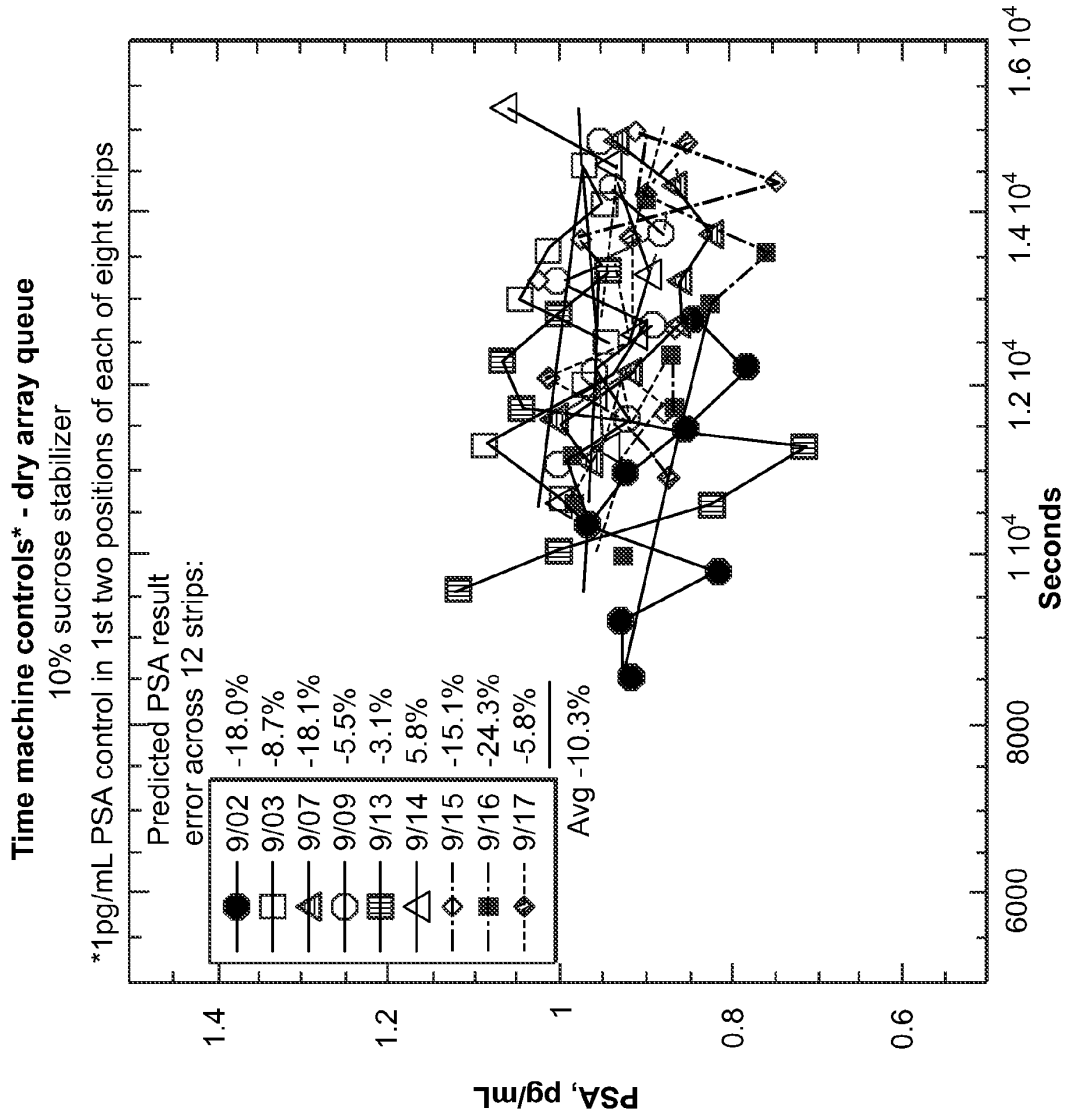


FIG. 9B

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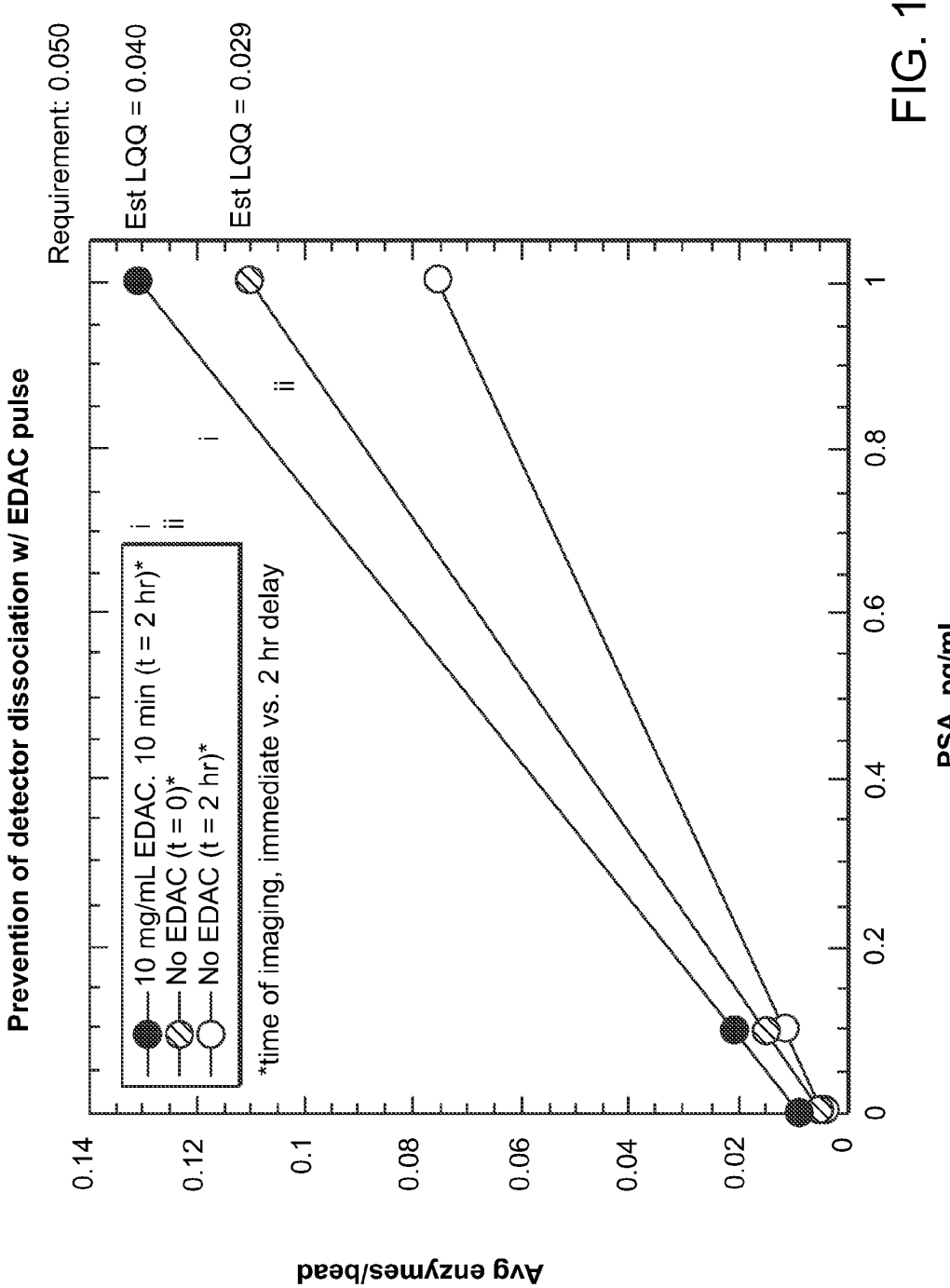


FIG. 10

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/026657

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/543
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, FSTA, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BHAT SOMANATH ET AL: "Single molecule detection in nanofluidic digital array enables accurate measurement of DNA copy number", ANALYTICAL AND BIOANALYTICAL CHEMISTRY, vol. 394, no. 2, May 2009 (2009-05), pages 457-467, XP002632899, ISSN: 1618-2642 abstract pages 458-464	1-5
Y	WO 2009/029073 A1 (TUFTS COLLEGE [US]; WALT DAVID R [US]; RISSIN DAVID M [US]) 5 March 2009 (2009-03-05) pages 2-6	1-46

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 April 2011	24/05/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Lunter, Pim

Form PCT/ISA/210 (second sheet) (April 2005)

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	AGRAWAL A ET AL: "Nanometer-scale mapping and single-molecule detection with color-coded nanoparticle probes", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA NATIONAL ACADEMY OF SCIENCES USA, vol. 105, no. 9, 4 March 2008 (2008-03-04) , pages 3298-3303, XP002632900, ISSN: 0027-8424 abstract -----	1-46

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2011/026657

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009029073 A1	05-03-2009	CA 2734029 A1	05-03-2009
		EP 2201374 A1	30-06-2010
		JP 2010538260 T	09-12-2010

Form PCT/ISA/210 (patent family annex) (April 2005)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
Q0052.70017		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US2012/033338	**12 APRIL 2012 (12.04.2012)**	12 APRIL 2011 (12.04.2011)

Applicant

QUANTERIX CORPORATION et al

This International search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of **4** sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language**, the international search was carried out on the basis of :
      ☒ the international application in the language in which it was filed
      ☐ a translation of the international application into _____ , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
   b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).
   c. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.
2. ☒ **Certain claims were found unsearchable** (See Box No. II)
3. ☐ **Unity of invention is lacking** (See Box No. III)
4. With regard to the **title,**
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established by this Authority to read as follows:
5. With regard to the **abstract,**
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.
6. With regard to the drawings,
   a. the figure of the **drawings** to be published with the abstract is Figure No. **1A**
      ☒ as suggested by the applicant.
      ☐ as selected by this Authority, because the applicant failed to suggest a figure.
      ☐ as selected by this Authority, because this figure better characterizes the invention.
   b. ☐ none of the figure is to be published with the abstract.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/033338

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

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

1. ☒ Claims Nos.: 10-12,14-16,18-20,22-98
because they relate to subject matter not required to be searched by this Authority, namely:

Claims 10-12,14-16,18-20,22-98 pertain to diagnostic methods for determining a patient's prognosis recovery from a brain injury following a hypoxic event, and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

2. ☒ Claims Nos.: 69-71,74,79,84,91,92
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

These claims are unclear because they are dependent claims referring to a dependent claim which do not comply with PCT Rule 6.4(a)

3. ☒ Claims Nos.: 41,66-68,72,73,75-78,80-83,85-90,93-98
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/033338

A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/68(2006.01)i, G01N 33/53(2006.01)i, G01N 33/58(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N 33/68; G06F 19/00; C12N 5/06; G01N 33/00; G01N 33/537; C07K 16/00; G01N 33/53; A61K 31/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: beta-amyloid, assay, concentration, recovery, brain injury, hypoxic, treatment protocol

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2006-0205024 A1 (ROGERS, J. et al.) 14 September 2006 See the whole document.	1-9,13,17,21,99
A	US 2008-0199879 A1 (TAKAYAMA SHIGEO et al.) 21 August 2008 See the whole document.	1-9,13,17,21,99
A	WO 2009-156747 A3 (THE NOTTINGHAM TRENT UNIVERSITY et al.) 30 December 2009 See the whole document.	1-9,13,17,21,99
A	US 2008-0234311 A1 (LI JINHE et al.) 25 September 2008 See the whole document.	1-9,13,17,21,99

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

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 NOVEMBER 2012 (29.11.2012)	**29 NOVEMBER 2012 (29.11.2012)**
Name and mailing address of the ISA/KR Korean Intellectual Property Office 189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea Facsimile No. 82-42-472-7140	Authorized officer Kang Yeon Mu Telephone No. 82-42-481-5516

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

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2012/033338

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2006-0205024 A1	14.09.2006	None	
US 2008-0199879 A1	21.08.2008	AU 2005-297854 A1	04.05.2006
		BR PI0516674A	16.09.2008
		CA 2585148 A1	04.05.2006
		CN 101048662 A0	03.10.2007
		EP 1813947 A1	01.08.2007
		EP 1813947 A4	11.06.2008
		IL182540D0	20.09.2007
		JP WO20-060466 44A1	04.05.2006
		KR 10-2007-0073778 A	10.07.2007
		KR20070073778A	10.07.2007
		MX 2007005053 A	19.06.2007
		NO20072206A	30.07.2007
		NO20072206B	30.07.2007
		WO 2006-046644 A1	04.05.2006
WO 2009-156747 A3	30.12.2009	GB 0811858 D0	30.07.2008
		WO 2009-156747 A2	30.12.2009
US 2008-0234311 A1	25.09.2008	WO 2009-067272 A1	28.05.2009

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PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:
GABERT ANDREA J.

WOLF, GREENFIELD & SACKS, P.C. 600 ATLANTIC AVENUE BOSTON MA 02210-2206 USA

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43bis.1)

Date of mailing *(day/month/year)*	**29 NOVEMBER 2012 (29.11.2012)**
Applicant's or agent's file reference Q0052.70017	**FOR FURTHER ACTION** See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US2012/033338	**12 APRIL 2012 (12.04.2012)**	12 APRIL 2011 (12.04.2011)

International Patent Classification (IPC) or both national classification and IPC

G01N 33/68(2006.01)i, G01N 33/53(2006.01)i, G01N 33/58(2006.01)i

Applicant

QUANTERIX CORPORATION et al

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☒ Box No. II Priority
- ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☒ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.
For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/KR	Date of completion of this opinion	Authorized officer
Korean Intellectual Property Office 189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea Facsimile No. 82-42-472-7140	29 NOVEMBER 2012 (29.11.2012)	Kang Yeon Mu Telephone No.82-42-481-5516

Form PCT/ISA/237 (cover sheet) (July 2011)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US2012/033338

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of :

   ☒ the international application in the language in which it was filed

   ☐ a translation of the international application into _____ , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of:

   a. a sequence listing filed or furnished
      ☐ on paper
      ☐ in electronic form

   b. time of filing or furnishing
      ☐ contained in the international application as filed.
      ☐ filed together with the international application in electronic form.
      ☐ furnished subsequently to this Authority for the purposes of search.

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additioanl copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
4192

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US2012/033338

Box No. II Priority

1. ☒ The validity of the priority claim has not been considered because the International Searching Authority does not have in its possession a copy of the earlier application whose priority has been claimed or, where required, a translation of that earlier application. This opinion has nevertheless been established on the assumption that the relevant date(Rules 43bis.1 and 64.1) is the claimed priority date.

2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid (Rules 43bis.1 and 64.1). Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if neccessary:

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
4193

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US2012/033338

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application

☒ claims Nos. 10-12,14-16,18-20,22-98

because:

☒ the said international application, or the said claims Nos. 10-12,14-16,18-20,22-98 relate to the following subject matter which does not require an international search *(specify)*:

Claims 10-12,14-16,18-20,22-98 pertain to diagnostic methods for determining a patient's prognosis for recovery from a brain injury following a hypoxic event, and thus relate to a subject matter which this International Searching Authority is not required, under Rules 43 bis.1(b), Rule 67.1 (iv)), to search.

☒ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. 69-71,74,79,84,91,92 are so unclear that no meaningful opinion could be formed *(specify)*:

Claims 69-71,74,79,84,91,92 are unclear because they are dependent claims referring to dependent claims which do not comply with PCT Rule 6.4(a).

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed *(specify)*:

☒ no international search report has been established for said claims Nos. 10-12,14-16,18-20,22-98

☐ a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:

☐ furnish a sequence listing on paper complying with the standard provided for in Annex C of the Administrative Istructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.

☐ furnish a sequence listing in electronic form complying with the standard provided for in Annex C of the Administrative Istructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.

☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13ter.1(a) or (b).

☐ See Supplemental Box for further details.

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US2012/033338

Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-9,13,17,21,99	YES
	Claims	NONE	NO
Inventive step (IS)	Claims	1-9,13,17,21,99	YES
	Claims	NONE	NO
Industrial applicability (IA)	Claims	1-9,13,17,21,99	YES
	Claims	NONE	NO

2. Citations and explanations :

Reference is made to the following documents:

D1: US 2006-0205024 A1 (ROGERS, J. et al.) 14 September 2006
D2: US 2008-0199879 A1 (TAKAYAMA SHIGEO et al.) 21 August 2008
D3: WO 2009-156747 A3 (THE NOTTINGHAM TRENT UNIVERSITY et al.) 30 December 2009
D4: US 2008-0234311 A1 (LI JINHE et al.) 25 September 2008

1. Novelty and Inventive Step

1.1. Independent Claims 1,13,17,21

1.1.1. Concerning claim 1

None of the documents cited in the ISR show that a method for determining a measure of the concentration of beta-amyloid peptide in a patient sample containing or not containing beta-amyloid peptide, wherein the limit of detection of beta-amyloid peptide of the assay is less than about 0.2 pg/mL. Accordingly, the feature of claim 1 is not anticipated by any of the documents, nor is it obvious by the documents, taken alone or in combination. Therefore, claim 1 is novel and involves an inventive step under PCT Article 33(2) and (3).

1.1.2. Concerning claims 13,17,21

None of the documents cited in the ISR show the methods for performing an assay and providing data for determining a treatment protocol for, or a prognosis of a patient's recovery from, a brain injury resulting from a hypoxic event, wherein the concentration of the biomarker and quantification of the assay are specifically limited. Accordingly, claims 13,17,21 are not anticipated by any of the documents, nor are they obvious by the documents, taken alone or in combination. Therefore, claims 13,17,21 are novel and involve an inventive step under PCT Article 33(2) and (3).

1.2. Dependent Claims 2-9,99

Claims 2-9,99 are dependent on claim 1. Consequently, Claims 2-9,99 are also considered to be novel and to involve an inventive step under PCT Article 33(2) and (3).

-Continued on Supplemental Box-

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US2012/033338

Box No. VII Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

Claims 41,66-68,72,73,75-78,80-83,85-90,93-98 do not comply with PCT Rule 6.4(a) because multiple dependent claims should not serve as a basis for any other multiple dependent claims.

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
4196

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US2012/033338

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

1. Claims 69-71,74,79,84,91,92 are unclear because they are dependent claims referring to dependent claims which do not comply with PCT Rule 6.4(a).
2. The terms "about" used in claims 1,11-22,44-46,54,55,62-67,70,71,73,93,94,99, "and/or" used in claims 10-13,15-22,62-64 are vague and unclear and leave the reader in doubt as to the meaning of the technical features to which they refer, thereby rendering the definition of the subject matter of the said claims unclear (PCT Article 6).

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US2012/033338

Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**
Continuation of :

Box V

2. Industrial Applicability

Claims 1-9,13,17,21,99 are industrially applicable under PCT Article 33(4).

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
4198

PATENT COOPERATION TREATY

ADVANCE E-MAIL

PCT

From the INTERNATIONAL BUREAU

NOTIFICATION CONCERNING TRANSMITTAL OF COPY OF INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY (CHAPTER I OF THE PATENT COOPERATION TREATY)
(PCT Rule 44bis.1(c))

To:

GABERT, Andrea J.
Wolf, Greenfield & Sacks, P.C.
600 Atlantic Avenue
Boston, MA 02210-2206
ETATS-UNIS D'AMERIQUE

Date of mailing *(day/month/year)*
24 October 2013 (24.10.2013)

Applicant's or agent's file reference
Q0052.70017

IMPORTANT NOTICE

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US2012/033338	12 April 2012 (12.04.2012)	12 April 2011 (12.04.2011)

Applicant
QUANTERIX CORPORATION et al

The International Bureau transmits herewith a copy of the international preliminary report on patentability (Chapter I of the Patent Cooperation Treaty)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. +41 22 338 82 70

Authorized officer
Athina Nickitas-Etienne

e-mail: pt04.pct@wipo.int

Form PCT/IB/326 (January 2004)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY
(Chapter I of the Patent Cooperation Treaty)

(PCT Rule 44*bis*)

Applicant's or agent's file reference Q0052.70017	**FOR FURTHER ACTION**	See item 4 below
International application No. PCT/US2012/033338	International filing date *(day/month/year)* 12 April 2012 (12.04.2012)	Priority date *(day/month/year)* 12 April 2011 (12.04.2011)
International Patent Classification (8th edition unless older edition indicated) See relevant information in Form PCT/ISA/237		
Applicant QUANTERIX CORPORATION		

1. This international preliminary report on patentability (Chapter I) is issued by the International Bureau on behalf of the International Searching Authority under Rule 44 *bis*.1(a).

2. This REPORT consists of a total of 9 sheets, including this cover sheet.

   In the attached sheets, any reference to the written opinion of the International Searching Authority should be read as a reference to the international preliminary report on patentability (Chapter I) instead.

3. This report contains indications relating to the following items:

☒	Box No. I	Basis of the report
☒	Box No. II	Priority
☒	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☒	Box No. VII	Certain defects in the international application
☒	Box No. VIII	Certain observations on the international application

4. The International Bureau will communicate this report to designated Offices in accordance with Rules 44*bis*.3(c) and 93*bis*.1 but not, except where the applicant makes an express request under Article 23(2), before the expiration of 30 months from the priority date (Rule 44*bis* .2).

	Date of issuance of this report 15 October 2013 (15.10.2013)
The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. +41 22 338 82 70	Authorized officer Athina Nickitas-Etienne e-mail: pt04.pct@wipo.int

Form PCT/IB/373 (January 2004)

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:
GABERT ANDREA J.

WOLF, GREENFIELD & SACKS, P.C. 600 ATLANTIC AVENUE BOSTON MA 02210-2206 USA

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43bis.1)

Date of mailing *(day/month/year)*	**29 NOVEMBER 2012 (29.11.2012)**
Applicant's or agent's file reference Q0052.70017	**FOR FURTHER ACTION** See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US2012/033338	**12 APRIL 2012 (12.04.2012)**	12 APRIL 2011 (12.04.2011)

International Patent Classification (IPC) or both national classification and IPC

G01N 33/68(2006.01)i, G01N 33/53(2006.01)i, G01N 33/58(2006.01)i

Applicant

QUANTERIX CORPORATION et al

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☒ Box No. II Priority
- ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☒ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.
For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/KR	Date of completion of this opinion	Authorized officer
Korean Intellectual Property Office 189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea Facsimile No. 82-42-472-7140	29 NOVEMBER 2012 (29.11.2012)	Kang Yeon Mu Telephone No.82-42-481-5516

Form PCT/ISA/237 (cover sheet) (July 2011)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US2012/033338

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of :

   ☒ the international application in the language in which it was filed

   ☐ a translation of the international application into _____ , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of:

   a. a sequence listing filed or furnished
      ☐ on paper
      ☐ in electronic form

   b. time of filing or furnishing
      ☐ contained in the international application as filed.
      ☐ filed together with the international application in electronic form.
      ☐ furnished subsequently to this Authority for the purposes of search.

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additioanl copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
4202

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US2012/033338

Box No. II Priority

1. ☒ The validity of the priority claim has not been considered because the International Searching Authority does not have in its possession a copy of the earlier application whose priority has been claimed or, where required, a translation of that earlier application. This opinion has nevertheless been established on the assumption that the relevant date(Rules 43bis.1 and 64.1) is the claimed priority date.

2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid (Rules 43bis.1 and 64.1). Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if neccessary:

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
4203

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US2012/033338

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application

☒ claims Nos. 10-12,14-16,18-20,22-98

because:

☒ the said international application, or the said claims Nos. 10-12,14-16,18-20,22-98 relate to the following subject matter which does not require an international search *(specify)*:

Claims 10-12,14-16,18-20,22-98 pertain to diagnostic methods for determining a patient's prognosis for recovery from a brain injury following a hypoxic event, and thus relate to a subject matter which this International Searching Authority is not required, under Rules 43 bis.1(b), Rule 67.1 (iv)), to search.

☒ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. 69-71,74,79,84,91,92 are so unclear that no meaningful opinion could be formed *(specify)*:

Claims 69-71,74,79,84,91,92 are unclear because they are dependent claims referring to dependent claims which do not comply with PCT Rule 6.4(a).

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed *(specify)*:

☒ no international search report has been established for said claims Nos. 10-12,14-16,18-20,22-98

☐ a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:

☐ furnish a sequence listing on paper complying with the standard provided for in Annex C of the Administrative Istructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.

☐ furnish a sequence listing in electronic form complying with the standard provided for in Annex C of the Administrative Istructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.

☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13ter.1(a) or (b).

☐ See Supplemental Box for further details.

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US2012/033338

Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-9,13,17,21,99	YES
	Claims	NONE	NO
Inventive step (IS)	Claims	1-9,13,17,21,99	YES
	Claims	NONE	NO
Industrial applicability (IA)	Claims	1-9,13,17,21,99	YES
	Claims	NONE	NO

2. Citations and explanations :

Reference is made to the following documents:

D1: US 2006-0205024 A1 (ROGERS, J. et al.) 14 September 2006
D2: US 2008-0199879 A1 (TAKAYAMA SHIGEO et al.) 21 August 2008
D3: WO 2009-156747 A3 (THE NOTTINGHAM TRENT UNIVERSITY et al.) 30 December 2009
D4: US 2008-0234311 A1 (LI JINHE et al.) 25 September 2008

1. Novelty and Inventive Step

1.1. Independent Claims 1,13,17,21

1.1.1. Concerning claim 1

None of the documents cited in the ISR show that a method for determining a measure of the concentration of beta-amyloid peptide in a patient sample containing or not containing beta-amyloid peptide, wherein the limit of detection of beta-amyloid peptide of the assay is less than about 0.2 pg/mL. Accordingly, the feature of claim 1 is not anticipated by any of the documents, nor is it obvious by the documents, taken alone or in combination. Therefore, claim 1 is novel and involves an inventive step under PCT Article 33(2) and (3).

1.1.2. Concerning claims 13,17,21

None of the documents cited in the ISR show the methods for performing an assay and providing data for determining a treatment protocol for, or a prognosis of a patient's recovery from, a brain injury resulting from a hypoxic event, wherein the concentration of the biomarker and quantification of the assay are specifically limited. Accordingly, claims 13,17,21 are not anticipated by any of the documents, nor are they obvious by the documents, taken alone or in combination. Therefore, claims 13,17,21 are novel and involve an inventive step under PCT Article 33(2) and (3).

1.2. Dependent Claims 2-9,99

Claims 2-9,99 are dependent on claim 1. Consequently, Claims 2-9,99 are also considered to be novel and to involve an inventive step under PCT Article 33(2) and (3).

-Continued on Supplemental Box-

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
4205

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US2012/033338

Box No. VII Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

Claims 41,66-68,72,73,75-78,80-83,85-90,93-98 do not comply with PCT Rule 6.4(a) because multiple dependent claims should not serve as a basis for any other multiple dependent claims.

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
4206

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US2012/033338

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

1. Claims 69-71,74,79,84,91,92 are unclear because they are dependent claims referring to dependent claims which do not comply with PCT Rule 6.4(a).
2. The terms "about" used in claims 1,11-22,44-46,54,55,62-67,70,71,73,93,94,99, "and/or" used in claims 10-13,15-22,62-64 are vague and unclear and leave the reader in doubt as to the meaning of the technical features to which they refer, thereby rendering the definition of the subject matter of the said claims unclear (PCT Article 6).

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
4207

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US2012/033338

Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**
Continuation of :

Box V

2. Industrial Applicability

Claims 1-9,13,17,21,99 are industrially applicable under PCT Article 33(4).

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
4208

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
Q0052.70018		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US2012/033343	**12 APRIL 2012 (12.04.2012)**	12 APRIL 2011 (12.04.2011)

Applicant

QUANTERIX CORPORATION et al

This International search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of **4** sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language**, the international search was carried out on the basis of :
      ☒ the international application in the language in which it was filed
      ☐ a translation of the international application into _____ , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
   b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).
   c. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.
2. ☒ **Certain claims were found unsearchable** (See Box No. II)
3. ☐ **Unity of invention is lacking** (See Box No. III)
4. With regard to the **title,**
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established by this Authority to read as follows:
5. With regard to the **abstract,**
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.
6. With regard to the drawings,
   a. the figure of the **drawings** to be published with the abstract is Figure No. **1A**
      ☒ as suggested by the applicant.
      ☐ as selected by this Authority, because the applicant failed to suggest a figure.
      ☐ as selected by this Authority, because this figure better characterizes the invention.
   b. ☐ none of the figure is to be published with the abstract.

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
4209

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/033343

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

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

1. ☒ Claims Nos.: 10-58,60-91
because they relate to subject matter not required to be searched by this Authority, namely:

Claims 10-58,60-91 pertain to methods for treatment of the human body by therapy, as well as diagnostic methods, and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

2. ☒ Claims Nos.: 34-36,43-50,52,53,69,70,72,77,84,85
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

These claims are unclear because they are dependent claims referring to a dependent claim which do not comply with PCT Rule 6.4(a) because multiple dependent claims should not serve as a basis for any other multiple dependent claims.

3. ☒ Claims Nos.: 21-33,37-42,51,54,61-68,71,73-76,78-83,86-91
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/033343

A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/68(2006.01)i, G01N 33/53(2006.01)i, G01N 33/52(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N 33/68; A61K 36/16; G06F 19/00; G01N 33/53; C12Q 1/68; A61P 25/28

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: tau protein, assay, brain injury, treatment protocol, recovery, concentration

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Shiiya, N. et al. Ann Thorac Surg. 2004, Vol.77, pp.2034-2038 See the whole document.	1-9,59
A	Bulut, m. et al. Advances in Therapy. Jan.2006, Vol.23, No.1 See the whole document.	1-9,59
A	WO 2011-032155 A2 (BANYAN BIOMARKERS, INC. et al.) 17 March 2011 See the whole document.	1-9,59
A	WO 2009-156747 A3 (THE NOTTINGHAM TRENT UNIVERSITY et al.) 30 December 2009 See the whole document.	1-9,59
A	US 2009-0022825 A1 (KERKMAN DANIEL et al.) 22 January 2009 See the whole document.	1-9,59

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

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 OCTOBER 2012 (25.10.2012)	**29 OCTOBER 2012 (29.10.2012)**
Name and mailing address of the ISA/KR Korean Intellectual Property Office 189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea Facsimile No. 82-42-472-7140	Authorized officer Kang Yeon Mu Telephone No. 82-42-481-5516

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

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2012/033343

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011-032155 A2	17.03.2011	EP 2478360 A2	25.07.2012
		WO 2011-032155 A3	17.03.2011
		WO 2011-032155 A3	06.10.2011
		WO 2011-160096 A2	22.12.2011
WO 2009-156747 A3	30.12.2009	GB 0811858 D0	30.07.2008
		WO 2009-156747 A2	30.12.2009
US 2009-0022825 A1	22.01.2009	AU 2003-291358 A1	03.06.2004
		AU 2003-291358 A8	03.06.2004
		CA 2505355 A1	27.05.2004
		EP 1626648 A2	22.02.2006
		EP 1626648 A4	28.04.2010
		JP 2006-517650 A	27.07.2006
		JP 2006-517650 T	27.07.2006
		MX PA05004828A	27.01.2006
		US 2004-166536 A1	26.08.2004
		WO 2004-043226 A2	27.05.2004
		WO 2004-043226 A3	05.07.2007

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PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:
GABERT ANDREA J.

WOLF, GREENFIELD & SACKS, P.C. 600 ATLANTIC AVENUE BOSTON MA 02210-2206 USA

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43bis.1)

Date of mailing *(day/month/year)*	**29 OCTOBER 2012 (29.10.2012)**
Applicant's or agent's file reference Q0052.70018	**FOR FURTHER ACTION** See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US2012/033343	**12 APRIL 2012 (12.04.2012)**	12 APRIL 2011 (12.04.2011)

International Patent Classification (IPC) or both national classification and IPC

G01N 33/68(2006.01)i, G01N 33/53(2006.01)i, G01N 33/52(2006.01)i

Applicant

QUANTERIX CORPORATION et al

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☒ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.
For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/KR	Date of completion of this opinion	Authorized officer
Korean Intellectual Property Office 189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea Facsimile No. 82-42-472-7140	25 OCTOBER 2012 (25.10.2012)	Kang Yeon Mu Telephone No.82-42-481-5516

Form PCT/ISA/237 (cover sheet) (July 2011)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US2012/033343

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of :

   ☒ the international application in the language in which it was filed

   ☐ a translation of the international application into _____ , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of:

   a. a sequence listing filed or furnished
      ☐ on paper
      ☐ in electronic form

   b. time of filing or furnishing
      ☐ contained in the international application as filed.
      ☐ filed together with the international application in electronic form.
      ☐ furnished subsequently to this Authority for the purposes of search.

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additioanl copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US2012/033343

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application

☒ claims Nos. 10-58,60-91

because:

☒ the said international application, or the said claims Nos. 10-58,60-91 relate to the following subject matter which does not require an international search *(specify)*:

Claims 10-58,60-91 pertain to methods for treatment of the human body by therapy, and thus relate to a subject matter which this International Searching Authority is not required, under Rules 43 bis.1(b), Rule 67.1 (iv)), to search.

☒ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. 34-36,43-50,52,53,69,70,72 are so unclear that no meaningful opinion could be formed *(specify)*:

Claims 34-36,43-50,52,53,69,70,72,77,84,85 are unclear because they are dependent claims referring to dependent claims which do not comply with PCT Rule 6.4(a) because multiple dependent claims should not serve as a basis for any other multiple dependent claims.

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed *(specify)*:

☒ no international search report has been established for said claims Nos. 10-58,60-91

☐ a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:

☐ furnish a sequence listing on paper complying with the standard provided for in Annex C of the Administrative Istructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.

☐ furnish a sequence listing in electronic form complying with the standard provided for in Annex C of the Administrative Istructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.

☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13ter.1(a) or (b).

☐ See Supplemental Box for further details.

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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US2012/033343

Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-9,59	YES
	Claims	NONE	NO
Inventive step (IS)	Claims	1-9,59	YES
	Claims	NONE	NO
Industrial applicability (IA)	Claims	1-9,59	YES
	Claims	NONE	NO

2. Citations and explanations :

Reference is made to the following documents:

D1: Shiiya, N. et al. Ann Thorac Surg. 2004, Vol.77, pp.2034-2038
D2: Bulut, m. et al. Advances in Therapy. Jan.2006, Vol.23, No.1
D3: WO 2011-032155 A2 (BANYAN BIOMARKERS, INC. et al.) 17 March 2011
D4: WO 2009-156747 A3 (THE NOTTINGHAM TRENT UNIVERSITY et al.) 30 December 2009
D5: US 2009-0022825 A1 (KERKMAN DANIEL et al.) 22 January 2009

1. Novelty and Inventive Step

1.1. Independent Claims 1,59

1.1.1. Concerning claim 1

The subject matter of claim 1 differs from these prior art documents in that the method of claim 1 further defines the limit of detection of tau protein as less than abou 0.2pg/mL. And it is not obvious to a person skilled in the art by the documents, taken alone or in combination. Therefore, claim 1 meets the requirements of PCT Article 33(2) and (3) with respect to novelty and inventive step.

1.1.2. Concerning claim 59

Claim 59 relates to the methods of performing an assay and providing data for determining a method of treatment for or a prognosis of a patients recovery from a brain injury using the curve of a graph of the tau protein concentration. None of the documents cited in the ISR show the feature of claim 1. Accordingly, claim 59 is not anticipated by any of the documents, nor is it obvious by the documents, taken alone or in combination. Therefore, claim 59 is novel and involves an inventive step under PCT Article 33(2) and (3)

1.2. Dependent Claims 2-9

Claims 2-9 are dependent on claim 1. Consequently, claims 2-9 are also considered to be novel and to involve an inventive step under PCT Article 33(2) and (3).

2. Industrial Applicability

Claims 1-9,59 are industrially applicable under PCT Article 33(4).

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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US2012/033343

Box No. VII Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

1. Claims 21-33,37-42,51,54,61-68,71,73-76,78-83,86-91 do not comply with PCT Rule 6.4(a) because multiple dependent claims should not serve as a basis for any other multiple dependent claims.
2. Claim 35 is not numbered consecutively in Arabic numbers, since claim 35 is worded as "fpg35".

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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US2012/033343

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claims 34-36,43-50,52,53,69,70,72,77,84,85 are unclear because they are dependent claims referring to dependent claims which do not comply with PCT Rule 6.4(a) .

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
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PATENT COOPERATION TREATY

ADVANCE E-MAIL

PCT

From the INTERNATIONAL BUREAU

NOTIFICATION CONCERNING TRANSMITTAL OF COPY OF INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY (CHAPTER I OF THE PATENT COOPERATION TREATY)
(PCT Rule 44bis.1(c))

To:

GABERT, Andrea, J.
Wolf, Greenfield & Sacks, P.C.
600 Atlantic Avenue
Boston, MA 02210-2206
ETATS-UNIS D'AMERIQUE

Date of mailing *(day/month/year)* 24 October 2013 (24.10.2013)	
Applicant's or agent's file reference Q0052.70018	**IMPORTANT NOTICE**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US2012/033343	12 April 2012 (12.04.2012)	12 April 2011 (12.04.2011)

Applicant
QUANTERIX CORPORATION et al

The International Bureau transmits herewith a copy of the international preliminary report on patentability (Chapter I of the Patent Cooperation Treaty)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. +41 22 338 82 70

Authorized officer
Athina Nickitas-Etienne

e-mail: pt04.pct@wipo.int

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY
(Chapter I of the Patent Cooperation Treaty)

(PCT Rule 44*bis*)

Applicant's or agent's file reference Q0052.70018	**FOR FURTHER ACTION**	See item 4 below
International application No. PCT/US2012/033343	International filing date *(day/month/year)* 12 April 2012 (12.04.2012)	Priority date *(day/month/year)* 12 April 2011 (12.04.2011)
International Patent Classification (8th edition unless older edition indicated) See relevant information in Form PCT/ISA/237		
Applicant QUANTERIX CORPORATION		

1. This international preliminary report on patentability (Chapter I) is issued by the International Bureau on behalf of the International Searching Authority under Rule 44 *bis*.1(a).

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   In the attached sheets, any reference to the written opinion of the International Searching Authority should be read as a reference to the international preliminary report on patentability (Chapter I) instead.

3. This report contains indications relating to the following items:

☒	Box No. I	Basis of the report
☐	Box No. II	Priority
☒	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☒	Box No. VII	Certain defects in the international application
☒	Box No. VIII	Certain observations on the international application

4. The International Bureau will communicate this report to designated Offices in accordance with Rules 44*bis*.3(c) and 93*bis*.1 but not, except where the applicant makes an express request under Article 23(2), before the expiration of 30 months from the priority date (Rule 44*bis* .2).

	Date of issuance of this report 15 October 2013 (15.10.2013)
The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. +41 22 338 82 70	Authorized officer Athina Nickitas-Etienne e-mail: pt04.pct@wipo.int

Form PCT/IB/373 (January 2004)

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:
GABERT ANDREA J.

WOLF, GREENFIELD & SACKS, P.C. 600 ATLANTIC AVENUE BOSTON MA 02210-2206 USA

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43bis.1)

Date of mailing *(day/month/year)*	**29 OCTOBER 2012 (29.10.2012)**
Applicant's or agent's file reference Q0052.70018	**FOR FURTHER ACTION** See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US2012/033343	**12 APRIL 2012 (12.04.2012)**	12 APRIL 2011 (12.04.2011)

International Patent Classification (IPC) or both national classification and IPC

G01N 33/68(2006.01)i, G01N 33/53(2006.01)i, G01N 33/52(2006.01)i

Applicant

QUANTERIX CORPORATION et al

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☒ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.
For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/KR	Date of completion of this opinion	Authorized officer
Korean Intellectual Property Office 189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea Facsimile No. 82-42-472-7140	25 OCTOBER 2012 (25.10.2012)	Kang Yeon Mu Telephone No.82-42-481-5516

Form PCT/ISA/237 (cover sheet) (July 2011)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US2012/033343

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of :

   ☒ the international application in the language in which it was filed

   ☐ a translation of the international application into _____ , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of:

   a. a sequence listing filed or furnished
      ☐ on paper
      ☐ in electronic form

   b. time of filing or furnishing
      ☐ contained in the international application as filed.
      ☐ filed together with the international application in electronic form.
      ☐ furnished subsequently to this Authority for the purposes of search.

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additioanl copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US2012/033343

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application

☒ claims Nos. 10-58,60-91

because:

☒ the said international application, or the said claims Nos. 10-58,60-91 relate to the following subject matter which does not require an international search *(specify)*:

Claims 10-58,60-91 pertain to methods for treatment of the human body by therapy, and thus relate to a subject matter which this International Searching Authority is not required, under Rules 43 bis.1(b), Rule 67.1 (iv)), to search.

☒ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. 34-36,43-50,52,53,69,70,72 are so unclear that no meaningful opinion could be formed *(specify)*:

Claims 34-36,43-50,52,53,69,70,72,77,84,85 are unclear because they are dependent claims referring to dependent claims which do not comply with PCT Rule 6.4(a) because multiple dependent claims should not serve as a basis for any other multiple dependent claims.

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed *(specify)*:

☒ no international search report has been established for said claims Nos. 10-58,60-91

☐ a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:

☐ furnish a sequence listing on paper complying with the standard provided for in Annex C of the Administrative Istructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.

☐ furnish a sequence listing in electronic form complying with the standard provided for in Annex C of the Administrative Istructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.

☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13ter.1(a) or (b).

☐ See Supplemental Box for further details.

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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US2012/033343

Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-9,59	YES
	Claims	NONE	NO
Inventive step (IS)	Claims	1-9,59	YES
	Claims	NONE	NO
Industrial applicability (IA)	Claims	1-9,59	YES
	Claims	NONE	NO

2. Citations and explanations :

Reference is made to the following documents:

D1: Shiiya, N. et al. Ann Thorac Surg. 2004, Vol.77, pp.2034-2038
D2: Bulut, m. et al. Advances in Therapy. Jan.2006, Vol.23, No.1
D3: WO 2011-032155 A2 (BANYAN BIOMARKERS, INC. et al.) 17 March 2011
D4: WO 2009-156747 A3 (THE NOTTINGHAM TRENT UNIVERSITY et al.) 30 December 2009
D5: US 2009-0022825 A1 (KERKMAN DANIEL et al.) 22 January 2009

1. Novelty and Inventive Step

1.1. Independent Claims 1,59

1.1.1. Concerning claim 1

The subject matter of claim 1 differs from these prior art documents in that the method of claim 1 further defines the limit of detection of tau protein as less than abou 0.2pg/mL. And it is not obvious to a person skilled in the art by the documents, taken alone or in combination. Therefore, claim 1 meets the requirements of PCT Article 33(2) and (3) with respect to novelty and inventive step.

1.1.2. Concerning claim 59

Claim 59 relates to the methods of performing an assay and providing data for determining a method of treatment for or a prognosis of a patients recovery from a brain injury using the curve of a graph of the tau protein concentration. None of the documents cited in the ISR show the feature of claim 1. Accordingly, claim 59 is not anticipated by any of the documents, nor is it obvious by the documents, taken alone or in combination. Therefore, claim 59 is novel and involves an inventive step under PCT Article 33(2) and (3)

1.2. Dependent Claims 2-9

Claims 2-9 are dependent on claim 1. Consequently, claims 2-9 are also considered to be novel and to involve an inventive step under PCT Article 33(2) and (3).

2. Industrial Applicability

Claims 1-9,59 are industrially applicable under PCT Article 33(4).

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US2012/033343

Box No. VII Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

1. Claims 21-33,37-42,51,54,61-68,71,73-76,78-83,86-91 do not comply with PCT Rule 6.4(a) because multiple dependent claims should not serve as a basis for any other multiple dependent claims.
2. Claim 35 is not numbered consecutively in Arabic numbers, since claim 35 is worded as "fpg35".

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US2012/033343

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claims 34-36,43-50,52,53,69,70,72,77,84,85 are unclear because they are dependent claims referring to dependent claims which do not comply with PCT Rule 6.4(a) .

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
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Electronic Acknowledgement Receipt

EFS ID:	22145976
Application Number:	14111326
International Application Number:	
Confirmation Number:	3274
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Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Foreign Reference	WO2011109372_QC.pdf	9258896 1cc08da727e904fd30289c26d216813fd29bdaeb	no	77

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Warnings:					
Information:					
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59	Non Patent Literature	Chang_et_al_Abstract_Poster_AACC_Mtg_2012_2pgs_QC.PDF	612220 568803f2d30c6cf78f58b3b6a9be5846434279ec	no	2
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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

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<u>New International Application Filed with the USPTO as a Receiving Office</u>
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Ohne internationalen Recherchenbericht und erneut zu veröffentlichen nach Erhalt des Berichts.

(54) Title: METHOD AND MEASURING DEVICE FOR DETERMINING A PLURALITY OF ANALYTES IN A SAMPLE

(54) Bezeichnung: VERFAHREN UND MESSEINRICHTUNG ZUR BESTIMMUNG EINER VIELZAHL VON ANALYTEN IN EINER PROBE

(57) Abstract

The invention relates to a method for determining a plurality of analytes in a sample which comprises the following steps: (a) bringing the sample into contact with; (i) a plurality of species of microparticles, whereby each species is suited for detecting certain analytes and a certain coding which can be optically differentiated from other species, and bringing in contact with; (ii) a plurality of soluble, analyte–specific analytical reagents which support a signal–emitting group or which can bind with a signal–emitting group; (b) subsequently depositing the microparticles on a support and at least; (c) determining the analytes by optically detecting the coding and the quantity, and determining the presence and/or the absence of the signal–emitting groups on at least one species of individual microparticles on the support. The invention also relates to a measuring device for carrying out the inventive method.

(57) Zusammenfassung

Es wird ein Verfahren zur Bestimmung einer Vielzahl von Analyten in einer Probe angegeben, umfassend die Schritte: (a) Inkontaktbringen der Probe mit (i) einer Vielzahl von Spezies von Mikropartikeln, wobei jede Spezies zum Nachweis von bestimmten Analyten geeignet ist und eine bestimmte, von anderen Spezies optisch unterscheidbare Codierung aufweist, und (ii) einer Vielzahl von löslichen analytspezifischen Nachweisreagenzien, die eine signalgebende Gruppe tragen oder mit einer signalgebenden Gruppe bindefähig sind; (b) anschließendes Aufbringen der Mikropartikel auf einen Träger und mindestens (c) Bestimmen der Analyten durch optisches Erfassen der Codierung und der Menge, des Vorhandenseins oder/und der Abwesenheit der signalgebenden Gruppen auf mindestens einer Spezies von individuellen Mikropartikeln auf dem Träger. Gegenstand der Erfindung ist ferner eine Meßeinrichtung zur Durchführung des Verfahrens.

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
4236

Method and measuring device for determining a plurality of analytes in a sample

Description

The invention relates to a method and a measuring device for determining a plurality of analytes in a sample. The invention further relates to a kit and an analyte support for use when carrying out the method.

1 Area of applications

1.1 Background

The precise detection of biologically relevant molecules in defined investigation material is of outstanding importance for basic research in the biosciences and for medical diagnostics, and some other disciplines. Every organism is, in the final analysis, composed of various biological macromolecules, frequently polymers such as nucleic acids and proteins, and substances derived therefrom, and the products of specific enzymatic synthesis outputs. The genetic information is moreover present in the form of an enormous diversity of different nucleic acid sequences, the DNA. Implementation of this information leads via the production of transcripts of the DNA in RNA usually to the synthesis of proteins which in turn are frequently involved in biochemical reactions. There is secondary building up and breaking down of numerous organic, in some cases low molecular weight, compounds thereby.

The result is thus an enormous diversity of substances in the body. In addition to these there are molecules supplied to the body from outside, such as, for example, pharmaceuticals or drugs of abuse.

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Knowledge about the occurrence, quality and conversion of at least the more important of these molecules is extremely useful for describing the healthy as well as the diseased state of an organism. As this knowledge increases there is an exponential rise in the understanding of the associations and benefits. In medicine it has been possible to a greatly increasing extent to develop, through in vitro diagnosis (IVD), instruments for determining important parameters of patients and to provide the treating physician therewith. Diagnosis of many disorders would be impossible at a sufficiently early time without these instruments (for example infectious diseases such as HIV, HBV and others). The same applies to the monitoring of specific parameters associated with a disorder (for example blood glucose levels in diabetes). It has been possible in biomedicine, with close intermeshing of basic research and clinical research, to elucidate the molecular causes and (pathological) associations of some pathological states as far as the level of the genetic information. This development is, however, still in its early stages, and greatly intensified efforts are needed in particular for conversion into therapeutic strategies.

1.2 Need

IVD methods have become an indispensable aid in clinical diagnosis. There are moreover increasing attempts to add to the number of parameters which can be determined. Novel technologies lead to so-called multiplex analysis, where it is attempted to integrate the analysis of an increased number or plurality of analytes in one method. It is attempted at the same time to reduce the size of or miniaturize the apparatuses. Most IVD methods

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measure only a few parameters, i.e. distinctly less than 100, in one run. Simultaneous detection of nucleic acids in order, for example, to identify a genetic predisposition, and other factors, for example auto-antibodies in the framework of an autoimmune disease such as rheumatoid arthritis, does not at present occur. Nucleic acid diagnosis able to detect, for example, HIV infections has to date been based on molecular biological methods such as the polymerase chain reaction (PCR) or sequencing, which either provide only limited information or are very complicated and thus costly. Thus, simply for reasons of cost, they are in some cases unsuitable for acquiring large quantities of data with broad applications. A novel approach which has received much attention is nucleic acid analysis by hybridization onto immobilized oligonucleotides (oligos) on a solid substrate, for example a silicon matrix. With such so-called DNA chips, a signal is generated through the binding of complementary strands to the immobilized oligos, and an extremely dense and miniaturized arrangement of the oligos on the chip is achieved for example by photolithographic synthesis, and thus the number of measurement points per experiment is very greatly increased. The production of such DNA chips is, however, very complicated and costly; in addition, the length of the oligos is limited. Every additional building block requires extensive synthesis and washing steps. The use of such biochips is currently confined to nucleic acid analysis. Analysis of the physiological associations only on the basis of the genes, i.e. nucleic acids, is not possible. So-called functional genomics attempts on the basis of analysis of genes, for example with biochips, to decipher the interplay of many genes (functional genomics). Analysis of the gene

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products (usually proteins) and their interaction as a whole is covered by the term proteomics. However, in this connection, in contrast to nucleic acid analysis, there are as yet hardly any approaches to highly integrated formats like the DNA chips, and the studies are carried out with less integrated methods which usually function serially. Nevertheless, knowledge which is very valuable for the diagnosis and therapy of disorders is already being produced on this basis. Novel requirements are necessary for methods and apparatus for these future studies. In basic biological and biomedical research at present manual operational steps generally prevail to a large extent, and particularly in the analysis of gene products and their importance in the context of cell and organism. These are on the one hand associated with variations for example in the carrying out of experiments and assays, but also corresponding staff costs for each item of information produced and usually a large expenditure of time.

The resulting picture overall is as follows:

In order to fulfill the promises of modern biomedicine for patient care, a highly parallel multiplex analysis of 10^2 to 10^6 parameters must be made possible, ideally the joint detection of very diverse analytes such as proteins, hormones or nucleic acids, without causing disproportionately high costs thereby. Such a technology is described in this patent application.

1.3 Fields of application of the invention

In vitro diagnostics and clinical diagnostics

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Merging a plurality of parameters for acute diagnosis and for routine investigation (preventive medicine); valuable, statistically analyzable knowledge will be derived therefrom for (extremely) early diagnostics of various pathological states; the quantity of data generated has become analyzable only through the developments in information technology.

DNA and RNA diagnostics

- infectious diseases (HIV, HBV etc.),
- early diagnosis of tumors,
- tumor classification (type and status),
- genetic diseases,
- oncology

Basic biological research, especially genomics and proteomics

Acquisition of very many measurement points in the system investigated, for example all expressed genes or all proteins and growth factors released into a culture medium; an enormous gain in knowledge in basic biological research (developmental biology, stem cell culture, tissue engineering, transplantation medicine, regeneration) will be derived from this and will also lead to important breakthroughs in biomedicine and to corresponding applications.

Forensics

Both genetic and immunological parameters are used for trace analysis.

Food analysis

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This invention ought to make rapid, cost-effective analysis of foodstuffs possible, for example for the presence of particular genes or proteins.

Screening of medicinal products

The manufacture of, for example, blood products is still associated with great complexity for the safety measures concerning purity. Rapid and cost-effective screening of such samples will be made possible by this invention.

2. Prior art

Biochips are miniaturized hybrid functional elements with biological and engineering components, for example biomaterials which are immobilized on the surface and which can act as specific interaction partners (for example antibodies, DNA oligonucleotides) and a silicon matrix. These functional elements are usually arranged in rows and columns, in which case they are referred to as biochip arrays. Since thousands of biochemical functional elements can be arranged on the biochip, they must be produced by microengineering methods.

Biochips disclosed to date can be classified according to the following criteria:

Detection principle

- Chromatographic methods
- Interaction of analytes with solid phase, usually immobilized interaction partners (for example hybridization of nucleic acids onto DNA oligonucleotides).
- Detection methods (optical, electrical).

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Marker-based (for example absorption, fluorescence or luminescence) or marker-free detection methods (light generation to detect the reaction).

- Assignment of the analyte to its support [solid phase]
(ARRAY with more than one immobilized interaction partner per support or SINGLE with only one immobilized interaction partner per support).

Production methods (for example synthesize oligonucleotides directly on the biochip with photoactivation, spot previously synthesized oligonucleotides, coat beads or tubes).

Types of support (glass chips, plastic chips, microtiter plates, tubes or beads).

- Presentation for detection (serial, parallel).

Optical detection (serially in a scanner or in parallel with a CCD camera).

In the area of biochips, the provision of polymer probes in the form of oligonucleotides on the surface of a planar support by means of photolithographic techniques is disclosed for example in US patent 5,744,305. It is possible in this case to achieve a particularly high degree of miniaturization with thousands of probes per square centimeter, the probes being arranged in an array.

Many methods are described in which different receptors or probes present in a fluid are applied in the form of small to very small drops to solid supports and, where appropriate, are immobilized there.

Technical solutions for this type of

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support production are in some cases derived from inkjet printing techniques. Methods of this type are limited through the manipulation of the finished probes in the fluid, which are stored in individual storage vessels, and in the number of different receptors on one support. Applications known to date reach a maximum of 10 000 different probes per support with very elaborate embodiments.

In methods using microparticles, the use of such particles has been described in connection with a cell counter (fluorescence activated cell sorting FACS). In addition, very small particles can be investigated in a method which is based on fluorescent labeling of the microparticles, by use of etched glass fiber microwells with back lighting, on which an analyte determination method is based.

US patent 4,767,205 describes a method for the coded identification of a selected item, with uniform microparticles with a size of 2 to 20 µm being coded in accordance with a particular combination of size, shape and color, and being introduced into the item to be identified. The patent contains no reference at all to a method for determining analytes.

German published specification 36 288 describes an immunological test method in which there is use of a reaction vessel along whose surface a plurality of analyte-specific antibodies are immobilized. Immobilization of the analyte present in the sample is followed by addition of labeling particles which likewise carry analyte-specific antibodies, in order to determine the immobilized analytes in this way. There is no reference in

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this document to the use of microparticles as particulate solid phase.

WO 98/00292 describes a method for detecting an antigen in a sample, using a coloured solid phase, for example a microtiter plate. There are no references at all to particulate solid phases.

US patent 4,494,830 describes a method for the immunological detection of analytes in biological fluids using test rods of different colours. There is no reference at all to the use of microparticles.

3. Aspects of the invention and advantages achieved thereby

The invention represents an important alternative to other parallel methods and multiplex formats as represented, for example, by biochips or coated tubes.

The invention relates according to claim 1 to a method for determining a plurality of analytes in a sample comprising the steps:

(a) contacting the sample with

(i) a plurality of species of microparticles, each species being suitable for detecting particular analytes and having a particular coding optically distinguishable from other species, and

(ii) a plurality of soluble analyte-specific detection reagents which carry a signal-emitting group or are capable of binding to a signal-emitting group,

(b) subsequently applying the microparticles to a support comprising a cavity for receiving the ensemble of microparticles between two plates whose flat sides are opposite to one another, where the distance between the plates is chosen so that in each case only one layer of microparticles lying side by side fits into the cavity and at least

(c) determining the analytes by optical detection of the coding and the amount, the presence or/and the absence of the signal-emitting groups or at least one species of individual microparticles on the support.

Preferred further developments of the method are indicated in claims 2-27.

The method of the invention can be used in particular for clinical parameter diagnostics, for example for nucleic acid diagnostics, for basic biological research, for forensics, for food analysis and for screening medicinal products.

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The invention further relates to a kit as claimed in claim 29, in particular for carrying out the method as claimed in any of claims 1-27. The kit comprises

(a) a plurality of species of microparticles in free form, each species being suitable for detecting particular analytes and having a particular coding optically distinguishable from other species,

(b) a plurality of soluble analyte-specific detection reagents and

(c) a support.

A further aspect is a measuring device as claimed in claim 30 for determining a plurality of analytes in a sample, in particular by the method of the invention. The measuring device comprises

- an electronic image acquisition sensor for essentially unmagnified image-wise detection of the optical behaviour of an ensemble of microparticles which have been applied to a support comprising a cavity for receiving the ensemble of microparticles between two plates whose flat sides are opposite to one another, where the distance between the plates is chosen so that in each case only one layer of microparticle lying side by side fits into the cavity and brought into contact with the sample,

- means for exciting signal emission by signal-emitting groups on microparticles in the ensemble of microparticles on the support,

- a data processing device which evaluates image data from the electronic image acquisition sensor for providing information about the presence of particular analytes in the sample on the basis of the detected optical behaviour of the ensemble of microparticles

- and wherein the support is aligned between the electronic image acquisition sensor and the means for exciting signal emission.

The electronic image acquisition sensor is preferably a colour-normal CCD image recorder. The image recorder can be brought into direct contact with the support, so that complicated optical image-conveying lenses, light guides and optical magnification elements can be dispensed with.

Preferred further developments of the measuring device of the invention indicated in claim 30 are indicated in claims 31-38.

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The invention further relates to a support for carrying out the method of the invention, where the support has a cavity, in particular an extended capillary slit for receiving the ensemble of microparticles between two plates whose flat sides are opposite to one another, of which at least one is transparent. The distance between the two plates is chosen so that in each case only one layer of microparticles lying side by side fits into the cavity.

The distance between the plates can, where appropriate, be adjustable as desired.

The invention further relates to a support for carrying out the method of the invention, where the support has plurality of capillary channels which in particular run parallel to one another, and is optically transparent at least in the region of the capillary channels. The last-mentioned support makes mobile presentation of the microparticles possible in a simple manner during the optical detection.

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It is possible with the support which is referred to as "fractal SMART chip - FSC" to carry out thousands of different assays (measurements) in one measuring operation. The principle of the fractal biochip is based on double coding of the solid phase composed of microparticles (beads) which acts as immobilization partner. Capture molecules (for example oligonucleotides) are immobilized on these coded beads (for example color-coded glass or polymer beads), resulting in "intelligent individual" SMART beads. The coding of the SMART beads means that the capture molecules on the surface of the SMART beads can be located and identified at any time. On the other hand, for example, the target analyte which is sought in the sample (for example a DNA analyte) can be coded (for example with fluorescent label). In the analysis system the target analyte molecules which fit in each case then become attached to the capture molecules on the SMART bead surface. This attachment preferably takes place in a three-dimensional vessel with appropriately favorable hydrodynamic mixing conditions.

For detection and evaluation of the respective molecule attachments, the SMART beads are put on a support which preferably forms a planar arrangement. The SMART beads need not in this case be introduced into a fixed grid or geometric matrix. Because of the planar alignment of the SMART beads and the optical double coding, detection of all the SMART beads is possible in parallel with the aid of CCD techniques, in particular both in the resting and in the moving state of the SMART beads.

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It is possible to dispense with optical imaging lens systems for the detection if parallel-directed light is employed and the size of the SMARTS is chosen so that they cover a sufficient number of pixel elements of the color CCD chips. This direct utilization (no optical system) of highly parallel CCD matrix chips with a large number (currently 8 million color pixels per 1 cm^2) of pixels (optical measurement points or sensors) makes it possible to detect a plurality of SMART beads on the basis of their specific color coding (spectral identity). Elaborate moving devices for the support (chip) or the detection unit, as are necessary in scanners, are entirely dispensed with. The predetermined dimensions of the CCD chips (more than 1 cm^2) make it possible to use a very large number of SMART beads (more than 100 000) with a moderate particle size (more than 10 µm), which also permits spectral identification by the absorption principle. This makes it possible to identify considerably more identities (colors) without error than would be possible with a fluorescent coloring (can also be used nevertheless). CCD tubes at present can recognize about 16 million colors. The maximum number of different spectral identities which can still be detected without error is determined by the necessary spectral gradation for the optical measurment and the possibility of reproducible coloring of the SMART beads in the production process.

The identification comprises both determination of the SMART bead class (color) and the exact location. This is made possible by the plurality of CCD pixels which are available for detection per SMART bead. This plurality of measurements forms a basis which permits an extensive mathematical multiplexing in the identification of

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the individual SMART beads in the two-dimensional array. The processing of the resulting quantities of data is possible without difficulty through the development in efficiency with, at the same time, fall in price of modern computer systems.

Detection of the detection reaction can provide both qualitative and quantitative information:

* Which capture molecules or polymer probes (evaluation via bead color or, where appropriate, characteristic shape contour) have found attachment partners (evaluation of the fluorescent labels on the localized beads).

* How many capture molecules of a class have found a hybridization partner.

The SMART beads - which are large relative to other bead methods - permit both during manufacture (production and biochemical coding) and in the analysis system, including detection unit, simple fluidic manipulation which can be optimized as far as the manipulation of a single microparticle. The same applies to the detection. In this case too identification of the individual SMART bead is possible in particular for applications in DNA analysis.

The invention thus covers the following requirements in clinical diagnostics:

* Plurality of parameters which can be determined simultaneously (achieved through miniaturized SMART beads and high-resolution optical detection).

* Rapid evaluation (achieved through parallel optical evaluation in planar application which can

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also be further expedited by detection of moving SMART beads).

* Cost-effective (achieved through the dispensation with costly microengineering and optical components, and complicated synthesis steps in the biochip production).

The value of the invention derives greatly from the suitable combination of technologies.

4 Main features of the achievement

The principal achievement in this system is derived from the coding (specific identity of the individual microparticles) via the biochemical coating of the surface for the detection method (labeling or coating) of the SMART beads via the mixing of the SMART beads with the sample to be investigated in the analysis system up to presentation and detection.

4.1 Fractal solid phase (SMART beads) and specific coding

In order to be able later to detect individual SMART beads unambiguously (microparticles of defined size and geometry) the beads are individually coded. This means that each SMART bead class acquires its own identity, resulting in identifiable individuals from the uniform support bodies, which makes it possible for the individual SMART bead classes (one class here are more than one SMART bead with the same identity) to be recognized again in the detection unit. The microparticles (beads or microspheres) become the actual SMART beads only through their individual coding.

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It is possible to use as coding in general, for example, the particle size, geometry, weight and physical properties such as, for example, magnetizability or the like. However, these principles point to only a very limited number of "gradations", so that only a few different codings can be generated.

The SMART beads preferably have an average diameter of at least 10 µm, preferably of at least 20 µm, particularly preferably of 25 to 200 µm, and most preferably of 25 to 80 µm. It is possible to employ optically transparent or optically nontransparent beads, with preference for beads with maximum optical transparency, which make simple analysis in a transmitted light method possible. Preference is also given to the use of SMART beads with a minimum variation in size, the diameter of at least 50%, particularly preferably at least 70% and most preferably at least 90% of the beads differing by a maximum of up to 20% and preferably up to 10% from the average diameter of the particular SMART bead species.

Conceivable methods for color-coding the SMART beads are, inter alia, a light-transmitting coloring of the material, a light-transmitting coloring of the surface, an opaque coloring of the material, an opaque coloring of the surface, an embedding of fluorescent dyes or a luminescence method.

Color or electromagnetic codings by contrast have a plurality of gradations, which makes it possible to identify very many SMART beads. The idea of the present invention is based on utilizing a color coding, that is to say a spectral identity of the SMART beads in combination with the continually growing efficiency of CCD matrix chips for optical

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detection. These chips are able at present to identify about 70 million colors.

4.2 Analyte determination - basic principle

The general principle to be used for analyte determination is the binding of the analyte to its specific immobilized interaction partner on the surface of a defined SMART bead.

In this case, the specificity of this interaction is intended to make selective binding of the analyte possible only to the SMART beads appropriately equipped therefor, i.e. carrying the immobilized interaction partner, and thus molecular recognition and subsequent detection of the analyte in a heterogeneous mixture. All the SMART beads defined as a class by their identical spectral properties (color) each receive the same equipment with an immobilized interaction partner, it being possible for the binding of the interaction partner preferably to be covalent, but also noncovalent via adsorptive or specific high-affinity interactions (for example biotin/avidin or streptavidin or hapten/anti-hapten antibody). The presence of the analyte in the investigation material therefore leads to binding and thus signal generation only on this spectrally identifiable class of SMART beads. If the particular class-specific equipment is known, this signal serves to identify the bound analyte and thus detect it in the investigation material.

The detection principle thus corresponds to established methods, for example in the development of ELISA tests (enzyme linked immunosorbent assay). The coating of beads with an immobilized interaction partner is "state of the art" (for example avidin coating of magnetic

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beads, Dynal; antibody coating in the ELISA format, Flowmetrix from Luminex).

4.3 Mixing of the SMART beads with the sample in the analysis system

The specific color- and analyte-coded SMART beads are mixed in the analysis system with the sample to be investigated. These operations are identical to those in other bead-based methods with a "movable" solid phase (for example Flowmetrix from Luminex). This means that a set of SMART beads which has been compiled for a specific application, for example composed of 10 000 microparticles for every 100 SMART beads of one of 100 classes, is brought together with the appropriately prepared sample in a vessel, incubated and mixed under suitable conditions, for example suitable hybridization conditions, until it can be assumed with sufficient probability that each SMART bead has had the opportunity to find its reactant.

4.4 Presentation of the SMART beads for detection

In all methods and techniques for presentation of SMART beads for detection, the aim is to achieve detection which is as fast as possible and, at the same time, reliable and thus efficient, because only accurately measured events contribute to the result of the measurement.

One possibility for achieving a high throughput in the detection is to present the SMART beads as quickly as possible at the detector - for example in analogy to the flow cytometer principle where the particles are forced at high speeds through a capillary and are measured at the exit "online" in the "particle jet". The possibility which is

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preferred according to the invention is parallelization of the detection for example by a two-dimensional presentation of the SMART beads at the detector (SMART bead array).

It is, of course, necessary for the detector used to be able to pick up the signals sufficiently quickly and in parallel. For this purpose, CCD chips should preferably be employed directly, that is to say without use of an optical system. This predetermines the dimensions of the area on which the SMART beads are to be provided for the detection. Thus, at present, high-resolution standard chips (not specially manufactured) have external dimensions of about 25 × 37 mm. If, for example, it is desired to arrange about 200 000 SMART beads on this area, each SMART bead could have a diameter of 60 µm, and each SMART bead would nevertheless still cover about 40 color pixels, which is sufficient to make reliable detection possible. With packing of maximum density through offsetting of rows it would in fact be possible to accommodate even more SMART beads, or the SMART beads could be even larger. Miniaturization and thus further parallelization is, of course, conceivable. Possible methods for planar distribution of the SMART beads on a support are the following techniques: two-dimensional capillary slit, parallel capillaries or tubes, spreading, stamp application (analogous to blood smear) or roller application or spotting on.

4.5 Excitation light source

In order to be able to identify the SMART beads in their planar distribution, an excitation light source which satisfies the requirements of the color coding method used just as much as the

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selected detection method for a reaction having taken place on the surface of the SMART beads. Where appropriate, two separate light sources should also be provided for the two steps, for example if the identity of the SMART beads is to be detected by an absorption measurement and the reaction detection is to be detected by a fluorescent signal. Possible types of light are parallel, flat light (continuous or as flash) or local, localized excitation for example by streaks of light or points of light.

These types of light may be, depending on requirements, monochromatic (for example laser light for fluorescence excitation) or heterogeneous (for example white light for absorption measurement).

4.6 Identification of the SMART beads

Optical recognition of the spectral identity of a single SMART bead in the SMART bead array provides two items of information:

(1) The SMART bead class (for example SMART beads with the same color) to which a particular SMART bead belongs, from which the analyte bound to its surface can be established (identity: "who am I and accordingly which analyte is bound to my surface").

(2) The exact location of the particular SMART bead in the planar SMART bead array (location: "where am I").

Modern, high-resolution CCD camera chips are preferably used for detecting SMART beads. These make both very rapid and highly parallel detection of the light signals possible.

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The second item of information of identification of position is necessary in the presentation method without accurately predetermined location (capillary slit, smear etc.) in order to be able to accurately assign the subsequent signal of the detection method to a SMART bead. In the methods with a predetermined location (spotting etc.) it is possible to use the item of information for quality assurance.

4.7 CCD detection of the specific detection reaction (analyte binding)

As described in detail in 6.2.3, the binding of an analyte is to lead directly or indirectly to a detectable signal, preferably a light signal. This can take place, for example, through an excitation light (fluorescence) or through photon emission (luminescence). The steps of detection of the analyte signal and recognition of the bead coding can take place in any sequence.

The light signal is detected on the CCD chip, and in particular differentiated both according to wavelength (color) and according to intensity. The recorded spectrum can be analyzed qualitatively or quantitatively. In addition, differentiation of wavelengths and intensities also permits signal sources to be differentiated.

5. Developed improvements and advantages created thereby compared with present systems

The fractal chip (FSC) of the invention shifts the information via the provision of a measurement point (for example of a DNA oligo of particular sequence) from the localized synthesis or coating (for example photolithography, spotting on) to the

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coding of the fractal components. This results in an extremely flexible arrangement with an enormous potential for multiplex analysis.

In addition, it is possible in this format to combine very diverse areas of biochemical diagnosis through separation of synthesis of the actual sensors (i.e. the SMART beads) and incubation with the investigation material. Specifically, the intention is to make it possible to analyze DNA, proteins and other parameters of interest simultaneously.

Through novel combinations of proven SMART classes for an investigational approach it is possible to provide the SMART beads, which are produced in a batch method, for a plurality of diagnostic and analytical problems. This results in great advantages in particular for production, which is reflected in the costs.

In this approach, the aim is, inter alia, through moderate size of the SMART beads, through direct detection and through utilization of the highly developed CCD technology, to realize 10^3 to 10^6 distinguishable SMART bead classes in one measurement run. This means a considerable improvement in the state of the art because, for example, great problems occur in the fluorescence-based coding of beads with only 64 or more distinguishable types . The first multiplex method which is able to compete, in density of the measurement points, with the DNA arrays which have just been developed, but without their restriction to DNA analysis and without the complicated and costly complete chip synthesis, is provided thereby. The flexibility and user-oriented extendability of the individual components are essential advantages of the invention.

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5.1 Color-coded SMART beads for absorption measurements

One advantage of parallel and direct detection of the SMART beads on an area which corresponds to the size of a CCD chip of, at present, about 25 x 37 mm is, from the viewpoint of coloring, the moderate particle size. Thus, SMART beads with a diameter of about 60 µm can be produced, color-coded, biochemically labeled and fluidically manipulated satisfactorily and they provide a strong light signal both in the SMART bead identification and in the subsequent reaction detection.

This makes absorption measurement as optical measurement method for SMART beads identification possible for the first time, the absorption measurement having the following characteristics: a very wide diversity of unambiguously measurable color variants (gradations) can be achieved, which is distinctly more limited for example in fluorescence methods, and a lower optical resolvability is necessary, i.e. the particles can be larger than is necessary, for example, in a fluorescence measurement.

These characteristics aresupported by the relative size of the SMART beads which is caused by the size of the CCD chips. Thus, a very large number of differently color-coded microsupports can be produced with the SMART beads, which is of particular interest for use of the FSC system in DNA analysis.

The achievable optimum of differently colored SMART beads in the FSC is determined by the following parameters: number of chemically

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possible colorings for unambiguous identification of a SMART bead, minimum permissible size for error-free identification (sufficiently strong light signal) of the SMART beads, and sufficient number of CCD pixels per SMART bead for error-free identification.

The potential of the system and, where appropriate, further miniaturization of the SMART beads is automatically advanced by the further miniaturization, with simultaneous increase in sensitivity, of the CCD sensors.

The absolute amount of sample is, for example, 3 ml for wetting about 200 000 of the 60 µm SMART beads, which is in a very reasonable volume range. It is possible in particular to include all SMART beads in the measurement, that is to say carry out a 100% measurement of all SMART beads which have a light-transmitting coloration of the material of the SMART beads, or a light-transmitting coloration of the surface of the SMART beads, for an absorption measurement.

Biochemical methodological advantages in the coating of the SMART beads are located in the aspects listed below:

* Separate synthesis of individual species makes it possible to couple very diverse, immobilized interaction partners onto the beads.

* It is possible for the first time to combine DNA, PNA, proteins etc. in one format.

* For DNA analytics, specifically determination of expressed genes, the potential coupling of all cDNAs or ESTs (expressed sequence tag) is a great advantage compared with conventional DNA chips

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because many fewer measurement points suffice for unambiguous identification of an RNA or DNA than is the case with arrays of short oligonucleotides (ideally only one measurement point).

5.2 Analyte-specific labeling (coating) of the colored SMART beads

The advantages of the biochemical coating of the SMART beads from the production technology viewpoint are the possibility of multiplexing starting materials and support particles and of parallelizable production in a high-throughput batch method. It is moreover possible to carry out all manipulation and production steps fluidically with utilization of physically advantageous flow processes. In addition, the method is simple and thus automatable (less work involved and thus cheaper to produce).

In the biochemical coating of the SMART beads in production it is possible to optimize the operations simply by the flexible multiplexing of starting materials (for example G, A, C, T or longer oligo sequences) or of the wash liquids, and of the SMART bead classes (microparticles of one color). This means that the most efficient combination is produced from parallelization of the SMART bead provision, parallelization of analyte provision and the freely selectable batch size on production in a batch method.

5.3 Optimal flow and manipulation processes

The favorable hydrodynamic mixing processes result in a very good wetting of the surfaces of the SMART beads with the binding partners (analytes) as well as defined washing and rinsing processes. These result in a distinctly smaller consumption

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of starting materials, especially by comparison with the hydrodynamically very unfavorable flat methods like those used with planar DNA chips on a support. Overall very good reproducibility and quality assurance is achieved thereby.

Because of the size of the SMART beads, manipulation of a single SMART bead is simplified, which entails the advantage of qualitative mixing and separation or sorting processes both in production and in analysis. With very small particles by contrast, only quantitative manipulation is possible. This means that amounts of fluid with a statistically determinable proportion of particles of one class are separated or mixed. The SMART beads make both methods possible, with their individual advantages.

The advantages of the system of the invention compared with biochips which are produced in two-dimensionally densely packed arrays even during production are therefore, on the one hand, superior flow processes so that less sample material is required and better wetting of the surfaces of the SMART beads with the sample material is achieved. This makes it possible to detect uncommon constituents of the sample with a low concentration in the sample. On the other hand, simple and automatable flow processes make it possible to achieve a greater information yield and more reliable results.

5.4 ARRAY arrangement and detection of the SMART beads

The FSC technique principle of measurement makes use of the continually growing potential of high-resolution image acquisition sensors and, in particular, of CCD chips for parallel detection of a plurality of SMART beads. The latter are to be

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provided preferably as densely as possible in a two-dimensional array on the optical CCD chip sensor for detection (presentation at the detector).

Owing to the use of a CCD chip, the system is focussed on parallelization of detection for increasing throughput. At the same time, this increases not only the speed of measurement but also the quality of measurement owing to a plurality of possible parallel reference measurements.

Thus, a planar array of SMART beads, which is as densely packed as possible and in which the individual SMART beads are preferably located directly next to one another, so that they can be detected simultaneously by a CCD camera, is produced.

5.4.1 Identification of identifier or/and location of the SMART beads

Identification of the identifier of the SMART beads:

For the purpose of the invention it is possible to provide for the microparticles (SMART beads) to be positioned in predetermined geometric arrangement on the support.

In this variant of the method, a geometric arrangement of the microbeads is imprinted on the array by the method step of positioning or presentation, utilizing the identification of the microbeads on the basis of their color only for differentiating the microbead classes but not for determining the location of the microbeads, because the latter is, after all, already predetermined by the lattice structure.

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A further novel method which positions the SMART beads in the correct location already during the presentation for the subsequent detection is the spotting of the SMART beads onto a support. This procedure is novel because single microparticles are spotted and no liquid. Conceivable supports are small glass or plastic plates (chips in the real sense), a moving belt, a sheet on the support etc.

An alternative possibility is to use a capillary, two-dimensional, planar slit (see Neubauer chamber) to the surfaces of which a hydrophobic grid is attached and directs the SMART beads distributed in the slit into an exact location in the slit.

The method of the invention which is considerably more elegant than the variant of the method mentioned above with a predetermined arrangement of the SMART beads in the correct location is an undefined, free and repeatedly renewing, i.e. fractal, arrangement of the SMART beads in a two-dimensional array, the "fractal SMART chip - FSC". In this case, the high resolution of the CCD chips [state of research in May 1998: 81 million, pixels] is also used for detecting the location. The presentation of the beads in this case can take place through a capillary, two-dimensional, planar slit (see Neubauer chamber), parallel capillaries (forced principle in two dimensions, detection of location necessary only in the direction of flow), a smear or stamp method (analogous to a blood smear) or a rolling out of the SMART beads.

This principle of measurement results in a new array each time in an undefined spatial

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arrangement on the surface of the detector. The identity of the individual binding sites is determined by the code intrinsic to the bead and not by a predetermined location on a chip.

5.5 Parallel and dynamic detection

A further potential for utilizing flat CCD chips for detection (no scanning process) of microparticles (SMART beads) is the possibility of simultaneous utilization of the "efficiency-increasing potential" of a dynamic detection of moving SMART beads. This combines the potentials of speed and parallelity in one system, which none of the three known systems described makes possible.

The dynamic (moving) presentation of the SMART beads at the CCD chip detector takes place in this case for example in a plurality of fluid compartments or subchambers which are preferably designed as parallel channels, for example as capillaries.

5.6 Direct detection without optically magnifying elements

The preferred direct detection of the transmitted light signals (without optical system between the SMART bead support and the detector, preferably a CCD image recorder) has the advantage of a considerably smaller amount of energy required by the light for error-free detection. Optical elements between the CCD image converter and SMART bead support would absorb light and thus reduce the sensitivity of the measuring device. The lower light intensity greatly reduces unwanted stray light effects as well as any need to cool the light source used. In addition, the omission of an

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optical system also means a great saving in space and a reduction in the production costs for the detection unit.

5.7 Identification and detection method in one

The use of modern, highly parallel CCD chips makes it possible to detect the particular detection reaction in one measurement together with the identification of the SMART beads according to class and location in the array.

Signal detection in this case is based on colocalization of a spectrally (color-)labeled SMART bead and of a signal generated by analyte binding. It is thus possible, in addition to the signal detection, also to minimize the nonspecific background. It is thus possible to detect a difference in light intensity or a color change owing to the reaction having taken place on the surface of a SMART bead. It is, of course, necessary for an unambiguous distinguishability nevertheless to be retained. Thus, the SMART beads without a reaction on their surface can in each case be distinguished by 10 of the current 4096 intensity levels in a color channel, and the SMART beads which have found their binding partner are, due to the biochemical reaction, located 2-3 intensity levels lower or higher.

5.8 Disposable chips - no cross-contamination

All the methods conceivable for the system of the invention for planar presentation of the SMART beads for direct detection with a CCD chip can, with the exception of the microparticle spotting, be designed simply as disposable methods (support chip as disposable article). Although DNA chips produced by photolithography are also offered as

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disposable articles, in this case the very high price, due to the elaborate production of the chips, is not in favor of throwing away after only one measurement.

5.9 Flexibility of use and scope of the areas of application

The scope of use extends from the search for an uncommon constituent of a sample, the search for the "needle in the haystack" with, in the extreme case, only a single SMART bead class (one color coding), to ultra-high throughput screening - UHTS - always with only a single SMART bead in each class. Thus all known and currently predictable applications are made possible, from "classical" immunology" up to modern, future-oriented DNA analysis, using the system of the invention.

And if the result of a measurement has not been satisfactory, or the first measurement reveals that further specific investigation is necessary, it is simple to add and detect additional SMART beads, which likewise confers on the system an enormous advantage in flexibility compared with known gene chips.

6 Summary of some preferred aspects of the invention

6.1 Individual coding of the SMART beads

Various methods are suitable for color coding, for example a light-transmitting coloration of the material (for example glass, polysaccharide or plastic) from which the SMART beads (microparticles) are produced, a light-transmitting coloration of the surface of the SMART beads and incubation of the beads with

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suitable dyes, embedding of fluorescent dyes (or markers) in the SMART beads, embedding of absorption dyes in the SMART beads or chemical coating of the particle surface with a luminescent marker.

Other ordering principles such as the shape or size of the particles or electromagnetic properties, and other material properties (magnetic, nonmagnetic), are also conceivable as supplement.

6.2 Analyte determination

6.2.1 Analytes

The method of the invention is suitable for determining any analytes. Examples which may be mentioned are nucleic acids (DNA, RNA, in specific cases also PNA), proteins, polypeptides and peptides in all manifestations, for example hormones, growth factors, enzymes, tumor antigens, serum factors, antibodies, carbohydrates in various combinations, for example various sugars in foods or agricultural crops, functional sugars, polymers, lipids, for example steroids, second messengers, membrane lipids, cholesterol, or other organic molecules, for example drugs of abuse, pharmaceuticals, metabolites, amino acids, transmitters, pesticides, insecticides, paints, various toxins etc. It is also possible in addition to determine cells, subcellular particles or viral particles.

6.2.2 Variants of the immobilized interaction partner

The binding of the analyte to the microparticles takes place via a specific binding to an immobilized interaction partner. On use of a

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plurality of classes of microparticles, during a single determination there is binding of different analytes to their respective interaction partners. The method permits the use of different types of analyte-binding reactions, for example hybridization of complementary nucleic acids, in which case it is possible to employ longer molecules such as cDNA, synthetic oligonucleotides, PNA, RNA, as interaction partners. The analyte can also be determined through receptor-ligand or antibody-antigen reactions, in which case, for example, natural antibodies, recombinant antibodies and their subunits, antigens, in general proteins and polypeptides of interest, are employed as immobilized interaction partner. It is also possible to use peptides, for example synthetic peptides, natural peptides, as immobilized interaction partner. A further possibility is to synthesize products of combinatorial chemistry directly on the beads.

6.2.3 Signal generation variants

Two principles of signal generation are employed predominantly: direct detection of an analyte which was labeled previously or during the reaction, and indirect detection through competition of the analyte with a labeled standard of the same substance. The first variant is well established for some applications, but is rather unsuitable for diagnostics of, for example, serum components. The second variant is therefore to be preferred for the latter applications, and, in principle, it permits sample preparation by the user to be simpler.

Direct detection can take place through labeling of the analyte with a fluorescent dye, a reporter enzyme and subsequent reaction (for example chemo-

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and bioluminescence), by selective labeling of the bound analyte, for example in the case of nucleic acids by intercalating (fluorescent) dyes, double strand-binding proteins or antibodies.

Secondary detection is possible by detecting the bound analyte with a second component, for example in the case of PNA-DNA hybrids by DNA-specific antibodies.

Labeled standards which can be employed for competitive detection reactions are dye-, fluorescence- or enzyme-coupled (for example chemo- and bioluminescence with alkaline phosphatase, peroxidase etc.) analyte molecules. Protein standards can be produced as fusion protein with reporter enzyme (see above) or autofluorescent protein (for example GFP), for example for recombinant antibodies, protein hormones, growth factors etc.

6.3 Presentation of the beads for detection

The following variants in particular are provided for the presentation of the SMART beads for optical detection with a CCD chip:

* Two-dimensional (planar) capillary slit (see Neubauer chamber or Thoma chamber):
Capillary forces are sufficient where appropriate for filling the slit; otherwise, the SMART beads must be conveyed into the slit with elevated pressure on the slit entry side and/or reduced pressure on the slit exit side. A further possibility is to enhance the capillary forces, which may theoretically reach 40 g, also by an appropriately absorbant material (fabric) on the slit exit side. Possible as material for the support with 2D capillary are, for example, glass

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or plastic, it being possible to design support with capillary, especially in the case of an injection molded version, cost-effectively and as disposable article.

* Parallel capillaries in a glass or plastic chip. The limiting conditions applying here are otherwise identical to those for the capillary slit, and this variant is particularly suitable for measuring moving SMART beads.

* Smear or stamp method based on the methods used for blood smears.

* Spotting onto supports, for example small glass or plastic plates (transmitted light measurement also possible), moving belt as suitable material, sheet over support etc.

The necessary single microparticle handling can also be employed for sorting processes for assembling the individual SMART bead kits.

6.4 Excitation and detection unit

The reading of the information from a SMART bead array is intended beneficially to take place in a combined excitation and detection unit, the preferred system being an arrangement of the excitational light source arranged directly over the SMART bead array and of the CCD chip arranged directly beneath (or vice versa) the array (sandwich architecture). This maximally compact architecture minimizes the distances traveled by the light and thus also the required intensity of light, as well as overlap effects of adjacent SMART beads. It is intended preferably to dispense with the use of an elaborate and costly optical system which takes up much space and consumes much

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light both on the excitation and on the detection side.

Another variant is a vertical alignment of the chip, so that gravitational forces can also be utilized for the loading and unloading of the chip with the SMART beads.

6.4.1 Excitation light source

Suitable light sources are, depending on the color coding of the SMART beads (absorption or fluorescence etc.) and the chosen detection method (fluorescence or luminescence etc.), for analyte binding, for example, highly parallel light from a lamp (white light), highly parallel light from a flash tube, highly parallel monochromatic light, a monochromatic streak of laser light (local excitation corresponds to the scanner principle and thus no longer makes use of the parallelity of the CCD camera), or a monochromatic laser beam (local excitation corresponds to the scanner principle and thus no longer makes use of the parallelity of the CCD camera).

It is moreover possible for an appropriate optical grating and/or an appropriate optical system to be present between excitation light source and SMART bead chip.

6.4.2 CCD camera detection

The detection unit comprises a CCD chip. These currently have about 2 000 × 3 000 color pixels on an area of about 25 × 37 mm, which corresponds to 6 million color pixels or 18 million single pixels (RGB-principle through miniaturized color filters in front of the pixels) (supplied by Canon). Arrangement of microparticles (SMART beads) with a

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diameter of 60 µm on such an area of 25 × 37 mm for direct detection results in the minimum of 200 000 microparticles (SMART beads). Each microparticle in this case covers about 40 square color pixels with an edge length of 9 to 10 µm. The result is thus 40 color or 120 black/white signals per SMART bead with a digital light intensity gradation of 4 096 discrete brightness levels per black/white pixel. Thus, an adequate amount of data for statistical signal verification is present in every case.

The maximally synchronously detectable, differently color-coded SMART beads is limited by the possibility of specific codability (chemical limit of reproducible color production) of the SMART beads and the possibility of optical detection of the color differences with a CCD chip. Dividing 256 intensity levels for each color (RGB) into 10 levels results in 25^3 = 15 625 possible colors which can be detected. The number of detectable colors can be increased further by expanding the number of color classes through further color filters. With four color filters (for example RGB and magenta) in front of the CCD chip described above it is possible theoretically to recognize 25 × 25^3 = 390 625 colors. Now, of course, only with about 30 four-fold color pixels. Because of the great advances in CCD technology, the stated numbers describe only the current state of the art. Initial prototypes already have 81 million pixels on the same area. There is thus a great growth potential also for the described application of CCD chip technology, and parallel detection of 10^6 individual SMART beads is technically feasible.

It is possible where appropriate for an appropriate optical grating and/or an appropriate

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optical system to be present between SMART bead array and CCD camera chip.

6.4.3 Parallel inspection unit

A particularly interesting aspect within the scope of the present invention is the use of a "parallel inspection unit".

This takes the form of a light emission detection device with a controllable light source matrix which can, for example, be a reflector matrix, a light valve matrix (for example LCD) or self-luminous illumination matrix, and with a light sensor matrix which is opposite to and faces the light source matrix, namely the CCD image recorder, in which case the support with the SMART beads present thereon or therein is to be arranged between the light source matrix and the CCD sensor.

One strength of this inspection unit are the flexible possibilities resulting from the combination of (positionally or/and temporally) targeted excitation and targeted detection in conjunction with modern high-efficiency computers for control and signal evaluation. This creates a novel technology platform in particular for optical detection methods. "Tuning through" or "scanning" individual points of light or lines of points of light in interplay with the CCD detection and suitable algorithms for signal evaluation makes extremely small changes in the individual measurement points in the "LCD-CCD light grid" (inspection unit) possible. In the area of DNA analysis, direct detection of a hybridization on a measurement device is conceivable within the framework of the invention. The LCD unit as two-dimensional light

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source matrix can be used both as light source for absorption measurements for determining particular positions and for fluorescence excitation in analyte determination (where appropriate with other background illumination).

7. Remarks

The following variants or modifications of the method of the invention have independent significance where appropriate:

The method of claim 1 can be modified in the following way: the step of applying the microparticles to a support in feature b) can take place before step a) of bringing the sample into contact with a plurality of species of microparticles. In the aforementioned variant of the method, the support used is a planar support or a chip, preferably with capillary slit, in which case, such a flat (where appropriate hollow) plate support should be coupled directly to the CCD image recorder in order to be able to carry out an unmagnified image-wise detection of what happens when the microparticles are brought into contact with the sample.

The aspect of detection or observation of the SMART beads by means of CCD image recorders in a moving ensemble of SMART beads also has independent significance where appropriate, and supports with capillaries are particularly suitable for such dynamic measurements.

8. Exemplary embodiments

8.1 Colored microparticles

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Particles of chromatography matrices, for example DEAE-Sepharose, Sephacryl, Sephadex or Sephacel, were stained by incubating with the dyes eosin red, Evans blue and light green for 5 min. This resulted in colored beads which could be unambiguously classified in the transmitted light method.

8.2 Light sources

Light sources which have proved suitable are xenon and halogen lamps, and cold light sources.

It was possible by incorporating an optical system between light source and support to improve the parallelity of the light impinging on the support. Suitable optical systems were, for example, an 80 to 200 nm telephoto optical system (Tokina photographic lens), and achromatic telephoto optical system (telephoto 300 mm achromatic lens) or a collimator (Melis-Criot).

In addition, the LCD matrix LCX017AL (Sony) with a chip area of 60 mm^2 (diagonal 46 mm, ratio of sides 5:4), a pixel number of 786 432 (corresponding to a pixel area of 830 μm^2) has been successfully tested.

8.4 CCD chip

The CCD chip ICX085AK (Sony) with a diagonal of 16.933 mm, a ratio of sides of 5:4 (10.57 mm × 8.5 mm) and a number of effective pixels of 1 300 × 3 030 (calculated pixel area 7.97 μm^2) has been successfully tested.

The invention is explained in detail hereinafter by means of an example with reference to the figures.

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Fig.1 shows a diagram to illustrate one embodiment of the method of the invention.

Fig. 2 shows a simplified perspective depiction of a detection unit composed of CCD image recorder and analyte support.

Fig. 3 shows a top view of the analyte support in fig. 2.

Fig. 4 shows a section through the analyte support along the plane of section indicated by IV-IV in fig. 3.

Fig. 5 shows diagrammatically an inspection unit composed of LCD matrix (liquid crystal display matrix), support chip as in fig. 2 and CCD matrix in a sandwich arrangement.

As shown in fig. 1, in a step A the sample material 1 is mixed with the prepared microparticles (SMART beads) in a mixing device 5. The ensemble of microparticles 3 introduced for this purpose comprises a plurality of differently colored, transparent microparticles 3, where microparticles 3 with the same coloring belong to one species which has the same capture molecules (for example oligonucleotides) immobilized on the microparticles 3. The presence of the target analytes in the sample is detected by using analyte-specific labeled detection reagents in soluble form, with whose aid it is possible to detect a binding of the target analytes to the beads.

During step A it is then possible under hybridization conditions for the appropriate target analyte molecules in each case to become attached to the capture molecules on the surface of the relevant microparticles (SMART beads).

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As shown in fig. 1, the mixing step A can be followed by an amplification step (for example PCR amplification etc.).

The analyte from the microparticles 3 which have been brought into contact with the sample 1 is brought into a planar arrangement with arbitrary distribution of the microparticles 3 on the analyte support 7. The analyte support 7 can in the simplest case be a glass plate or transparent plastic plate on which the microparticle-analyte 3 is, for example, smeared, rolled or, where appropriate, stamped.

In step C in fig. 1 there is in a first measurement operation an image-wise absorption measurement of the ensemble, arranged on the analyte support 7, of microparticles 3, using a CCD image recorder 9 as image acquisition sensor. In the example shown, the analyte support 7 is located between the CCD image recorder and a light source 11 which emits a spectral broadband and is indicated in fig. 1 by a light-bulb symbol. In the absorption measurement in step C there is preferably transillumination of the analyte support 7 and the analyte layer provided thereon with parallel light from the light source 11. The CCD image recorder 9 in this case acquires an image of the ensemble of microparticles 3 on the analyte support 7, and a data processing and control device 13 evaluates the image data from the CCD image recorder in order to file the image point locations of the CCD image recorder 9 on which microparticles 3 with a particular color were recorded.

As indicated in fig. 1, the size of the microparticles 3 is chosen so that each microparticle 3 covers more than one pixel (image point sensors) 15 of the CCD image recorder 9 in plan view projection.

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After the various microparticle species have been identified with assignment to their location in the recorded image in step C, detection of the detection reaction takes place in step D. In the exemplary case, the reaction is detected by fluorescent signals which are emitted by microparticles 3 to which analyte-specific detection reagents have become attached. A UV light source 17 which is depicted in fig. 1 by a light-bulb symbol and which emits parallel light serves as fluorescence excitation light source. The fluorescent radiation is recorded by the image recorder 9 with assignment to the location of the respective fluorescent microparticles 3. The data processing system is then able to establish from the results of steps C and D the particular species of microparticles 3 to which the microparticle(s) 3 which have emitted a relevant fluorescent signal belong. Since microparticles 3 identified in this way, or the capture molecules immobilized thereon, can have reacted only with a particular target analyte from the sample, it is thus established that the target analyte was present in the sample.

Fig. 2 shows an analyte support chip 7a in the measurement arrangement on a CCD image recorder 9. The analyte support chip 7a (also referred to as fractal SMART chip or FSC) is also depicted in plan view in fig. 3. The chip 7a has a disk-shaped capillary slit or cavity 19 which is limited at the top and bottom in the depiction in fig. 2 by transparent plate sections 21, 23. The height of the slit h (cf. fig. 4) is chosen so that it is larger than the average diameter D of the microparticles 3 and smaller than twice the diameter (2D) of the microparticles 3, so that in each case there is room for only one layer of microparticles 3 lying side by side between the plate sections 21, 23 in the capillary slit 19.

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The analyte support chip 7a has at its right-hand end in fig. 3 and 4 a sample input 25 for introducing the analyte (microparticles 3 after mixing with the sample). The sample input 25 is connected to the capillary slit 19. As is evident from fig. 3, the capillary slit 19 extends in area over a detection region 27 which, as shown in fig. 2, is in alignment with the active image acquisition field, indicated by 29, of the CCD image recorder 9 when image recorder 9 and support chip 7a are joined together to give the detection unit shown in fig. 2.

At the exit side, located on the left in fig. 2 and 3, of the capillary slit 19 there is provided in the example shown an absorbent material 30 in a chamber of the chip 7a, which enhances the capillary forces to fill the slit 19 with analyte through its suction effect. The analyte support 7a is preferably made of plastic in an injection molded version so that it can be used cost-effectively as disposable article.

It should be added that the size of the detection region 27 on the chip 7a essentially corresponds to the size of the active image-acquisition field 29 on the CCD sensor. In one embodiment, the detection region has the dimensions 2.5 cm × 3.7 cm.

Fig. 5 shows diagrammatically the arrangement of fig. 2 with an LCD matrix 35 as two-dimensional measurement and excitation light source whose light source elements can be controlled in a location- and time-dependent manner specifically according to the intensity and color of the light, and the relevant control device is the joint control device for the LCD matrix and the CCD matrix.

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It is to be understood that a reference herein to a prior art document does not constitute an admission that the document forms part of the common general knowledge in the art in Australia or in any other country.

In the claims which follow and in the preceding description of the invention, except where the context requires otherwise due to express language or necessary implication, the word "comprising" and grammatical variations thereof, is used in the sense of "including" i.e. the features specified may be associated with further features in various embodiments of the invention.

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Claims

1. A method for determining a plurality of analytes in a sample comprising the steps:

(a) contacting the sample with

(i) a plurality of species of microparticles, each species being suitable for detecting particular analytes and having a particular coding optically distinguishable from other species, and

(ii) a plurality of soluble analyte-specific detection reagents which carry a signal-emitting group or are capable of binding to a signal-emitting group,

(b) subsequently applying the microparticles to a support comprising a cavity for receiving the ensemble of microparticles between two plates whose flat sides are opposite to one another, where the distance between the plates is chosen so that in each case only one layer of microparticles lying side by side fits into the cavity and at least

(c) determining the analytes by optical detection of the coding and the amount, the presence or/and the absence of the signal-emitting groups or at least one species of individual microparticles on the support.

2. The method as claimed in claim 1, characterised in that the analytes are selected from cells, subcellular particles, nucleic acids, polypeptides, peptides, glycoproteins, lipoproteins, mono-, oligo- and polysaccharides, lipids, metabolites, hormones, mediator substances, neurotransmitters, pesticides, insecticides, toxic substances, pharmaceutically active substances and combinations thereof.

3. The method as claimed in claim 1 or 2, characterised in that the sample used is a biological sample selected from body fluids such as serum, blood, plasma, urine, lymph, cerebrospinal fluid, sperm, saliva and the like, tissues, tissue extracts, cell, cell extracts, plant samples, environmental samples and fecal samples.

4. The method as claimed in any of claims 1-3, characterised in that microparticles selected from organic particles such as organic polymer latices, or polysaccharide particles, inorganic particles such a magnetic particles and glass particles, and organic-inorganic composite particles are used.

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5. The method as claimed in any of the preceding claims, characterised in that optically transparent or optically nontransparent microparticles are used.

6. The method as claimed in any of the preceding claims, characterised in that a number of up to 10^6 species of microparticles is used.

7. The method as claimed in any of the preceding claims, characterised in that each species of microparticles comprises on its surface at least one immobilised, different, analyte-specific receptor.

8. The method as claimed in claim 7, characterised in that the immobilised, analyte-specific receptors are selected from nucleic acids such as DNA and RNA, nucleic acid analogs such a peptidic nucleic acids (PNA), antibodies, antibody fragments, antigens, peptides, haptens and synthetic receptors.

9. The method as claimed in any of the preceding claims, characterised in that the coding of the microparticles is produced by colour coding, for example by colouring the material, colouring the surface or/and embedding dyes.

10. The method as claimed in any of the preceding claims, characterised in that the determination takes place via at least 2 species of different microparticles.

11. The method as claimed in any of the preceding claims, characterised in that at least one soluble analyte-specific detection reagent selected from analyte-specific receptors and analyte analogs is used for the determination of each analyte.

12. The method as claimed in claimed 11, characterised in that the soluble analyte-specific detection reagents are selected from nucleic acids such as DNA and RNA, nucleic acids analogs such as peptidic nucleic acids (PNA), antibodies, antibody fragments, antigens, peptides, haptens and synthetic receptors.

13. The method as claimed in any of the preceding claims, characterised in that the soluble analyte-specific detection reagents are directly coupled to a signal-emitting group.

14. The method as claimed in any of claims 1-12, characterised in that the soluble analyte-specific detection reagents carry a group which is capable of binding to a signal-emitting group, preferably via a high-affinity biological interaction.

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15. The method as claimed in claim 13 or 14, characterised in that an identical signal-emitting group is used for determining a plurality of analytes.

16. The method as claimed in any of the preceding claims, characterised in that a luminescent, fluorescent or phosphorescent group is used as signal-emitting group.

17. The method as claimed in any of the preceding claims, characterised in that a planar support is used.

18. The method as claimed in any of the preceding claims, characterised in that a support which has a surface selected from plastics, glass, semiconductor materials and metals is used.

19. The method as claimed in claim 18, characterised in that a chip is used as support.

20. The method as claimed in any of the preceding claims, characterised in that an optically transparent support is used.

21. The method as claimed in any of the preceding claims, characterised in that a support with structured surface regions is used.

22. The method as claimed in any of the preceding claims, characterised in that the application comprises a spreading on, rolling out, stamp application or spotting of a suspension containing the microparticles onto the support.

23. The method as claimed in any of the preceding claims, characterised in that the application comprises a migration of the microparticles on the support via capillary action.

24. The method as claimed in any of the preceding claims, characterised in that the determination takes place on a static or dynamic arrangement of the microparticles on the support.

25. The method as claimed in any of the preceding claims, characterised in that the determination takes place by means of high-resolution image-acquisition means, for example a CCD sensor.

26. The method as claimed in claim 25, characterised in that the size of the microparticles is selected so that each microparticle covers an area of at least 2,

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preferably of at least 4, particularly preferably of at least 9 and most preferably of at least 16 individual pixels.

27. The method as claimed in any of the preceding claims, characterised in that the microparticles have an average diameter of at least 10 µm, preferably of at least 20 µm and particularly preferably of 25 µm – 80 µm.

28. The use of the method as claimed in any of claims 1-27 for diagnostics of clinical parameters, for example for nucleic acid diagnostics, for biological basic research, for forensics, for food analysis and for screening medicinal products.

29. A kit for carrying out the method as claimed in any of the claims 1-27, comprising:

(a) a plurality of species of microparticles in free form, each species being suitable for detecting particular analytes and having a particular coding optically distinguishable from other species,

(b) a plurality of soluble analyte-specific detection reagents and

(c) a support.

30. A measuring device for determining a plurality of analytes in a sample by the method as claimed in at least one of claims 1-27, comprising

- an electronic image acquisition sensor for essentially unmagnified image-wise detection of the optical behaviour of an ensemble of microparticles which have been applied to a support comprising a cavity for receiving the ensemble of microparticles between two plates whose flat sides are opposite to one another, where the distance between the plates is chosen so that in each case only one layer of microparticle lying side by side fits into the cavity and brought into contact with the sample,

- means for exciting signal emission by signal-emitting groups on microparticles in the ensemble of microparticles on the support,

- a data processing device which evaluates image data from the electronic image acquisition sensor for providing information about the presence of particular analytes in the sample on the basis of the detected optical behaviour of the ensemble of microparticles

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- and wherein the support is aligned between the electronic image acquisition sensor and the means for exciting signal emission.

31. The measuring device as claimed in claim 30, where the image acquisition sensor is a CCD image recorder.

32. The measuring device as claimed in claim 30 or 31, where the microparticles are colour coded, and where the image acquisition sensor is suitable for detecting differences in colour of image points in the particular recorded image of an ensemble of microparticles in assignment to the location of the detected image points, in order to be able to determine the location of microparticles each of the same colour coding on the support for image-wise detection of the optical behaviour of the ensemble of microparticles.

33. The measuring device as claimed in any of claims 30 to 32, comprising at least one light source for providing in particular spectrally broad band light for parallel measurement of the optical absorption behaviour or reflection behaviour of the individuals in the ensemble of microparticles by means of the image acquisition sensor.

34. The measuring device as claimed in any of claims 30 to 33, where the device for exciting signal emission comprises at least one light source for fluorescence excitation of at least one species of microparticles with signal-emitting group, and where the image acquisition sensor is suitable for detecting the fluorescent light in the assignment to the location of the image points of any fluorescent individuals in the image-wise detection of the optical behaviour of the particular ensemble of microparticles.

35. The measuring device as claimed in any of claims 30 to 34, where the microparticles are coded according to their shape and where the optical measuring device composed of image acquisition sensor and data processing device is suitable for distinguishing individual species of microparticles on the basis of the different shape contours in the image recorded in each case.

36. The measuring device as claimed in any of claims 30 to 35, where the support is a planar window of the image acquisition sensor, which covers the image recording field of the image acquisition sensor toward the outside.

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37. The measuring device as claimed in any of claims 30 to 36, where the support and the image acquisition sensor can be fixed in defined positions relative to one another on a joint retaining means or in a directly mutually exchangeable manner.

38. The measuring device as claimed in any one of claims 30 to 37, where the support has at least one flow path for the microparticles and where the measuring device is set up to carry out the image-wise detection of the optical behaviour of the microparticles during the movement of the ensemble of microparticles along the flow path.

39. Use of a support for carrying out the method as claimed in any of claims 1 to 27, for a measuring device as claimed in any of claims 30 to 38, where the support has an extended capillary slit for receiving the ensemble of microparticles between two plates whose flat sides are opposite to one another, of which at least one is transparent, and wherein the distance between the two plates is chosen so that in each case only one layer of microparticles lying side by side fits into the extended capillary slit.

40. Use of the support according to claim 39 where the distance between the plates can be adjusted as desired.

41. Use of a support for carrying out the method as claimed in any of claims 1 to 27 for a measuring device as claimed in any of claims 30 to 38, where the support has a plurality of capillary channels which run parallel to one another, and is optically transparent at least in the region of the capillary channels.

42. A support for carrying out the method as claimed in any of claims 1 to 27 for a measuring device as claimed in any of claims 30 to 38, where the support has an extended capillary slit for receiving the ensemble of microparticles between two plates whose flat sides are opposite to one another, of which at least one is transparent, and wherein the distance between the two plates can be adjusted as desired so that in each case only one layer of microparticles lying side by side fits into the extended capillary slit.

43. A method according to claim 1, substantially as described herein with reference to the Figures.

44. A kit according to claim 29, substantially as described herein with reference to the Figures.

45. A measuring device according to claim 30, substantially as described herein with reference to the Figures.



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46. A support according to claim 39, substantially as described herein with reference to the Figures.

Dated this 20th day of February 2003

Febit Ferrarius Biotechynology GmbH

by its attorneys

Freehills Carter Smith Beadle

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WO 00/12123 PCT/EP99/06315

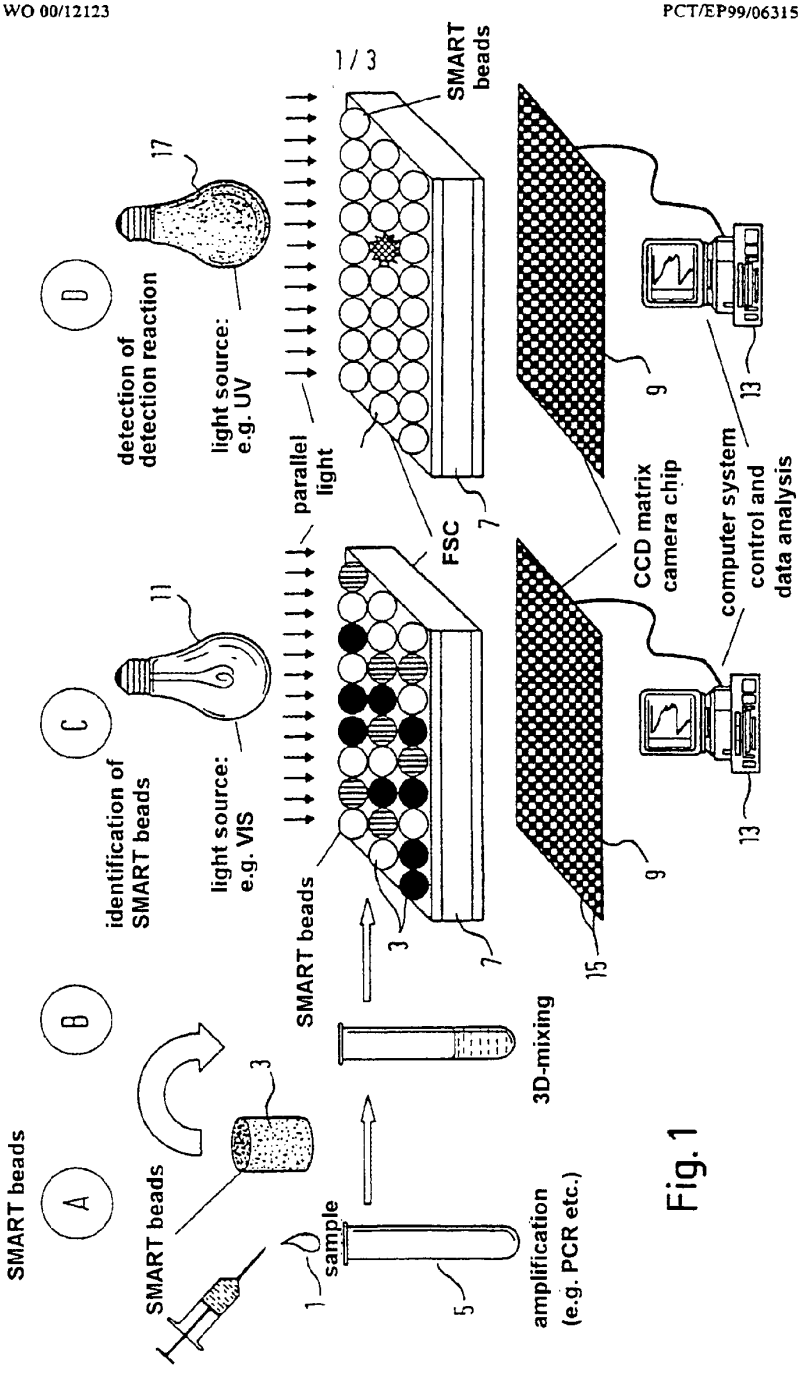
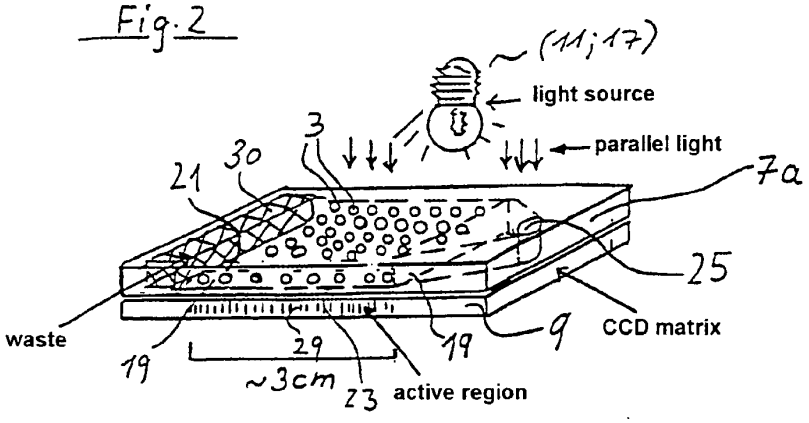


Fig. 1

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Fig. 2



Detection in the transmitted light method
(direct detection)

Fig. 3

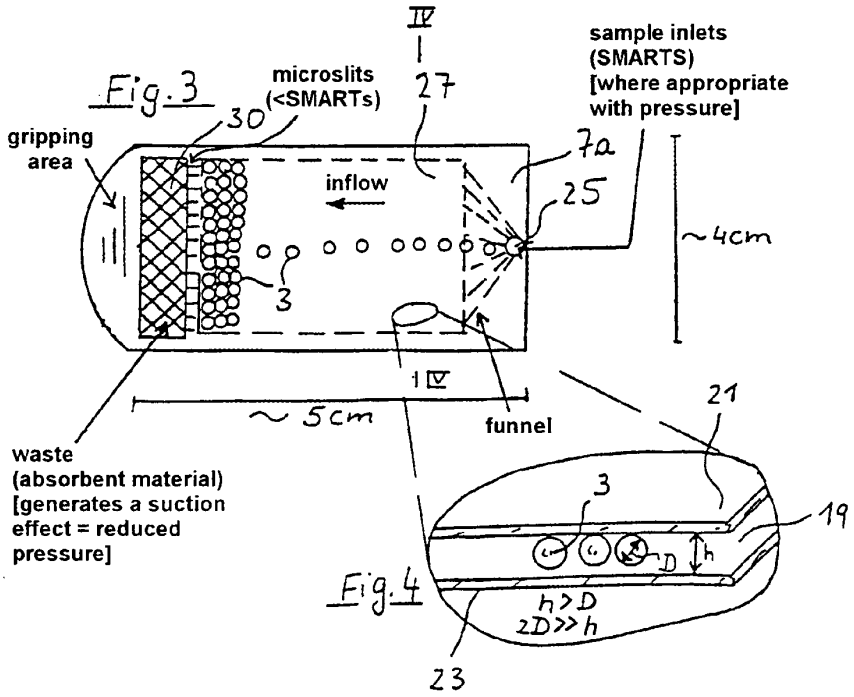
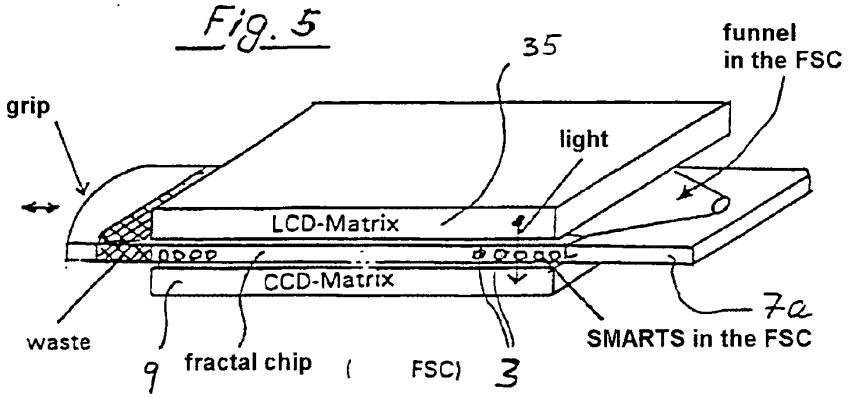


Fig. 4

Fig. 5


35
funnel
in the FSC
grip
light
LCD-Matrix
CCD-Matrix
7a
waste
9
fractal chip
FSC)
3
SMARTS in the FSC

Espacenet

Bibliographic data: CN1635146 (A) — 2005-07-06

One-dimensional biological chip and application in gene, protein expression analysis

No documents available for this priority number.

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Disclosed is an one-dimensional biochip belonging to a serial analytic technique for micro -total analysis systems. The one-dimensional biochip is provided with a plurality of cellules on the micro-separation channel of micro flow control chip, microparticles modified by different biomolecules on surface are arranged in different cellules; in the gene or protein analysis, when the sample flows through the microchannel with a plurality of cellules, the microparticles can specifically identify and capture multiple target molecules, then a reagent with fluorescence labels can be introduced, and finally the microparticles will specifically combine with the fluorescence labels on surface to be detected by fluorescent imaging.; The one-dimensional biochip provided by the invention not only has advantages of micro flow control technology and array analysis, but also improves the detection sensitivity and identification capability of target molecules, and provides an effective research measure for the gene and protein expression analysis at single cell level, and tumor research and drug screening.

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[54] 发明名称 一维生物芯片及其在基因、蛋白表达分析中的应用

[57] 摘要

本发明涉及微全分析系统的串行分析技术。一维生物芯片是在微流控芯片微分离通道上设置多个小室，不同的小室放置表面修饰了不同生物分子的微颗粒；在进行基因或蛋白分析时，当样品流经设置了多个小室的微通道，微颗粒特异性地识别和捕获多种目标分子，然后引入带荧光标记的试剂，微颗粒表面最终特异性地结合上荧光标记物，再用荧光成像检测。本发明设计的一维生物芯片不仅具微流控技术与阵列分析的优点，而且提高了检测灵敏度和目标分子特异性识别能力，为实现单细胞水平上的基因、蛋白表达分析，为肿瘤研究和药物筛选提供一个强有力的研究手段。

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1、一种一维生物芯片，包括聚二甲基硅氧烷片基和玻片片基，其特征在于阳模板上用聚二甲基硅氧烷浇注的一维生物芯片 PDMS 片基中的微通道（2）的两端分别与储液池（1）、（6）相通，微通道（2）上设置了若干个互相连通的小室（3），小室（3）内放置表面可以修饰各种相同或不相同生物分子的微颗粒（5）。

2、按权利要求书 1 所述的一种一维生物芯片，其特征在于小室（3）内放置的可以修饰各种生物分子的微颗粒（5）可以是硅颗粒，也可以是聚苯乙烯类有机聚合物颗粒。

3、一种如权利要求 1 所述的一维生物芯片在基因、蛋白表达分析中的应用，其特征在于该一维生物芯片用于基因、蛋白表达的分析方法包括微颗粒中的硅颗粒用碱活化或氨基化、羧基化处理，微颗粒在修饰前用生物素与亲和素或亲和素与戊二醛或单一的戊二醛或溴化氰进行预处理，然后加入各种已知的生物分子，以及将修饰了不同生物分子的微颗粒逐个移入微通道的小室后，用洁净的玻片粘合封装，其特征在于：

（1）、蛋白的表达分析为：按待分析蛋白要求，选取已知的一维蛋白芯片，取微量的待分析的目标分子置于芯片一侧储液池（1）或（6）中，以平均 20mm/min 的压力驱动或 300v/cm 的电驱动方式，使样品流经微通道（2）进入小室（3），经 30～10 分钟后，用二次水冲洗储液池和微通道中的多余样品溶液，然后以同样条件的压力驱动或电驱动方式将待分析目标分子的一抗兔抗多克隆抗体与荧光标记的二抗山羊抗兔 IgG，以 1：100 比例的抗体稀释液稀释后依次由储液池进入微通道与小室中的微颗粒作用，20 分钟后用二次水洗净储液池和微通道，反应后的芯片用荧光成像检测；

（2）、基因的表达分析为：按待分析基因要求，选取已知的一维基因芯片，取微量待分析的靶 DNA 的 TM 杂交缓冲液置于芯片的一侧储液池（1）或（6）中，以平均 20mm/min 的压力驱动方式使样品溶液流经微通道（2），进入小室（3），20 分钟后用二次水冲洗储液池和微通道，洗去多余样品溶液，以同样条件的压力驱动方式将荧光标记的报告探针的 Mg^{2+}浓度为 200mM 的 10mM TM 杂交缓冲液流经微通道，与小室中的靶 DNA 杂交，20 分钟后用二次水洗净储液池和微通道，将反应后的芯片用荧光成像检测。

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一维生物芯片及其在基因、蛋白表达分析中的应用

技术领域：

本发明涉及微全分析系统领域，具体涉及一种基于微流控的基因或蛋白串性分析技术。

背景技术

微全分析系统（μ-TAS，又称芯片实验室，Lab-on-chip）现已成为当今生命科学研究领域发展最快的重要前沿之一。具备微型化、集成化、多功能以及高通量等特性的微全分析系统使得系统研究一个生物体系的基因表达及蛋白表达成为可能，并将革命性地改变生物医学检验与疾病诊断。目前国内外芯片技术大致朝着两个方向发展：一类是微流控芯片，主要以分析化学和分析生物化学为基础，以微机电加工技术为依托，以微管道网络为结构特征，是当前微全分析系统发展的重点；一类是基于二维点阵式的、高通量大规模并行分析方法的微阵列芯片，主要以生物技术为基础，以亲和结合技术为核心，以在芯片表面固定一系列高密度的、有序并且可寻址的识别分子阵列为结构特征，是高通量地获取相关生物信息的一种新型技术手段。从目前的文献报道看，这两种芯片技术拥有各自的技术优势和研究领域，虽然发展迅速，却没有实现交叉和融合，一些相关技术的发展仍待跟进。如今，生命科学的发展已经深入到单细胞、亚细胞、单分子这样的层次，实现单细胞水平上的基因、蛋白表达分析已成为生命科学研究的一个重大挑战。这一重大科学目标的实现亟需适合其需求的相关平台技术的突破和发展。因此，发展芯片技术、构建新型芯片技术平台，提高检测灵敏度和目标分子特异性识别能力，实现单细胞水平上的基因、蛋白表达分析，是当前研究者们不懈努力的一个方向。

发明内容

本发明旨在提出和构建一种新型生物芯片，将微流控芯片与基于高通量并行分析方法的阵列芯片原理结合起来，成为基于微流控串行分析技术的一种新型芯片平台技术，以提高检测灵敏度和目标分子特异性识别能力，实现单细胞水平上的基因、蛋白表达谱分析。

本发明是通过以下技术方案实现发明目的的。一维生物芯片，包括聚二甲基硅氧烷片基和玻片片基，在阳模板上用聚二甲基硅氧烷浇注的一维生物芯片 PDMS 片基中的微通道的两端分别与储液池相通，微通道上设置了若干个互相连通的小室，小室内放置表面可以修饰各种相同或不相同生物分子的微颗粒。小室内放置的可以修饰各种生物分子的微颗粒可以是硅颗粒，也可以是聚苯乙烯类有机物聚合的颗粒。一维生物芯片用于基因、

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蛋白表达的分析方法包括微颗粒中的硅颗粒用碱活化或氨基化、羧基化处理，微颗粒在修饰前用生物素与亲和素或亲和素与戊二醛或单一的戊二醛或溴化氰进行预处理，然后加入各种已知的生物分子，以及将修饰了不同生物分子的微颗粒逐个移入微通道的小室后，用洁净的玻片粘合封装。

（1）、蛋白的表达分析为：按待分析蛋白要求，选取已知的一维蛋白芯片，取微量的待分析的目标分子置于芯片一侧储液池中，以平均 20mm/min 的压力驱动或 300v/cm 的电驱动方式，使样品流经微通道进入小室，经 30～10 分钟后，用二次水冲洗储液池和微通道中的多余样品溶液，然后以同样条件的压力驱动或电驱动方式将待分析目标分子的一抗兔抗单或多克隆抗体与荧光标记的二抗山羊抗兔 IgG，以 1：100 比例的抗体稀释液稀释后依次由储液池进入微通道与小室中的微颗粒作用，20 分钟后用二次水洗净储液池和微通道，反应后的芯片用荧光成像检测；

（2）、基因的表达分析为：按待分析基因要求，选取已知的一维基因芯片，取微量待分析的靶 DNA 的 TM 杂交缓冲液置于芯片的一侧储值池中，以平均 20mm/min 的压力驱动方式使样品溶液流经微通道进入小室，20 分钟后用二次水冲洗储液池和微通道，洗去多余样品溶液，以同样条件的压力驱动方式将荧光标记的报告探针的 Mg^{2+}浓度为 200mM 的 10mM TM 杂交缓冲液流经微通道，与小室中的靶 DNA 杂交，20 分钟后用二次水洗净储液池和微通道，反应后的芯片用荧光成像检测。

下面结合附图进一步详述本发明。

附图说明

图 1 为一维生物芯片的结构示意图；

图 2 为 CNE2 细胞中 p53 蛋白的表达结果；

图 3 为不同细胞中 p53 蛋白的表达结果；

图 4 为一维生物芯片检测 p53 蛋白的校正曲线；

图 5 为一维生物芯片用于基因表达谱的研究。

首先制作一维生物芯片的掩膜和阳模板，然后在阳模板上采用聚二甲基硅氧烷（PDMS）浇注的方式制作成一维生物芯片的 PDMS 片基。一维生物芯片 PDMS 片基上的微管道网络设计区别于传统的微流控芯片，在于微分离通道上设置了多个小室，不同的小室放置表面修饰了不同生物分子的微颗粒，修饰的生物分子可以是不同的抗体，也可以是不同的分子探针（如分子信标，三明治探针等），分别构成一维蛋白质芯片和一维基因芯片。采用显微操纵方式将修饰了生物分子的微颗粒放置到微通道的小室里，首先，用一套进口的拉针、烧针和磨针系统制作微吸管，然后将微吸管装在固定在倒置显微镜上

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的显微操纵系统上，在显微条件下，将微颗粒逐个移入微通道的小室，每个小室对应某种已知的、特定的修饰微颗粒，不同的芯片可以进行不同的编码，识别和捕获样品溶液中的多种目标分子。

芯片最后的封装是将放置了修饰颗粒的 PDMS 片基与玻片片基进行粘合。将待分析目标分子和试剂溶液进入芯片微通道可采用压力驱动或电驱动两种方式，当样品流经设置了多个小室的微通道时，微颗粒可以特异性地识别和捕获多种目标分子，在一维生物芯片中，这些目标分子是无须进行电泳分离的，因此也无须考虑电泳分离条件的一系列优化问题。反应后的芯片用荧光成像系统进行检测。

如图 1 所示的一维生物芯片的结构，1、6 是直径为 1～2mm 的圆形储液池，一端作样品池，另一端作废液池，微通道 2 由多个小室 3 构成，小室宽为 80μm，微通道深均为 60μm，卡口 4 宽 20μm。小室中放置的微颗粒 5 为微米级硅颗粒或有机聚合物颗粒（如聚苯乙烯颗粒），直径约 40μm，颗粒表面用实验室方法修饰上特定的生物分子，如抗体，分子探针等。

微颗粒修饰生物分子的方法如下：

微颗粒表面修饰分子信标探针(Molecular beacon，简称 MB)或三明治探针(Sandwich probe)：

二氧化硅颗粒：取直径 40μm 二氧化硅颗粒约 10mg 置于 0.5ml Ep 管中，加入 500μl 0.01M NaOH 活化 20min，用二次水清洗 3 次；活化后的颗粒，加入 80μl 0.1～1.0mg/ml biotin-BSA，置于低温摇床（4℃，>300rpm）摇荡 12～48h，然后用二次水清洗 3 次，这样颗粒上结合了 biotin 分子；加入 80μl 0.1～1.0mg/ml streptavidin（或 avidin），置于低温摇床(4℃，>300rpm)摇荡 1～4h，用二次水清洗 3 次，streptavidin(或 avidin)因与颗粒上的 biotin 分子作用而结合到了颗粒表面；加入 50μl 0.1～1.0 M 的连接了 biotin 的 MB 或三明治探针，置于低温摇床（4℃，>300rpm）摇荡 1～4h， MB 或三明治探针通过 biotin 与 streptavidin（或 avidin）的作用修饰到颗粒表面；用 TM 缓冲液清洗 3 次，之后悬浮于 TM 中备用（4℃）。

氨基化二氧化硅颗粒：取直径 40μm 氨基化二氧化硅颗粒约 10mg 置于 0.5ml Ep 管中，加入 80μl 0.1～1.0mg/ml streptavidin（或 avidin），置于低温摇床（4℃，>300rpm）摇荡 4～24h，用二次水清洗 3 次；加入 100μl 4% 戊二醛，置于低温摇床（4℃，>300rpm）摇荡 1～4h，用二次水清洗 3 次，这样通过戊二醛交联的方法将 streptavidin(或 avidin)结合到了颗粒表面；加入 50μl 0.1～1.0 M 的连接了 biotin 的 MB 或三明治探针，置于低温摇床（4℃，>300rpm）摇荡 1～4h，MB 或三明治探针通过 biotin 与 streptavidin

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（或 avidin）的作用修饰到颗粒表面；用 TM 缓冲液清洗 3 次，之后悬浮于 TM 中备用（4℃）。

有机聚合物颗粒：取直径 40μm 聚苯乙烯颗粒约 10mg 置于 0.5ml Ep 管中，加入 80μl 0.1～1.0mg/ml biotin-BSA，置于低温摇床（4℃，>300rpm）摇荡 12～48h，然后用二次水清洗 3 次，这样颗粒上结合了 biotin 分子；加入 80μl 0.1～1.0mg/ml streptavidin（或 avidin），置于低温摇床（4℃，>300rpm）摇荡 1～4h，用二次水清洗 3 次，streptavidin（或 avidin）因与颗粒上的 biotin 分子作用而结合到了颗粒表面；加入 50μl 0.1～1.0μM 的连接了 biotin 的 MB 或三明治探针，置于低温摇床（4℃，>300rpm）摇荡 1～4h，MB 或三明治探针通过 biotin 与 streptavidin（或 avidin）的作用修饰到颗粒表面；用 TM 缓冲液清洗 3 次，之后悬浮于 TM 中备用（4℃）。

微颗粒表面修饰单克隆抗体：

二氧化硅颗粒：取直径 40μm 二氧化硅颗粒约 10mg 置于 0.5ml Ep 管中，加入 200μl 2M Na_2CO_3 溶液，活化 15～30min，再加入 100μl 1g/ml CNBr 乙腈溶液，继续反应 30min。将反应后的颗粒用冰水充分洗涤 3 次，10mM PBS 缓冲液充分洗涤 3 次。往 Ep 管中加入 100μl 4～100μg/ml 鼠抗单克隆抗体，置于低温摇床（4℃，>300rpm）摇荡 24～48h，这样通过 CNBr 交联的方法将鼠抗单克隆抗体修饰到了颗粒表面。将反应后的颗粒用 10mM PBS 缓冲液清洗 3 次，加入含 0.1%～3% BSA 的 10mM PBS 缓冲液，置于低温摇床（4℃，>300rpm）摇荡 6～24h，将颗粒表面的多余活性位点进行封闭。取出，于 4℃保存备用。

氨基化二氧化硅颗粒：取直径 40μm 氨基化二氧化硅颗粒约 10mg 置于 0.5ml Ep 管中，加入 100μl 4% 戊二醛，置于低温摇床（4℃，>300rpm）摇荡 1～4h，用二次水清洗 3 次；加入 100μl 4～100μg/ml 鼠抗单克隆抗体，置于低温摇床（4℃，>300rpm）摇荡 6～24h，这样通过戊二醛交联的方法将鼠抗单克隆抗体修饰到了颗粒表面。将反应后的颗粒用 10mM PBS 缓冲液清洗 3 次，加入含 0.1%～3% BSA 的 10mM PBS 缓冲液，置于低温摇床（4℃，>300rpm）摇荡 6～24h，将颗粒表面的多余活性位点进行封闭。取出，于 4℃保存备用。

有机聚合物颗粒：取直径 40μm 聚苯乙烯颗粒约 10mg 置于 0.5ml Ep 管中，加入 100μl 4～100μg/ml 鼠抗单克隆抗体，置于低温摇床（4℃，>300rpm）摇荡 12～48h，这样鼠抗单克隆抗体就修饰到了颗粒表面。用 10mM PBS 缓冲液清洗 3 次，加入含 0.1%～3% BSA 的 10mM PBS 缓冲液，置于低温摇床（4℃，>300rpm）摇荡 6～24h，将颗粒表面的多余活性位点进行封闭。取出，于 4℃保存备用。

本发明将微流控芯片与基于高通量并行分析方法的阵列芯片原理结合起来，发展成

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基于微流控串行分析技术的一种新型芯片平台技术，这种芯片设计的特点，兼具有微流控技术与阵列分析的优点，如试样用量极微，自动化程度高，不易污染，高通量等。化学与生物修饰过程中运用了生物纳米技术和分子探针技术，目的是拓展芯片中的化学、生物信号放大技术和信号传感技术，大大提高检测灵敏度和目标分子特异性识别能力，为在“芯片实验室”上实现单细胞水平上的基因、蛋白表达谱分析，为肿瘤研究和药物筛选提供一个强有力的研究手段。

具体实施方式

实施例 1（一维生物芯片的构造及其在细胞蛋白表达谱的研究应用）

有机聚合物颗粒表面修饰单克隆抗体：取直径 40μm 聚苯乙烯颗粒约 10mg 置于 0.5ml Ep 管中，加入 100μl 20μg/ml 鼠抗单克隆抗体，置于低温摇床（4℃，>300rpm）摇荡 24h。用 10mM PBS 缓冲液清洗 3 次，加入含 0.1%～3% BSA 的 10mM PBS 缓冲液，置于低温摇床（4℃，>300rpm）摇荡 12h。取出，于 4℃保存备用。

在已经制作好的阳模板上用聚二甲基硅氧烷（PDMS）浇注一维生物芯片的 PDMS 片基，置于 75° C 烘箱 40 分钟左右，待固化后取出，将 PDMS 片基从阳模板上剥离下来。将 PDMS 片基置于 Leica 倒置显微镜下，在显微条件下，采用显微操纵方式将上述表面修饰一系列相关表达蛋白的鼠抗单克隆抗体的直径为 40μm 聚苯乙稀颗粒放置到 PDMS 片基的微通道的小室里，每个小室对应已知的、特定的修饰微颗粒，用以识别和捕获样品溶液中的多种目标分子。将放置了鼠抗单克隆抗体修饰颗粒的 PDMS 片基与非常洁净的玻片片基进行粘合完成芯片的封装。

取 3μl 从肿瘤细胞中抽提出来的蛋白质样品溶液，置于样品池中，在压力驱动下以平均 20mm/min 的流速从芯片的微通道中流过，与微颗粒发生作用，20 分钟后用二次水冲洗样品池和微通道，洗去多余的样品溶液。然后同样以压力驱动方式分别将目标分子的兔抗多克隆抗体（一抗，Santa Cruz 产品，浓缩液按 1：100 用抗体稀释液稀释，一次消耗仅需 3μl）、Cy3 标记的山羊抗兔 IgG（二抗，Sigma 试剂，浓缩液按 1：100 用抗体稀释液稀释，一次消耗仅需 3μl）依次引入微通道中与微颗粒发生作用，试剂每次在微通道中停留 20 分钟，然后用二次水洗去。最终在微颗粒表面特异性地结合上 Cy3 荧光标记物。通过荧光成像检测，可以获得肿瘤细胞中一系列相关蛋白的表达谱信息。

实施例 2（一维生物芯片检测肿瘤细胞 CNE2 中 p53 蛋白的表达）：

氨基化二氧化硅颗粒表面修饰单克隆抗体：取直径 40μm 氨基化二氧化硅颗粒约 10mg 置于 0.5ml Ep 管中，加入 100μl 4% 戊二醛，置于低温摇床（4℃，>300rpm）摇荡 2h，用二次水清洗 3 次；加入 100μl 20μg/ml 鼠抗 P53 单克隆抗体，置于低温摇床（4℃，

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>300rpm）摇荡 12h。将反应后的颗粒用 10mM PBS 缓冲液清洗 3 次，加入含 0.1%～3% BSA 的 10mM PBS 缓冲液，置于低温摇床（4℃，>300rpm）摇荡 12h。取出，于 4℃保存备用。

在已经制作好的阳模板上用聚二甲基硅氧烷(PDMS)浇注一维生物芯片的 PDMS 片基，置于 75° C 烘箱 40 分钟左右，待固化后取出，将 PDMS 片基从阳模板上剥离下来。将 PDMS 片基置于 Leica 倒置显微镜下，在显微条件下，采用显微操纵方式将直径为 40μm 左右表面修饰鼠抗 P53 的氨基化二氧化硅颗粒放置到 PDMS 片基的微通道的小室里，再将放置了鼠抗 p53 修饰颗粒的 PDMS 片基与非常洁净的玻片片基进行粘合完成芯片的封装。

在显微条件下，用微吸管移取 50 个 CNE2 细胞到样品池中，加入 0.1%SDS 溶液将细胞溶膜裂解。样品以电驱动方式引入微通道中，与微颗粒发生作用，电压控制在约 300v/cm，进样时间 10 分钟。然后用二次水冲洗储液池和微通道，洗去多余的样品溶液。样品池换上 3μl 兔抗 p53 多克隆抗体（一抗，Santa Cruz 产品，浓缩液按 1：100 用抗体稀释液稀释），在 300v/cm 电压控制下一抗进入微通道中与微颗粒发生作用，10 分钟后用二次水冲洗干净储液池和微通道。最后样品池换上 3μl FITC 标记的羊抗兔 IgG（二抗，Santa Cruz 产品，浓缩液按 1：100 用抗体稀释液稀释），在 300v/cm 电压控制下二抗进入微通道中与微颗粒发生作用，10 分钟后用二次水冲洗干净储液池和微通道。这样在微颗粒表面最终特异性地结合上 FITC 荧光标记物。另用一块芯片做对照实验，除了不加样品外，其余步骤同上一致。将反应后的芯片置于荧光倒置显微镜增强型 CCD（ICCD）成像系统下，对微颗粒成像，可获得高灵敏的荧光图像，如图 2 所示。结果表明，加样后用一维生物芯片灵敏地检测出了 CNE2 细胞的 p53 蛋白，芯片中的鼠抗 p53 修饰颗粒因特异性地识别 CNE2 细胞的 p53 蛋白而发出荧光，检测的细胞个数为 50 个，而未加样的一维生物芯片检测结果呈阴性，此结果与传统的 western blotting 的实验结果一致。

实施例 3（一维生物芯片检测几种细胞中 p53 蛋白的表达）

二氧化硅颗粒表面修饰单克隆抗体：取直径 40μm 二氧化硅颗粒约 10mg 置于 0.5ml Ep 管中，加入 200μl 2M Na_2CO_3 溶液，活化 15～30min，再加入 100μl 1g/ml CNBr 乙腈溶液，继续反应 30min。将反应后的颗粒用冰水充分洗涤 3 次，10mM PBS 缓冲液充分洗涤 3 次。往 Ep 管中加入 100μl 20μg/ml 鼠抗 P53 单克隆抗体，置于低温摇床（4℃，>300rpm）摇荡 24h。将反应后的颗粒用 10mM PBS 缓冲液清洗 3 次，加入含 0.1%～3% BSA 的 10mM PBS 缓冲液，置于低温摇床（4℃，>300rpm）摇荡 12h。取出，于 4℃保存备用。

在已经制作好的阳模板上用聚二甲基硅氧烷(PDMS)浇注一维生物芯片的 PDMS 片基，置于 75° C 烘箱 40 分钟左右，待固化后取出，将 PDMS 片基从阳模板上剥离下来。将 PDMS 片基置于 Leica 倒置显微镜下，在显微条件下，采用显微操纵方式将直径为 40μm 左右表

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面修饰鼠抗 p53 单克隆抗体的二氧硅颗粒放置到 PDMS 片基的微通道的小室里，再将放置了鼠抗 p53 修饰颗粒的 PDMS 片基与非常洁净的玻片片基进行粘合完成芯片的封装。

对 4 种培养的细胞 CNE2、A549、敲除了 p53 基因的 CHO（以 CHO- p53 表示）以及正常成纤维细胞(Fibroblast)分别进行蛋白质的抽提，分别取 3μl 从 4 种细胞中抽提出来的蛋白质样品溶液，各置于 4 块芯片的样品池中，在压力驱动下以平均 20mm/min 的流速从芯片的微通道中流过，与微颗粒发生作用，20 分钟后用二次水冲洗样品池和微通道，洗去多余的样品溶液。然后同样以压力驱动方式分别将兔抗 p53 多克隆抗体（一抗，Santa Cruz 产品，浓缩液按 1：100 用抗体稀释液稀释，一次消耗 3μl）、Cy3 标记的山羊抗兔 IgG（二抗，Sigma 试剂，浓缩液按 1：100 用抗体稀释液稀释，一次消耗 3μl）依次引入微通道中与微颗粒发生作用，试剂每次在微通道中停留 20 分钟，然后用二次水洗去。最终在微颗粒表面特异性地结合上 Cy3 荧光标记物。将反应后的芯片置于荧光倒置显微镜 CCD 成像系统下，对微颗粒成像，并用荧光图像分析软件进行分析，可获得如图 3 所示的不同细胞中 p53 蛋白的表达情况。从图 3 可以看出，敲除了 p53 基因的 CHO 细胞，p53 蛋白的表达呈阴性，结果与理论相符，而正常成纤维细胞(Fibroblast)和肿瘤细胞 CNE2、A549 均有 p53 蛋白的表达。这些结果与传统的 western blotting 的实验结果一致。

配制一系列不同浓度的 p53 蛋白的标准溶液（浓度单位是 nM）：0.1；0.5；1.0；5.0；10；50；100。按同上的方法将不同浓度的 p53 蛋白的标准溶液用压力驱动的方式分别引入芯片中进行检测，可获得如图 4 所示的一维生物芯片检测 p53 蛋白的校正曲线。图 4 表明，实施例 3 在 0.1～10nM 的 p53 蛋白的检测范围内具有很大的响应斜率，灵敏度高。以 3 倍信噪比作为信号检出限，检测下限达到了 0.05nM。

实施例 4（一维生物芯片应用于基因表达谱研究）

二氧化硅颗粒表面修饰三明治探针（Sandwich probe）：取直径 40μm 二氧化硅颗粒约 10mg 置于 0.5ml Ep 管中，加入 500μl 0.01M NaOH 活化 20min，用二次水清洗 3 次；活化后的颗粒，加入 80μl 1mg/ml biotin-BSA，置于低温摇床（4℃，>300rpm）摇荡 24h，然后用二次水清洗 3 次；加入 80μl 1mg/ml streptavidin（或 avidin），置于低温摇床（4℃，>300rpm）摇荡 2h，用二次水清洗 3 次；加入 50μl 1μM 的连接了 biotin 的三明治探针，置于低温摇床（4℃，>300rpm）摇荡 2h，用 TM 缓冲液清洗 3 次，之后悬浮于 TM 中备用（4℃）。

在已经制作好的阳模板上用聚二甲基硅氧烷(PDMS)浇注一维生物芯片的 PDMS 片基，置于 75° C 烘箱 40 分钟左右，待固化后取出，将 PDMS 片基从阳模板上剥离下来。将 PDMS 片基置于 Leica 倒置显微镜下，在显微条件下，采用显微操纵方式将直径为 40μm 左右的

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二氧化硅颗粒放置到 PDMS 片基的微通道的小室里，二氧化硅颗粒为三种不同修饰颗粒，表面修饰三明治 DNA 捕获探针分别是：p53（5’-ACACGCACCTCAAAGCAAT-Biotin-3’）、p21（5’-CCATCAATGACCAC-biotin-3’）和 nm23（5’-ATGAAGGTACGCTC- biotin-3’）。将放置了修饰颗粒的 PDMS 片基与非常洁净的玻片片基进行粘合完成芯片的封装。

取 3μl 样品溶液置于样品池中，样品溶液是含 0.1nM p53（5’-GCTTTGAGG TGCGTGTTTGTGCCTGTCCTGG-3’）、p21（5’- GTGGTCATTGATGGGGAG ACGTGCCTGT- 3’）和 nm23（5’- GAGCGTACCTTCATTGCGATCAAACCAG -3’）靶 DNA 的 TM 杂交缓冲液（10mM），杂交缓冲液中 Mg^{2+}浓度为 200mM。在压力驱动下样品溶液以平均 20mm/min 的流速从芯片的微通道中流过，与微颗粒上的 DNA 捕获探针进行杂交，20 分钟后用二次水冲洗样品池和微通道，洗去多余的样品溶液。样品池换上 3μl 四甲基罗丹明(TAMRA)标记的 0.1nM p53、p21 和 nm23 荧光报告探针： p53（5’-(TAMRA)- AACCAGGACAGGCACAA-3’）、p21（5’-TAMRA- ACAGGCACGTCTCC -3’）和 nm23（5’-TAMRA- CTGGTTTGATCGCA-3’）（10mM 的 TM 杂交缓冲液，Mg^{2+}浓度为 200mM），在压力驱动下进入微通道中与微颗粒上结合的靶 DNA 进行杂交，20 分钟后用二次水冲洗干净储液池和微通道。这样在微颗粒表面最终特异性地结合上 TAMRA 荧光标记物。另用一块芯片做对照实验，除了不加样品外，其余步骤同上一致。将反应后的芯片置于荧光倒置显微镜 ICCD 成像系统下，对微颗粒成像，并用荧光图像分析软件进行分析，可获得如图 5 所示的三种基因的表达结果。结果表明，用一维生物芯片灵敏地检测出了三种目标 DNA，芯片中三种修饰颗粒上的 DNA 捕获探针和荧光报告探针因特异性地识别 p53、p21 和 nm23 靶 DNA 使修饰颗粒发出荧光，与各自的浓度对应，荧光强度基本一致。在本实施例中获得的检测下限达到了 0.01nM。

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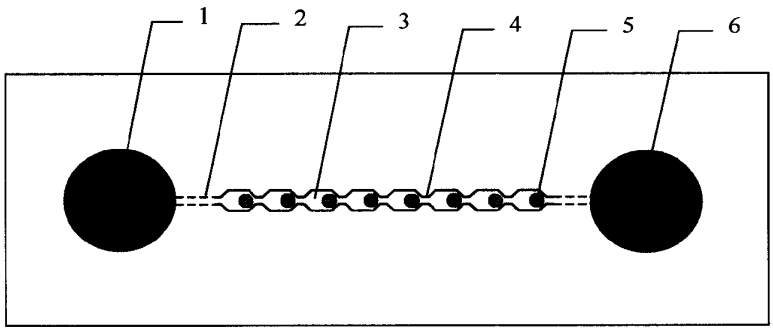


图1

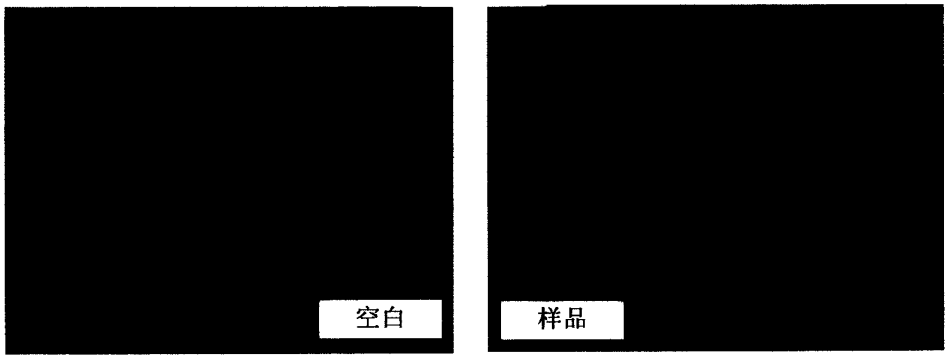


图2

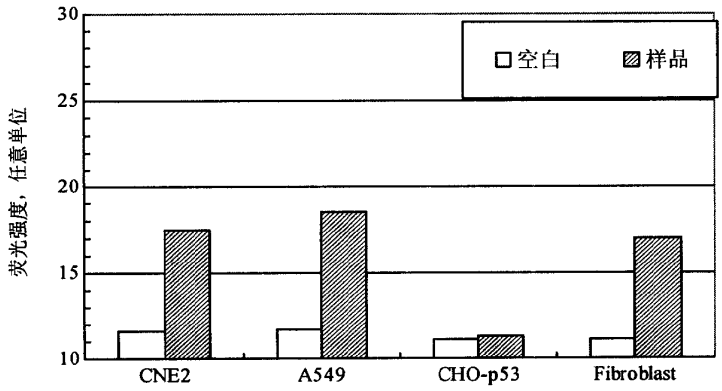


图3

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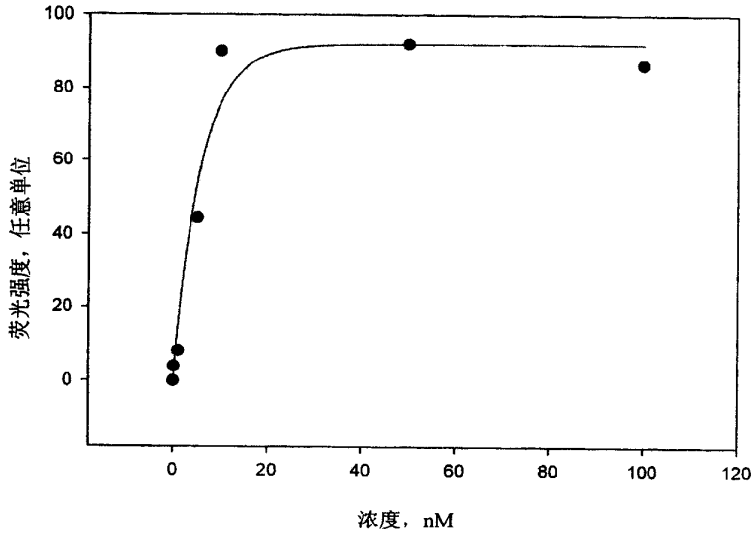


图4

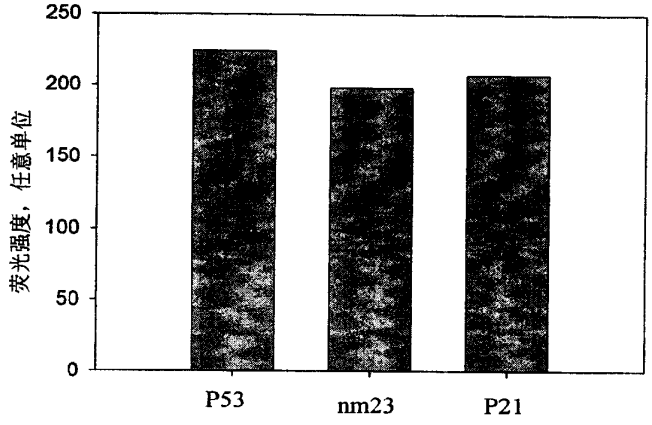


图 5

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Espacenet

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Method and assembly for DNA isolation with dry reagents

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Applicant(s): SIEMENS AG [DE] ± (SIEMENS AG)

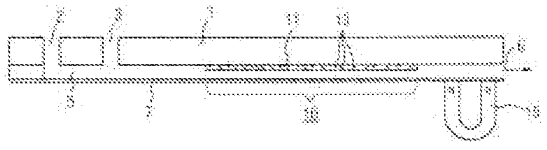
Classification: - international: ***B01L3/00; C12N15/10; C12Q1/68;*** *B01L7/00*
- cooperative: **B01L3/502761; C12N15/1013; C12Q1/6806;** B01J2219/00286; B01J2219/00459; B01J2219/005; B01J2219/00648; B01J2219/00722; B01L2200/0668; B01L2200/082; B01L2200/10; B01L2200/16; B01L2300/0816; B01L2400/0406; B01L2400/0487; B01L7/52 more

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Abstract of CN1950520 (B)



The invention relates to a DNA isolation method by removal of constituents, interfering with a subsequent PCR. According to the method, all substances and method steps are fully integrated into a closed unit (cartridge) for single use, which allows entry of a DNA-containing sample and DNA-binding substrates are used for isolating the released DNA. In particular, when the method is applied to the DNA isolation from the whole blood by disruption of white blood cells, the reagents, required for carrying out the cell disruption and other reactions, are stored in a form which is stable at room temperature. For disrupting the white blood cells and for DNA isolation, a dry stored lysis reagent is dissolved in an aqueous solution and brought into contact with the white blood cells.; The corresponding assembly comprises a unit, for housing

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DNA-containing biological containers and/or reagents, whereby at least one microchannel (5) is provided to contain reagents, whereby the reagent is present in the microchannel as a dry mixture with a negligible vapor pressure, which forms a stable substance at room temperature.

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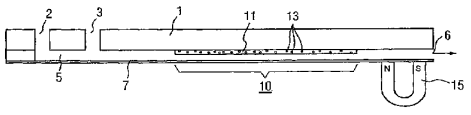
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权利要求书 3 页 说明书 5 页 附图 3 页

[54] 发明名称

用于试剂分离 DNA 的方法和装置

[57] 摘要

本发明涉及一种在去除后续 PCR 的干扰成分情况下分离 DNA 的方法，其具有下列措施：所有物质和方法步骤完全整合在一个密闭的一次性使用的单元(所谓的药筒)中，该单元允许加入含 DNA 的样品；为分离该释出的 DNA 使用结合 DNA 的基底。特别是在应用通过白细胞的细胞分解从全血中分离 DNA 的方法时，用于实施细胞分解和其它反应所需的试剂以室温下稳定的形式储存，并且为分解白细胞和分离 DNA，使干储存的分解－试剂溶于水溶液中，并与白细胞接触。在相关的装置中包括用于接受含 DNA 的生物容器和/或试剂的单元，因而至少提供一个微通道(5)以容纳试剂，而在微通道中试剂以具有可忽略蒸汽压的干混合物存在，其在室温下形成稳定物质。



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1. 一种在去除后续 PCR 的干扰成分情况下分离 DNA 的方法，其具有下列措施:

·所有物质和方法步骤完全整合在一个一次性使用的单元(所谓的药筒)中，该单元允许加入含 DNA 的样品;

· 应用干试剂释放 DNA;

· 应用结合 DNA 的基底以分离该释出的 DNA;

· 收集分离的 DNA，并将其输送到后续的 PCR 的位置。

2. 权利要求 1 的方法，其中分解含 DNA 的生物容器，特别是细胞、细菌或病毒，为实施分解和其它的方法步骤所需的试剂以在室温下稳定的形式储存在该单元中，其特征在于，为分解含 DNA 的生物容器，使干储存的分解-试剂与生物容器，特别是细胞、细菌或病毒相接触，并结合所释出的 DNA 并将其输送到 PCR 的位置。

3. 权利要求 2 的方法，其特征在于，应用白细胞作为含 DNA 的容器。

4. 权利要求 3 的方法，其特征在于，该干储存的和储存稳定的分解-试剂借助于水而和白细胞接触。

5. 权利要求 1 和 2 的方法，其特征在于，样品的稀释、干试剂的溶解或悬浮、生物容器的分解和 DNA 的结合是在一个单一的方法步骤中完成。

6. 权利要求 1 的方法，其特征在于，应用所谓的磁-珠作为结合 DNA 的基底。

7. 权利要求 1 的方法，其特征在于，应用一种在细胞分解通道出口处的不移动的含 DNA-捕集物质的水凝胶作为结合 DNA 的基底。

8. 权利要求 6 的方法，其特征在于，该释出的 DNA 通过磁-珠结合，并输送到收集位置或反应位置。

9. 权利要求 6 的方法，其特征在于，该磁-珠通过应用磁场收集。

10. 权利要求 9 的方法，其特征在于，流动的磁-珠通过位置固定的磁场收集。

11. 权利要求 1 的方法，其特征在于，在反应位置该 PCR 是通过与干储存的水可溶的试剂的热循环来进行。

12. 权利要求 1 的方法，其特征在于，稀释加入到单元中的血样品，并

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通过试剂通道泵入适于 PCR 的反应通道。

13. 权利要求 12 的方法，其特征在于，经 DNA-分离后，残余的血、血成分和试剂废物保留在单元中，以致不可能发生与有害健康的物质相接触。

14. 上述权利要求之一的方法，其特征在于，应用一次性产品作为实施反应的单元，该单元可以小量并低成本批量制备。

15. 一种实施权利要求 1 或权利要求 2-14 之一的装置，其具有接受样品和/或试剂的单元，其中有至少一个微通道(5)以接受试剂，在微通道(5)中的试剂以具有可忽略蒸汽压的干混合物存在，其在室温下形成稳定物质。

16. 权利要求 15 的装置，其特征在于，通过加入成膜剂该干试剂以层(11)结合。

17. 权利要求 15 的装置，其特征在于，该含 DNA 的生物容器特别是细胞、细菌、病毒。

18. 权利要求 17 的装置，其特征在于，为实施生物容器的分解，在微通道(5)中的具有可忽略蒸汽压的干物质是分解-试剂，其具有适于白细胞的特异性质。

19. 权利要求 18 的装置，其特征在于，适于分解-物质的通道(5)通到 PCR-空腔(20)内。

20. 权利要求 19 的装置，其特征在于，在微通道(5)或微空腔(20)中存在具有结合 DNA 的特性的基底。

21. 权利要求 20 的装置，其特征在于，该结合 DNA 的基底是所谓的磁-珠(10)。

22. 权利要求 21 的装置，其特征在于，该分解-试剂(11)和磁-珠(13)包含于一个共同的干基体中。

23. 权利要求 15-22 之一的装置，其特征在于，存在一种用于全血-样品的入口(3)。

24. 权利要求 15-23 之一的装置，其特征在于，存在用于加入水的手段(2)。

25. 权利要求 15-24 之一的装置，其特征在于，存在用于调节开确定的离子强度的干缓冲物质。

26. 权利要求 15-25 之一的装置，其特征在于，存在用于混合血液样品

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和水或缓冲溶液的手段。

27. 权利要求 15-26 之一的装置，其特征在于，存在具有血、血/水混合物或血/缓冲液混合物通流用的以分解试剂/珠试剂涂敷过的微通道(5)或微空腔(20)的手段。

28. 权利要求 15-27 之一的装置，其特征在于，存在用于产生磁场以在 PCR-空腔(20)中固定 DNA/磁-珠-复合体的手段。

29. 权利要求 15-28 之一的装置，其特征在于，存在用于热循环的手段。

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用干试剂分离 DNA 的方法和装置

本发明涉及一种用干试剂分离 DNA 的方法。此外本发明还涉及一种用于实施该方法的相关装置。

在核酸分析要回答如全血的白细胞中人类基因的问题时,在样品制备步骤中首先必需破裂细胞，并接着分离释放出的 DNA。这时必需去除能阻碍后续 PCR 的血液成分如血红蛋白、免疫球蛋白和乳铁蛋白。

在实验室这种操作步骤是按熟知的现有技术进行的。除其它方法外，细胞还可用碱性溶液(NaOH)破裂(所谓溶胞)，并接着将 DNA 结合到涂有硅胶的磁-珠上。通过施加磁场固定载有 DNA 的磁-珠并洗涤之。其后如此分离出的 DNA 可以有珠或无珠地用于 PCR (“聚合酶链反应”)。

在现有技术中，该结合 DNA 的磁-珠以悬浮体混入细胞-溶解-缓冲液中。所有的操作步骤例如均在 1.5 ml-反应容器 (所谓的“Eppendorf”-容器，即微量离心管) 中进行。将如 10 μl 的全血加到给定体积的珠-悬浮物中(例如，200μl)。由此血细胞分解并释出 DNA。该磁-珠结合 DNA 并形成一种 DNA/珠-复合体。接着该 DNA/珠-复合体通过磁场固定在“Eppendorf 管”的容器壁上，以致可进行洗涤步骤以去除阻碍 PCR 的物质。最后可进行 PCR。

实施上述方法是依赖于现有的实验室设备如“ Eppendorf”-容量(管)、管-支承-设备包括磁体、移液管-设备、适于试剂的冷却容器，并必需由经培训过的人员在遵守安全规定(传染危险、废物处置等)下进行。部分对健康有危害的物质(如 NaOH)的各种容积和高准确的计量必需通过移液管进行。这些操作步骤也是很耗时的。

由 US 2002/0022261 A1 已知一种适于微型遗传分析的体系和相关的操作方法，其中应用一种具有至少一个入口和一个多孔性输送道的药筒，该药筒应有结合 DNA 的特性。这时进行细胞分解，为此需要时在容器壁上提供有试剂。此外，该壁的结构区可配置有结合 DNA 的材料，需要时还有磁珠。总之其中给出了一系列用于进行遗传分析测量的各种建议，但仍未实现连续的方法。此外，由 EP 0723549 B1 已知一种用于分离 DNA 的混合物，

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该混合物特别是含硅胶、玻璃细粒和至少一种 kaotropisches 盐的混合物。

基于最近现有技术，本发明的目的在于，无例外地在整合的微型药筒中实施 DNA-分离，并提供一种相关的装置。

本发明的目的是通过权利要求 1 的措施实现的。相关设备示于权利要求 13。该方法的其他方面和相关装置的扩展方案列于从属权利要求中。

在本发明范围内，权利要求 2 包括具有含 DNA 的生物容器如细胞、细菌或病毒的分解方法。由此可在特别有利的和实用的实践中以 DNA-信息检测全血样品。

本发明除了基于上述现有技术外还基于 WO 02/072262 A1 中的“分析设备”附图。其中描述了干式储存在“嵌片”的微通道或微空腔中的在室温下稳定的试剂，该试剂在使用前加水而变成溶液。该干试剂按预定剂量提供，以在溶解后产生定量的分析介质。与此相反，在本发明中涉及一种生物容器的细胞分解，例如从全血-样品中分离 DNA 以进行其后的 PCR 和/或分析。由样品中分离出的 DNA 以合适的方法提供。

与至今应用的实验室方法相比，用本发明方法具有下列优点：

· 所有物质和方法完全整合在一个密封的一次性使用的药筒中；

· 确保该试剂以安全、对健康无害及在室温下可稳定储存的方式储存；

· 除注入血液样品外，无需手工操作步骤；

· 与有害健康的物质即在药筒中的血液和试剂-废物无直接接触；该药筒是小量并可低成本批量生产。

本发明的装置具有下列特征：

· 存在至少一个微通道或微空腔。

· 在微通道或微空腔中，该溶解-介质特别是在有适于 DNA 的基底的成膜剂包体下安置在通道的壁上。

引入到微通道或微空腔中的溶解试剂具有下列特性：

· 适于白细胞和/或其它细胞、细菌、病毒的溶解特性；

· 具有可忽略的蒸汽压的固体或液体；

· 在室温下稳定；

· 在微通道壁上或微空腔壁上的优良粘附性。

本发明中重要的是，在连贯的方法链中释放出含于样品中的 DNA，收集分离的 DNA 并以合适的方法送到 PCR 或测定的地点。

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本发明的其它详情和优点由下面按实施例的附图描述并结合权利要求给出。

附图简介

图 1 示出分析设备(药筒)的俯视图

图 2-图 6 各为图 1 中沿线 II-II 的截面图，用于说明由全血中的 DNA-分离，其中图 2-6 各包括不同的功能阶段。

下面的描述是阐明含 DNA 的样品。这类样品可以是在液体中的 DNA 的溶液，但是也可以是含 DNA 的生物容器的悬浮物。在本文中例如细胞、细菌或病毒均视为生物容器。为释放 DNA 必需分解该生物容器。如果相应于人类基因目的使用全血样品，则需对 DNA 位于其中的白细胞进行细胞分解。

图 1 中示出分析设备图，随后也可形成“药筒”和作为集中的或分散的测量设备。特别是形成嵌片(“嵌片实验室”)类的分析设备，该嵌片含有用于处理和分析测量样品的所有介质，本设备按规定操作所需要的相关控制设备/读出设备在本文中未给出。

详述之，该设备由塑料制成的嵌片体 1 组成，其具有流体的入口和出口。特别是有引入水的入口 2(孔)和引入测量样品如血液的入口 3(孔)。重要的是通过合适的液流设备 2–10，特别是不同几何形状的通道和空腔以传输和汇集测量样品和溶液介质。

详述之，该液流-设备除上述水入口和样品入口 2，3 外还包括两个试剂通道 4，4’以及有出口 6 的流道 5、废物收集通道 8 和其它液流通道 9。用 10 表示适于样品处理的流道 5 中的集中混合区。

该样品在混合段 10 中经处理并经分离含于测量样品中的 DNA 后，收集释放出的 DNA，并送入 PCR-腔 20 以进行 PCR(聚合酶链反应)。

在图 1 装置中特别是用干试剂进行 PCR 的方法详述于本申请人的具有同样申请优先权的同时申请并名称为“借助用干试剂的 PCR 的扩增 DNA 的方法和装置”中。

该嵌片 1(“药筒”)除含用于 PCR 的介质外,还包含具有入口 32 和出口 31 的探测模块 30 以及相关的用于传感器信号读出的电连接。此外，还有用于接纳探测用的试剂的手段如通道 4 和 4’以及用于接纳废物如血和用过的

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试剂溶液的手段如通道 8 和 9。因此保证了整合性，这样无有害健康的物质向外排出。

由图 2-6 可看出，图 1 的具有水入口 2 和血液入口 3 的塑料嵌片 1 包含穿流通道 5，通过该通道，可将作为冲洗液的水和作为测量样品的例如血或 DNA-溶液或细胞悬浮液/细菌悬浮液/病毒悬浮液引入其它封闭的体系中。经出口 6 可将废物转送到废物-通道 8 或废物空腔中。穿流通道 5 是在塑料嵌片 1 上，其相对面通过胶粘膜 7 所覆盖。在穿流通道 5 的中心区形成混合段 10，其中特别是制备用于从全血中 DNA-分离的测量样品。

从全血中的 DNA-分离是通过白细胞的分解而实现。为此，该细胞用分解-试剂进行化学破裂，并将其中所含的 DNA 释出和收集。特别是利用已知的前述方法即经所谓的磁-珠，它至少暂时结合细胞分解后的 DNA，并通过外磁场浓集。

特别是由图 2 可看出，在塑料嵌片 1 的穿流通道 5 的区域 10 中安置一个有可溶于水的储存稳定的干试剂 11。本文中储存稳定性意指该固体在室温下可储存几个月并还保持对样品起分解作用的特性。

在图 2 和 3 中，该干物质 11 具有在穿流通道壁上的优良粘附性。例如这可通过加入成膜剂来实现。详述之，在混合段 10 中呈大面积地安置由含磁-珠 13 的已知分解-试剂组成的混合物 11。此外，磁铁 15 以图例表示，其表明应用磁性以收集其上结合有 DNA 的磁-珠 13。

由图 3 和图 4 看出，在图 2 的装置中经移液管尖 17 将全血 12 加入到测量体系中。加入样品后，该入口 3 用胶粘膜 18 封闭。

经入口 2 引入水或缓冲溶液。水或缓冲溶液沿混合段与血液样品 12 混合，以同样方式溶解或悬浮包括磁-珠 13 的分解-试剂 11，并发生作用。为此该白细胞的细胞壁首先通过溶降-试剂而破裂，接着将这时释放出的 DNA 结合在磁-珠的表面。

图 4 是通过水/缓冲溶液稀释该血液样品，并同时溶解该分解-试剂，该分解-试剂用于确保白细胞分解。以同样方式还使存在于通道壁上混合物 11 的磁-珠进入或悬浮于溶液中，并经磁-珠 13 结合 DNA。由此下列 4 个重要的方法步骤可在单一过程中实现：

· 样品稀释，

· 试剂溶解/磁珠悬浮，

· 细胞分解，

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· DNA 结合。

最后按图 5 进行说明。这时可收集经磁-珠 13 可受磁性作用的 DNA，而 PCR-阻碍物质由水或缓冲溶液洗去，并经出口 6 以废物排出。

作为上述措施的结果，图 6 中通过磁-珠 13 结合的 DNA 在磁铁的磁极上的浓集。该 DNA 可用于分析，这时首先特别要进行 PCR。

另外，在与磁场组合下作为结合 DNA 的介质可应用移动的即悬浮的磁粒，也可使用不移动的即定位在细胞分解-通道的出口的位置固定的结合 DNA 的介质。在此实施方案中，由细胞释出的 DNA 在离开细胞分解通道时通过溢流越过位置固定的结合 DNA 的介质由液体流中引出，并结合在结合 DNA 的介质上。作为结合 NDA 的介质可使用如有结合 DNA 的捕集分子的水凝胶或类似物质。

通过本发明的方法和相关装置特别可以以一步法实现简单而快速的 DNA-分离，这仅需要最小的微流体介质、试剂和方法步骤。

接着可对经分离的 DNA 进行 PCR。采用 PCR 可将 DNA 浓度提高到可分析可检测值的水平。这时可将 PCR 组合到分析过程中。该分析在相应于图 1 的探测模块 30 中进行，有关这方面这里不再详述。

总之在本发明中实现了下列措施:

· 在微通道或微空腔中引入的物质具有结合 DNA 的特性。为此应用例如结合 DNA 的磁-珠;

· 该分解-试剂和磁-珠特别是一同包含在一个单一的干基体中;

· 有适于全血-样品、细胞悬浮物/细菌悬浮物/病毒悬浮物的入口;

· 有加水的手段。例如可以是连接到外水源上的输入口;

· 需要时存在用于计量加入如调节所确定的离子强度用的盐的手段;

· 需要时存在用于稀释血液样品的手段;

· 需要时存在其它手段，特别是通道和空腔，以用于制备所确定的盐溶液/缓冲溶液以洗涤所结合的 DNA，如 DNA-磁-珠-复合体;

· 优选在药筒外部存在以血或血/水混合物、血/缓冲液混合物通流具有分解试剂/珠-试剂涂敷过的微通过或微空腔的手段;

· 存在产生磁场以例如在微通道的出口处固定 DNA/磁-珠-复合体的手段，该手段优选存在于药筒的外部。

由此确保包括样品制备的整个分析过程是在封闭的体系中完成，该体系是由环境相容的塑料制成的一次性药筒。

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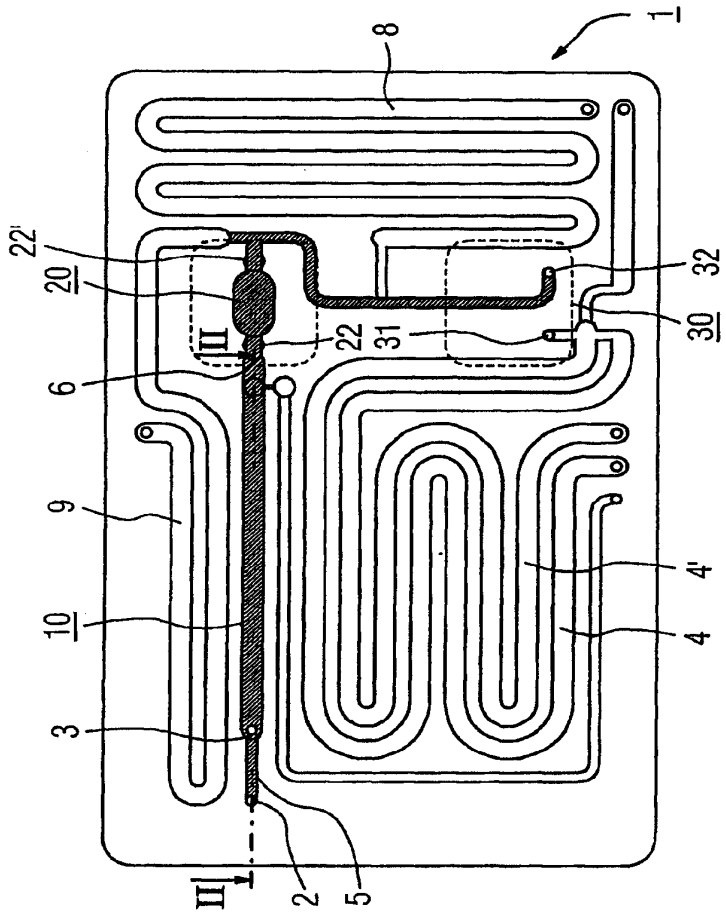


图 1

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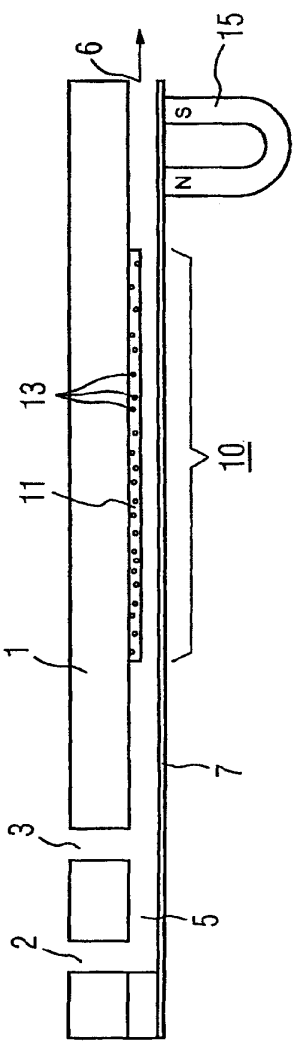


图 2

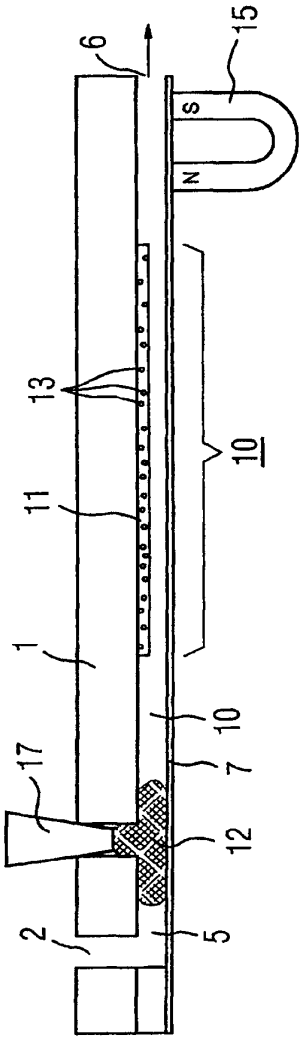


图 3

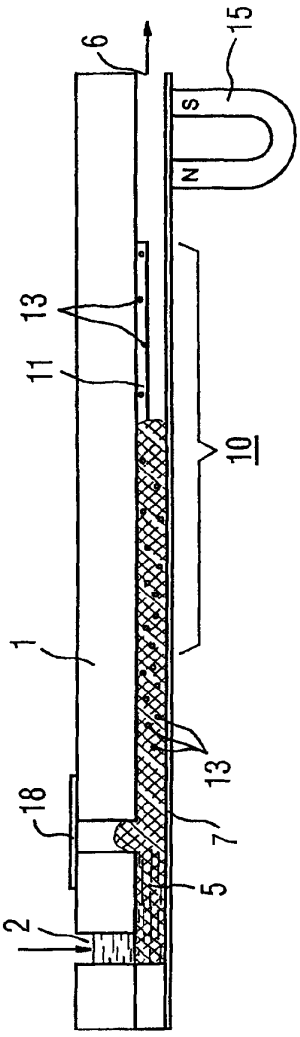


图 4

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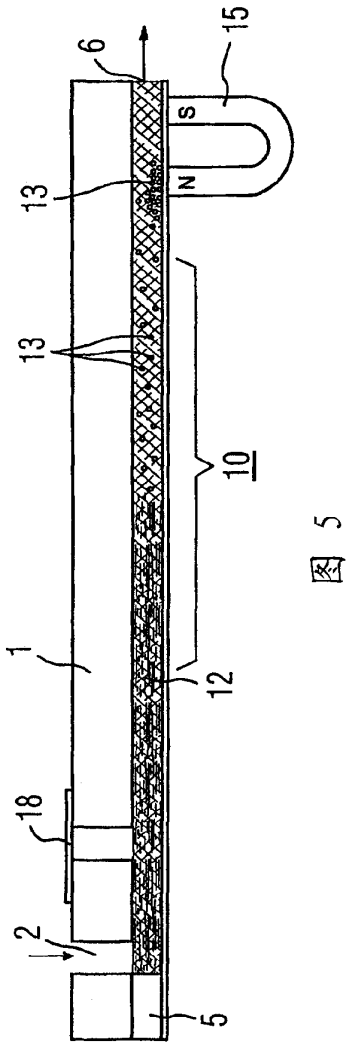


图 5

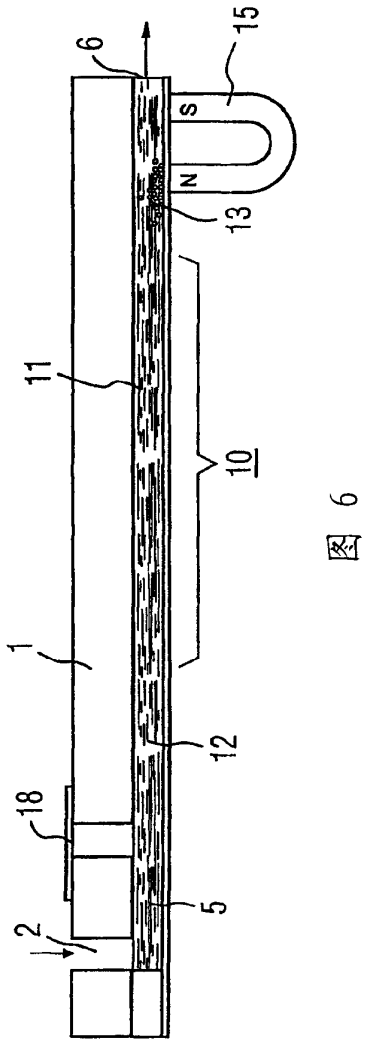


图 6

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KARUBE, Isuc et al: Biosensors for environmental control. In: trends in analytical chemistry, Vol. 14, No. 7 (1995), S. 295-299;

Prüfungsantrag gem. § 44 PatG ist gestellt

(54) Verfahren und Mehrkanalbiosensor zur Mehrkomponentenanalyse von Mischungen und/oder Gemischen

(57) Die Erfindung bezieht sich auf ein Verfahren zur simultanen Mehrkomponentenanalyse von Mischungen und/oder Gemischen und einen Mehrkanalbiosensor für dieses Verfahren.
Der erfindungsgemäße Mehrkanalbiosensor besteht aus einem oder mehreren zumindest teilweise für die einzelnen zu bestimmenden Substanzen nichtselektiven biologischen Sensorelementen, die als biologische Komponente Zellen und/oder Zellorganellen verwenden, und einer Auswerteeinheit, die die von den Sensorelementen zur Verfügung gestellten Daten zeitlich und/oder bezüglich der einzelnen Sensorelemente analysiert.

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Die folgenden Angaben sind den vom Anmelder eingereichten Unterlagen entnommen

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Beschreibung

Die Erfindung bezieht sich auf ein Verfahren zur simultanen Mehrkomponentenanalyse von Mischungen und/oder Gemischen mit einem Mehrkanalbiosensor sowie auch auf den dazu verwendeten Mehrkanalbiosensor. Derartige Verfahren werden verwendet, um Mischungen und/oder Gemische gleichzeitig auf mehrere verschiedene Analyte zu untersuchen. Besondere Bedeutung besitzen diese Verfahren und derartige Mehrkanalbiosensoren im Bereich der Umweltanalytik, beispielsweise Abwasseranalytik, der medizinischen Diagnostik, beispielsweise Blut- und Harnanalytik, in der biologischen und chemischen Prozeßanalytik, beispielsweise Fermentationstechnik, Lebensmittel- oder Kosmetikindustrie, sowie in der Gasanalytik.

Herkömmliche Biosensoren zur Analyse von Mischungen und/oder Gemischen besitzen Sensorelemente, die für die einzelnen zu bestimmenden Analyte möglichst selektiv sind. Dabei bedienen sich die Sensorelemente auf Basis von Zellen unterschiedlicher Verfahren, um ihre Spezifität bzw. Selektivität bezüglich eines einzelnen Analyten zu vergrößern. Dies kann entweder im Bereich der Zufuhr der Mischungen und/oder Gemische zu dem biologischen Sensorelement, beispielsweise durch Ausschluß interferierender Substrate durch selektiv permeable Membranen, geschehen oder, indem die in dem biologischen Sensorelement eingesetzten Mikroorganismen bezüglich eines bestimmten Analyten sensibilisiert werden. Dies kann beispielsweise durch gentechnisch veränderte Mikroorganismen mit speziellen Abbaufähigkeiten für den gewünschten Analyten, durch Induktion von spezifischen Transportsystemen oder Stoffwechselwegen in der Mikroorganismenzelle, durch spezifische Inhibierung von Transportsystemen oder Stoffwechselwegen oder auch durch Bildung eines Hybridsensors mit Hilfe der Kopplung der Mikroorganismen mit Enzymen erreicht werden. Nur durch den Ausschluß interferierender Substrate durch selektiv permeable Membrane ist es möglich, einzelne Analyte selektiv mit einem einzelnen Biosensor zu bestimmen. Bisher war auch keine simultane Bestimmung mehrerer Analyte mit einem einzelnen biologischen Sensorelement möglich.

Das Meßprinzip der meisten mikrobiellen Biosensoren auf der Basis von Zellen beruht darauf, daß die Assimilation von organischen Substraten sowie teilweise der Stofftransport in die Zelle sauerstoffverbrauchende Prozesse sind. Mit Hilfe einer Sauerstoffelektrode als elektrischem Signalwandler läßt sich eine dadurch hervorgerufene Änderung des Sauerstoffpartialdrucks auf einfache und empfindliche Weise elektrochemisch detektieren. Durch das Multirezeptorverhalten der Mikroorganismen ist dieses Signal jedoch bei Vorlage eines Substanzgemisches ohne die obengenannten Verfahren zur Selektivitätssteigerung der Sensorelemente nicht selektiv.

Aufgabe der vorliegenden Erfindung ist es, ein Verfahren zur simultanen Mehrkomponentenanalyse von Mischungen und/oder Gemischen mit einem Mehrkanalbiosensor zur Verfügung zu stellen, durch das gleichzeitig mehrere in den Mischungen und/oder Gemischen vorliegende Analyte quantitativ und/oder qualitativ analysiert werden können. Dabei soll dieses Verfahren auf einfache Art und Weise zu realisieren sein und gleichzeitig eine hohe Nachweisgenauigkeit bezüglich der einzelnen zu bestimmenden Analyte aufweisen. Weiterhin ist es Aufgabe der Erfindung, einen Mehrkanalbiosensor für Gemische und/oder Mischungen zur Verfügung zu stellen, der die für das erfindungsgemäße Verfahren genannten Vorteile besitzt und sich zum Einsatz in dem erfindungsgemäßen Verfahren eignet.

Diese Aufgabe wird durch ein Verfahren und einen Mehrkanalbiosensor nach den Oberbegriffen der unabhängigen Ansprüche in Verbindung mit ihren kennzeichnenden Merkmalen gelöst.

Danach besteht der Mehrkanalbiosensor aus mindestens einem bzw. mehreren biologischen Sensorelementen. Die biologischen Sensorelemente enthalten als biologische Komponente Zellen und/oder Zellorganellen. Die biologische Komponente kann beispielsweise Mikroorganismen, Zellkulturen, Gewebeschnitte oder auch isolierte Mitochondrien oder Chloroplasten enthalten. Diese erzeugen bei Kontakt mit mindestens einem der Analyte das Signal durch Veränderung eines physikalischen und/oder chemischen Parameters.

Erfindungsgemäß werden in dem Mehrkanalbiosensor biologische Sensorelemente verwendet, die zumindest teilweise in unspezifischer und/oder nicht selektiver, jedoch meist gering unterschiedlicher Weise auf die einzelnen zu bestimmenden Analyte des Gemischs und/oder der Mischung ansprechen. Dieses zumindest gering unterschiedliche Ansprechen auf die einzelnen zu bestimmenden Substanzen kann beispielsweise in einem unterschiedlichen zeitlichen Ansprechverhalten und/oder einer zumindest gering unterschiedlichen Empfindlichkeit der einzelnen Sensorelemente bezüglich der einzelnen zu bestimmenden Substanzen bestehen.

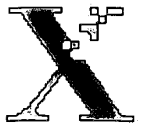
Die gering unterschiedlichen partiellen Empfindlichkeiten der biologischen Sensorelemente bezüglich der gleichzeitig vorliegenden Analyte ergeben sich beispielsweise aus geringen Unterschieden bezüglich metabolischer Prozesse oder auch bezüglich aktiver und/oder passiver Transportprozesse in den biologischen Sensorelementen bzw. in den Zellen oder Zellorganellen, die in den biologischen Sensorelementen verwendet werden.

Bei dem erfindungsgemäßen Verfahren wird nun die Mischung und/oder das Gemisch mit einem derartigen biologischen Sensorelement in Kontakt gebracht und die Signale zeitlich aufgelöst und/oder im Falle mehrerer Sensorelemente des Mehrkanalbiosensors bezüglich der einzelnen Sensorelemente analysiert. So läßt sich das Gesamtsignal des Sensorelements bzw. lassen sich die Gesamtsignale der verschiedenen Sensorelemente analysieren, und es kann simultan auf die Konzentrationen der einzelnen Analyte geschlossen werden.

Weiterhin besitzt der Mehrkanalbiosensor eine Auswerteeinheit, die die Signale der biologischen Sensorelemente zeitlich und/oder im Falle mehrerer Sensorelemente bezüglich der einzelnen Sensorelemente analysiert.

Die erfindungsgemäßen Mehrkanalbiosensoren lassen sich kostengünstig und einfach entwickeln und herstellen. Im Gegensatz zu den bisherigen Verfahren zur Mehrkomponentenanalyse und den herkömmlichen Biosensoren entfällt die Suche nach bzw. Entwicklung von auf die einzelnen Analyte selektiv ansprechenden Sensorelementen. Damit ergeben sich große Vorteile bezüglich der benötigten Entwicklungszeit und der Entwicklungskosten.

Gegenüber herkömmlichen aufgereinigten Nachweissubstanzen, wie beispielsweise aufgereinigten Enzymen oder Enzymgemischen, ist auch die Gewinnung der in den erfindungsgemäßen biologischen Sensorelementen verwendeten Zellen und/oder Zellorganellen



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erheblich leichter und kostengünstiger durchzuführen.

In herkömmlichen Biosensoren, die als biologische Komponente isolierte Enzyme verwenden, sind diese Enzyme aus ihrer natürlichen biologischen Umgebung entfernt und daher nicht physiologischen Bedingungen ausgesetzt. In den erfindungsgemäßen Mehrkanalbiosensoren werden als biologische Komponenten intakte Zellen und/oder Zellorganellen verwendet. Die biologischen Komponenten liegen daher in einem Zustand und in einer Umgebung vor, die dem physiologischen Zustand ähnlich oder gleich ist. Dies führt insbesondere zu einer Verlängerung der Lebensdauer der erfindungsgemäßen Mehrkanalbiosensoren verglichen mit herkömmlichen Biosensoren auf Enzymbasis.

Die Analyse der gewonnenen Mehrkanalbiosensordaten bezüglich ihres zeitlichen Verlaufs und/oder bezüglich der einzelnen Sensorelemente ist sehr rasch und zuverlässig und weist im Ergebnis bezüglich der einzelnen zu bestimmenden Analyte eine hohe Empfindlichkeit auf. Insbesondere können auch organische Analyte in Mischungen und/oder Gemischen bestimmt werden, für die bisher noch kein spezifischer, selektiver Einkanalsensor existiert bzw. für die bisher kein Trennverfahren vorhanden ist.

Vorteilhafte Weiterbildungen des erfindungsgemäßen Verfahrens und des erfindungsgemäßen Mehrkanalbiosensors werden in den abhängigen Ansprüchen gegeben.

Die in den biologischen Sensorelementen eingesetzten Zellen und/oder Zellorganellen können zusätzlich immobilisiert werden. Dadurch ergeben sich Vorteile bezüglich der Reproduzierbarkeit der Signale und der Lebensdauer der Sensorelemente.

Der Mehrkanalbiosensor kann aus mehreren biologischen Sensorelementen bestehen, die unterschiedliche biologische Komponenten besitzen. Diese unterschiedlichen Zellen und/oder Zellorganellen, beispielsweise unterschiedliche Stämme, Arten oder Gattungen von Mikroorganismen, sprechen in zumindest gering unterschiedlichen partiellen Empfindlichkeiten auf die zu bestimmenden Analyte in der Mischung und/oder in dem Gemisch an. Bei Einwirkung derselben Mischung und/oder desselben Gemischs auf die unterschiedlichen Sensorelemente erzeugen diese entsprechend ihrer jeweiligen partiellen Empfindlichkeiten für die einzelnen Analyte unterschiedliche Signale. Die Signale dieses Sensor-Arrays und/oder der Signal-Zeit-Verläufe können dann anschließend, beispielsweise chemometrisch, analysiert werden, so daß sich aus dieser Analyse die Konzentration der einzelnen Analyte quantitativ oder deren Anwesenheit qualitativ ergibt.

In den einzelnen biologischen Sensorelementen können verschiedene Zellen und/oder Zellorganellen als biologische Komponenten eingesetzt werden. Weiterhin kann auch in einem einzelnen Sensorelement die biologische Komponente aus einer bestimmten Mischung unterschiedlicher Zellen und/oder Zellorganellen bestehen.

Das Verfahren bzw. der Mehrkanalbiosensor, der aus einem Array aus biologischen Sensorelementen mit zumindest gering unterschiedlichen Empfindlichkeiten bzw. unterschiedlichem zeitlichen Ansprechverhalten besteht, kann auch mit der Analyse der Signale der Sensorelemente bezüglich ihres zeitlichen Verlaufs verbunden werden. Damit ergeben sich insgesamt drei verschiedene Möglichkeiten, das erfindungsgemäße Verfahren und den erfindungsgemäßen Mehrkanalbiosensor anzuwenden.

Zum einen kann der Mehrkanalbiosensor aus nur einem biologischen Sensorelement bestehen, dessen Signal zu unterschiedlichen Zeitpunkten (Zeitkanälen) analysiert wird. Zum anderen kann der Mehrkanalbiosensor aus mehreren verschiedenen biologischen Sensorelementen bestehen und dessen Signale bezüglich der einzelnen Sensorelemente (Sensorkanäle) analysiert werden. Schließlich können diese beiden Verfahren auch kombiniert werden, so daß sowohl ein zeitlich als auch bezüglich der einzelnen Sensorelemente aufgelöstes und analysiertes Signal zur Verfügung gestellt wird. Alle diese drei Verfahren eignen sich zur Mehrkomponentenanalyse von Mischungen und/oder Gemischen.

Zur Erzeugung des Signals der biologischen Sensorelemente können metabolische Leistungen der biologischen Komponenten, wie beispielsweise Stofftransport oder Verstoffwechselung mit elektrochemischen oder optischen Verfahren gemessen werden.

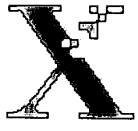
Besonders geeignet ist die Messung des Sauerstoffverbrauchs der Zellen und/oder Zellorganellen, der beispielsweise vom Stofftransport bzw. von der Metabolisierung der zu bestimmenden Analyte abhängt. Besonders einfach kann der Sauerstoffverbrauch bestimmt werden, wenn ein elektrochemisches Verfahren, wie beispielsweise eine Clark-Sauerstoffelektrode, zur Bestimmung der Sauerstoffkonzentration verwendet wird. Sauerstoffelektroden zur Bestimmung des Sauerstoffverbrauchs können auch mit Dünnschichttechnik oder Dickschichttechnik hergestellt werden.

Da bei vielen metabolischen Prozessen eine Protonenfreisetzung oder -aufnahme erfolgt, kann vorteilhafterweise auch eine durch die biologische Komponente bewirkte Änderung der Protonenkonzentration ausgewertet werden. Derartige Änderungen der Protonenkonzentration können auf einfache, rasche und sichere Weise potentiometrisch bestimmt werden.

Selbstverständlich können innerhalb eines biologischen Sensorelemente auch verschiedene Parameter, beispielsweise Sauerstoffverbrauch und Änderung der Protonenkonzentration, zugleich bestimmt und in die Auswertung einbezogen werden. Weiterhin können innerhalb eines Mehrkanalbiosensors mehrere biologische Sensorelemente angeordnet sein, die jeweils unterschiedliche Parameter bestimmen bzw. unterschiedliche Kombinationen von Parametern bestimmen. Auch derartige Anordnungen können in dem erfindungsgemäßen Verfahren bzw. als erfindungsgemäße Mehrkanalbiosensoren für die simultane Bestimmung mehrerer Analyte verwendet werden.

Zur Analyse der zeitlich und/oder bezüglich der einzelnen Sensorelemente aufgelösten Signaldaten eignen sich multivariate Auswerteverfahren, wie beispielsweise Mustererkennungsverfahren, multivariate Kalibrationsmethoden und/oder künstliche neuronale Netzwerke. Dabei kann sich die Auswertung sowohl auf bestimmte Zeitpunkte (Zeitkanäle), auf die einzelnen Sensorelemente (Sensorkanäle) als auch auf abgeleitete Größen der Meßdaten beziehen. Insbesondere können abgeleitete Größen wie Signalhöhe, -anstieg oder -abfall, sowie Fläche unter der Signalkurve oder Halbwertsbreite des Signals analysiert werden.

Durch diese neue Verbindung von Mehrkanalbiosensoren mit multivariaten Auswerteverfahren lassen sich die Signale der einzelnen Kanäle sehr schnell und mit hoher Genauigkeit analysieren und es kann simultan rasch und zuverlässig auf die Konzentrationen der einzelnen Analyte geschlossen werden. Die Auswertung mit multivariaten Verfahren ist eine besonders sichere,



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elegante und schnelle Weise, um mehrdimensionale Sensordaten zu analysieren.

Eine weitere Präzisierung der quantitativen Bestimmung der einzelnen Analyte durch den Mehrkanalbiosensor und das erfindungsgemäße Verfahren kann dadurch erreicht werden, daß der Mehrkanalbiosensor mit Hilfe vorherbestimmter Mischungen und/oder Gemische der zu bestimmenden Analyte, beispielsweise durch ein multivariates Kalibrationsmodell, kalibriert wird.

Im folgenden werden beispielhafte Ausführungsformen des erfindungsgemäßen Verfahrens und des erfindungsgemäßen Mehrkanalbiosensors gegeben. Es zeigen:

Fig. 1 — die Bestimmung von Natriumacetat-Trihydrat; und

Fig. 2 — die Bestimmung von Natriumgluconat.

Es wurden Mischungen aus Natriumacetat-Trihydrat (Konzentrationsbereich 0—10 mg/l) und Natriumgluconat (Konzentrationsbereich 0—40 mg/l) untersucht. Es wurden unterschiedliche Mischungen beider Analyten hergestellt und ihre Signale mit einem amperometrischen mikrobiellen Sensor, der aus einer Clark-Sauerstoffelektrode und immobilisierten Alcaligenes eutrophus KT02 Zellen (entsprechend einem Sensorkanal) bestand, in einem Durchflußmeßsystem aufgenommen. Dabei wurden die Signal-Zeit-Verläufe erfaßt. Als Meßkanäle dienten neunzehn stets gleich gewählte Zeitpunkte (Zeitkanäle), bei denen die immobilisierten Zellen des Sensorelementes gering unterschiedliche partielle Empfindlichkeiten je Analyt aufwiesen. Es muß jedoch betont werden, daß das Sensorelement keineswegs selektiv auf nur einen der Analyten ansprach. Die Unterschiede in den Empfindlichkeiten bezüglich jedes dieser Analyten waren sehr gering.

Es wurden insgesamt zwanzig unterschiedliche Mischungen aus Acetat und Gluconat hergestellt. Sechzehn dieser zwanzig unterschiedlichen Mischungen wurden als Kalibrationsproben benutzt. Die verbliebenen vier Mischungen wurden anschließend als unbekannte Proben analysiert.

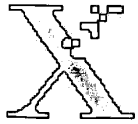
Die gemessenen Signal-Zeit-Verläufe der Kalibrationsproben wurden gegen ihre bekannten Konzentrationen mit einem numerischen Verfahren (Partial Least Squares Regression) angepaßt. Das erhaltene multivariate Kalibrationsmodell wurde anschließend validiert, indem sowohl die Konzentrationen der Kalibrationsproben in einer Rückrechnung als auch die Konzentrationen der Analysenproben als Vorhersage ermittelt wurden. Die Ergebnisse dieser Auswertungen sind in den Fig. 1 und 2 dargestellt. Fig. 1 zeigt einen Vergleich zwischen den wahren Konzentrationen an Natriumacetat-Trihydrat und den mit Hilfe des mikrobiellen Sensorelementes bestimmten Natriumacetat-Trihydrat-Konzentrationen in Gegenwart von Natriumgluconat. Auf der x-Achse ist der wahre Wert, der sich aus der vorgelegten Zusammensetzung der Mischung ergibt, aufgetragen. Auf der y-Achse ist die mit dem Sensorelement und der anschließenden numerischen Auswertung bestimmte Natriumacetat-Trihydrat-Konzentration aufgetragen. Dabei symbolisieren Kreise die als Kalibrationsproben verwendeten Acetatkonzentrationen, während Kreuze die als Analysenproben verwendeten Acetatkonzentrationen bezeichnen. Sämtliche Werte liegen weitgehend auf der Diagonalen des Schaubildes und zeigen so die sehr gute Übereinstimmung zwischen den wahren Werten der Acetatkonzentrationen und den Werten der Acetatkonzentrationen, die mit Hilfe des mikrobiellen Mehrkanalbiosensors bestimmt wurden. Trotz der geringen Unterschiede in den partiellen Empfindlichkeiten für die beiden Analyte gelang also eine sehr genaue, simultane Analyse bezüglich der beiden Analyte in Mischung bereits mit einem einzigen biologischen Sensorelement (Sensorkanal). Dieses Ergebnis zeigt eindrucksvoll die Leistungsfähigkeit des erfindungsgemäßen Verfahrens und des erfindungsgemäßen Mehrkanalanalysators.

Fig. 2 zeigt dieselbe Auswertung für den zweiten in der Mischung vorhandenen Analyten, Natriumgluconat. Auch hier ergibt sich wieder eine hervorragende Übereinstimmung zwischen den wahren Konzentrationswerten und den aus den mikrobiellen Sensordaten berechneten Konzentrationen von Natriumgluconat in Gegenwart von Natriumacetat-Trihydrat.

Auch in einem weiteren, hier nicht dargestellten Fall, bei dem nur fünf Kalibrationsproben und fünfzehn Analysenproben eingesetzt wurden und zugleich fünfzehn Zeitkanäle (Zeitpunkte) verwendet wurden, zeigten sich nur geringe Abweichungen der bestimmten von den vorhergesagten Konzentrationen.

Patentansprüche

1. Verfahren zur Mehrkomponentenanalyse von Mischungen und/oder Gemischen mit biologischen Sensorelementen, die als biologische Komponente Zellen und/oder Zellorganellen enthalten, **dadurch gekennzeichnet,** daß mindestens ein bzw. mehrere biologische Sensorelemente, die auf die zu analysierenden Substanzen der Mischung und/oder des Gemisches zumindest teilweise nichtselektiv, jedoch in unterschiedlicher Weise ansprechen, mit der Mischung oder dem Gemisch in Kontakt gebracht werden und die von den Sensorelementen erzeugten Signale zeitlich und/oder bezüglich der einzelnen Sensorelemente analysiert werden.
2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die Zellen und/oder Zellorganellen immobilisiert sind.
3. Verfahren nach mindestens einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß die verschiedenen biologischen Sensorelemente aus unterschiedlichen biologischen Komponenten und/oder unterschiedlichen Kombinationen mehrerer unterschiedlicher Komponenten bestehen.
4. Verfahren nach mindestens einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß die biologischen Sensorelemente metabolische Leistungen der biologischen Komponente messen.
5. Verfahren nach Anspruch 4, dadurch gekennzeichnet, daß die Messung der metabolischen Leistung durch ein elektrochemisches oder optisches Verfahren erfolgt.
6. Verfahren nach mindestens einem der Ansprüche 4 und 5, dadurch gekennzeichnet, daß die biologischen Sensorelemente den Sauerstoffverbrauch der biologischen Komponente messen.
7. Verfahren nach Anspruch 6, dadurch gekennzeichnet, daß die Messung des Sauerstoffverbrauchs mit einer clark-Elektrode erfolgt.
8. Verfahren nach mindestens einem der Ansprüche 4 bis 7, dadurch gekennzeichnet, daß die biologischen Sensorelemente eine Änderung der Protonenkonzentration messen.
9. Verfahren nach Anspruch 8, dadurch gekennzeichnet, daß die Messung der Änderung der Pro-



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tonenkonzentration potentiometrisch erfolgt.

10. Verfahren nach mindestens einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß die Daten qualitativ mit Hilfe von Mustererkennungsverfahren und/oder quantitativ mit multivariaten Kalibrationsverfahren analysiert werden.

11. Verfahren nach mindestens einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß die Daten mit multivariaten Auswerteverfahren analysiert werden.

12. Verfahren nach Anspruch 11, dadurch gekennzeichnet, daß die Daten mit multivariater linearer Regression, kanonischer Korrelationsanalyse, Partial Least Squares Regression, Hauptkomponentenanalyse, Hauptkomponentenregression, Diskriminanzanalyse und/oder künstlichen neuronalen Netzwerken analysiert werden.

13. Verfahren nach mindestens einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß mit Hilfe vorgelegter Mischungen und/oder Gemische eine Kalibration der gemessenen Signale erfolgt.

14. Mehrkanalbiosensor für Mischungen und/oder Gemische, gekennzeichnet durch mindestens ein bzw. mehrere biologische Sensorelemente, die als biologische Komponente Zellen und/oder Zellorganellen enthalten und die auf die zu analysierenden Substanzen der Mischung und/oder des Gemisches zumindest teilweise nichtselektiv, jedoch in unterschiedlicher Weise ansprechen, sowie durch eine Auswerteeinheit, die die Signale der biologischen Sensorelemente zeitlich und/oder bezüglich der einzelnen Sensorelemente analysiert.

15. Mehrkanalbiosensor nach Anspruch 14, dadurch gekennzeichnet, daß die biologischen Komponenten immobilisiert sind.

16. Mehrkanalbiosensor nach mindestens einem der Ansprüche 14 und 15, dadurch gekennzeichnet, daß verschiedene biologische Sensorelemente unterschiedliche biologische Komponenten und/oder unterschiedliche Kombinationen verschiedener biologischer Komponenten enthalten.

17. Mehrkanalbiosensor nach mindestens einem der Ansprüche 14 bis 16, dadurch gekennzeichnet, daß die biologischen Sensorelemente eine Vorrichtung zur Messung der metabolischen Leistung der biologischen Komponente enthalten.

18. Mehrkanalbiosensor nach Anspruch 17, dadurch gekennzeichnet, daß die Vorrichtung zur Messung der metabolischen Leistung der biologischen Komponente ein elektrochemisches oder optisches Verfahren verwendet.

19. Mehrkanalbiosensor nach mindestens einem der Ansprüche Anspruch 17 und 18, dadurch gekennzeichnet, daß die biologischen Sensorelemente eine Vorrichtung zur Messung des Sauerstoffverbrauchs enthalten.

20. Mehrkanalbiosensor nach Anspruch 19, dadurch gekennzeichnet, daß in der Vorrichtung zur Bestimmung des Sauerstoffverbrauchs eine Clark-Elektrode angeordnet ist.

21. Mehrkanalbiosensor nach Anspruch 19, dadurch gekennzeichnet, daß in der Vorrichtung zur Bestimmung des Sauerstoffverbrauchs eine mit Dünnschichttechnik oder Dickschichttechnik hergestellte Sauerstoffelektrode angeordnet ist.

22. Mehrkanalbiosensor nach mindestens einem der Ansprüche 17 bis 21, dadurch gekennzeichnet, daß die biologischen Sensorelemente eine Vorrichtung zur Messung von Änderungen der Protonenkonzentration enthalten.

23. Mehrkanalbiosensor nach Anspruch 22, dadurch gekennzeichnet, daß die Vorrichtung zur Messung von Änderungen der Protonenkonzentration ein potentiometrisches Verfahren verwendet.

24. Mehrkanalbiosensor nach mindestens einem der Ansprüche 14 bis 23, dadurch gekennzeichnet, daß die Auswerteeinheit Mustererkennungsverfahren und/oder multivariate Kalibrationsverfahren verwendet.

25. Mehrkanalbiosensor nach mindestens einem der Ansprüche 14 bis 24, dadurch gekennzeichnet, daß die Auswerteeinheit multivariate Auswerteverfahren zur Auswertung der Daten verwendet.

26. Mehrkanalbiosensor nach Anspruch 25, dadurch gekennzeichnet, daß die Auswerteeinheit multivariate lineare Regression, kanonische Korrelationsanalyse, Partial Least Squares Regression, Hauptkomponentenanalyse, Hauptkomponentenregression, Diskriminanzanalyse und/oder künstliche neuronale Netzwerke zur Auswertung der Daten verwendet.

Hierzu 2 Seite(n) Zeichnungen



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– Leerseite –

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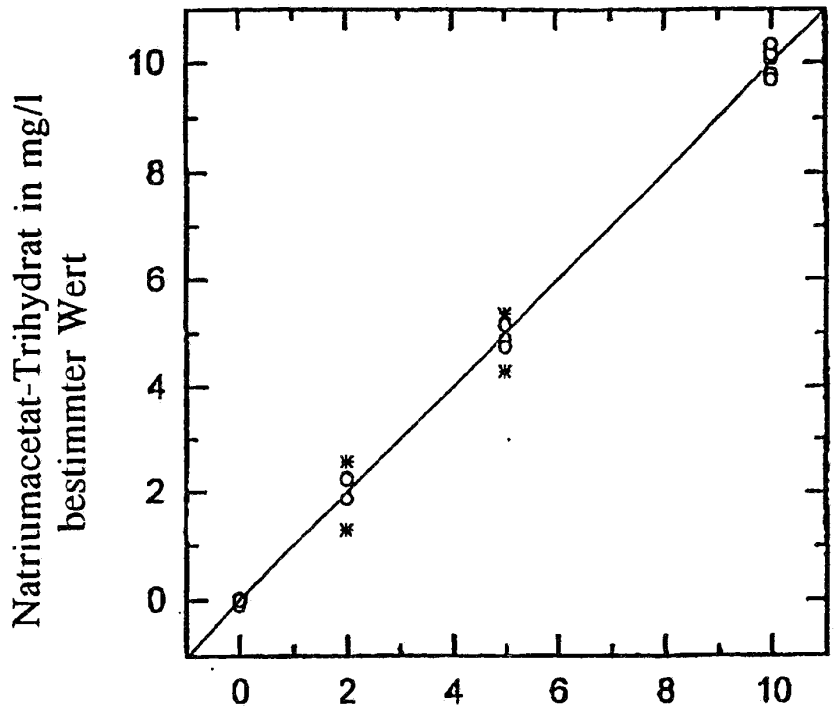


Fig. 1

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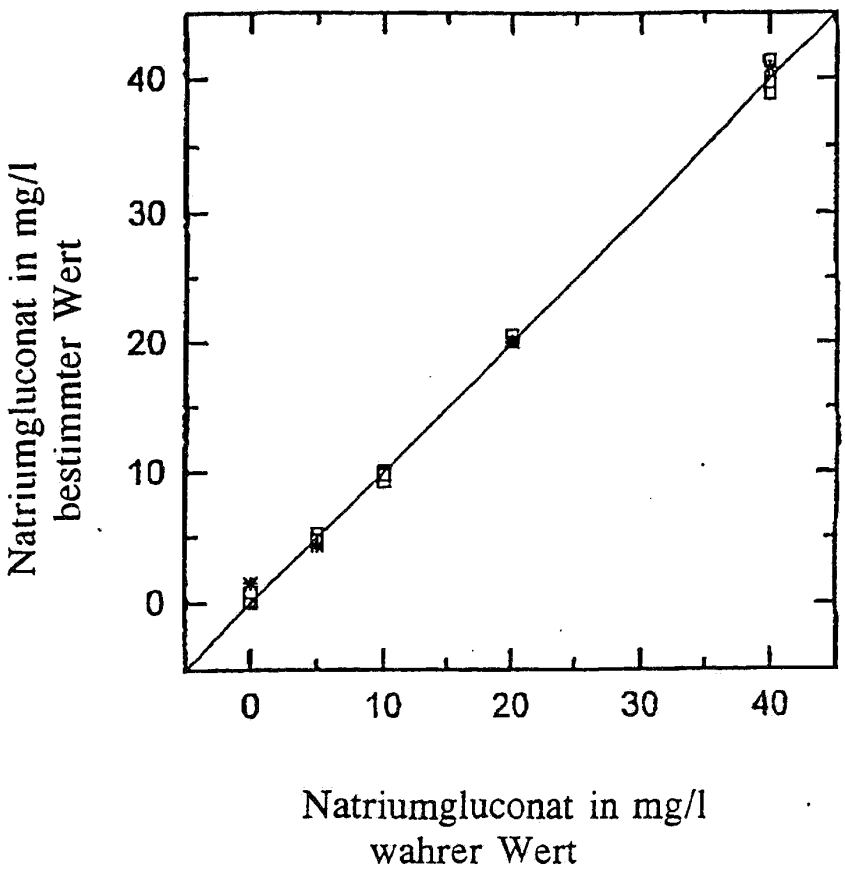


Fig. 2

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Method and multi-channel bio-sensor for multi-component analysis of mixtures

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Abstract of **DE19540098**

Multi-component analyses are carried out on mixtures and/or blends using \- 1 biological sensor element, which is at least partly non-selective to the substances in the mixture to be analysed, but which responds in a different way. The sensor(s) is brought into contact with the mixture and signals from the sensor elements are analysed over time and/or with respect to the individual sensor elements. The sensor elements contain cells and/or cell organelles which are preferably immobilised. The sensor elements preferably measures the oxygen consumption of the biological components using a Clark electrode. Also claimed is a multi-channel biosensor with an evaluation unit for carrying out the method.

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(54) Diagnostic methods and probes

(57) The invention features methods for detecting a target analyte in a sample. In these methods, a sample is contacted with a detector probe and a capture probe to form a detector probe-analyte-capture probe complex. The detector probe includes a new moiety, having a predetermined pI and containing a detectable label. The complex is isolated from detector probe that is not bound in the complex, the moiety is released from the complex, and the released moiety is concentrated, *e.g.*, by isoelectric focusing or other methods. Detection of the moiety, *e.g.*, at a position in a pH gradient corresponding to its pI, can be used as a measure of the presence and concentration of the analyte in the sample.

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Description

This invention relates to methods and probes for detecting a target analyte in a sample.

Detection of analytes present in trace amounts in a sample requires sensitive and specific methods. Otherwise, detection of such analytes may be hindered by the presence of substances found at higher concentrations in the sample. This problem is compounded if the analyte does not have a physical or chemical property that renders it easy to detect.

Isoelectric focusing (IEF) is an electrophoretic method in which an electric field is applied to a molecule in a pH gradient to localize the molecule to a position in the gradient at which its net charge is zero, i.e., at its isoelectric point (pI). IEF is often used to fractionate proteins in complex mixtures and as a tool for characterizing biomolecules of unknown composition. The pH gradients used in IEF are commercially available, and consist of mixtures of multi-charged ampholytes, which are low molecular weight compounds (*e.g.*, polyaminopolycarboxylic acids) having closely spaced, known pI values and high conductivity. The mixture of ampholytes forms a pH gradient upon application of an electric field.

Capillary tubes have been used in various electrophoretic methods, including IEF (see, *e.g.*, Novotny *et al.*, Electrophoresis, 11:735-749, 1990). One application of capillary IEF has been for detecting target analytes in samples. For example, Afeyan *et al.*, U.S. Patent No. 5,376,249 (1994), describes a method in which a complex formed between an analyte and a labeled analyte-specific binding moiety is fractionated by capillary IEF. Similarly, Karger *et al.*, U.S. Patent No. 5,348,633 (1994), describes a method in which a complex formed between an analyte and a labelled Fab fragment of an immunoglobulin is concentrated by capillary IEF. In both of these methods, the pI of an analyte-probe complex for each analyte to be detected must be determined empirically. If there are more than a few analytes, this empirical determination can become difficult.

Summary of the Invention

The present invention is based on the discovery that a new detectable and isolable moiety can be used in simple methods to detect a wide variety of analytes in complex samples. The special moiety is bound to a probe that specifically binds to the targeted analyte, the sample is mixed with the analyte-specific probe, the probe is bound to the analyte to form an analyte-probe complex, unbound probe is removed from the complex, and the moiety is released from the complex and isolated, *e.g.*, by fractionation in a pH gradient by IEF. Detection of the moiety, *e.g.*, at the position in the gradient corresponding to its pI, is used as a measure of the presence of the analyte in the sample.

Accordingly, in one aspect, the invention features a method for detecting an analyte in a sample. In this method, the sample is contacted with a detector probe, which contains a moiety (*e.g.*, phycoerythrin) containing a detectable label, and a capture probe to form a detector probe-analyte-capture probe complex. The complex is then separated from any detector probe that is not bound in the complex, the moiety is released from the complex, the released moiety is isolated from the complex, and, optionally, concentrated. The isolated moiety can then be detected as a measure of the presence of the analyte in the sample.

In addition to the detectable label, the moiety has a property, *e.g.*, a predetermined pI, that facilitates its isolation and/or concentration. In the case where the moiety has a predetermined pI, the isolating and concentrating of the released moiety can be carried out by isoelectric focusing. The focused moiety can then be detected at a position in a pH gradient corresponding to the pI of the moiety, as a measure of the presence of the analyte in the sample. The moiety may also be isolated and/or concentrated on the basis of size, *e.g.*, by the use of column chromatography or a molecular sieve.

The sample, detector probe, and capture probe can be contacted with one another in any order. For example, the sample can be contacted with the detector probe prior to its contact with the immobilized capture probe in the lumen of the fractionation chamber. Alternatively, the sample and the detector probe can be independently contacted with the capture probe in the lumen of the fractionation chamber, or the sample can be contacted with the capture probe in the lumen of the fractionation chamber prior to the detector probe.

The capture probe for use in the method of the invention can contain the first member of a specific binding pair, and the separation step can be carried out by contacting the complex with the second member of the specific binding pair, which is immobilized on a solid support (*e.g.*, an agarose or glass bead or a support in the lumen of a fractionation chamber, such as a capillary tube). The specific binding pair can be, *e.g.*, a pair of complementary nucleic acids, a pair of polypeptides that specifically bind to one another (*e.g.*, a polypeptide containing an antigen binding site of an antibody (in the context of, *e.g.*, an antibody, such as a single chain antibody) and an antigen to which the antigen binding site binds), or avidin and biotin.

The capture probe used in the method of the invention can be immobilized in the lumen of a fractionation chamber (*e.g.*, a capillary tube or a groove in the surface of a plate) and the isolating, releasing, and concentrating steps can thus be carried out in the lumen of the fractionating chamber.

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Target analytes that can be detected using the method of the invention include, *e.g.,* nucleic acids *(e.g.,* a chromosome containing a translocation, or a pre-mRNA or mRNA transcript thereof), polypeptides, carbohydrates, lipids, metabolites, and drugs. The capture probes and detector probes can be, *e.g.*, nucleic acids or polypeptides (*e.g.*, a polypeptide containing an antigen binding site of an antibody, such as a single chain antibody).

The moiety can be released from the detector probes using methods including, *e.g.*, chemical cleavage. For example, in the case where the moiety is linked to the detector probe by a disulfide bond, the moiety can be released by reduction of the disulfide bond. The moiety may also be released by enzymatic cleavage, *e.g.*, by the use of a restriction enzyme, DNAse, RNAse, or a ribozyme.

In a second aspect, the invention features a method for detecting the presence of a chromosome containing a chromosomal translocation (*i.e.*, a chromosome containing a portion of a first chromosome and a portion of a second chromosome) in a sample. In this method, the sample is contacted with a capture probe and a detector probe to form a detector probe-analyte-capture probe complex. The capture probe binds to the portion of the first chromosome, or a corresponding RNA transcript thereof, and contains the first member of a specific binding pair. The detector probe binds to the portion of the second chromosome, or a corresponding RNA transcript thereof, and contains a detectable label. The complex is then isolated from any detector probe that is not bound in the complex by contacting the complex with the second member of the specific binding pair, immobilized on a solid support. The detectable label present in the complex can then be detected as a measure of the presence of the chromosome containing the chromosomal translocation in the sample.

The invention also features probes (*e.g.*, nucleic acid or polypeptide (*e.g.*, antibody) probes) containing releasable moieties, which contain labels and have properties (*e.g.*, predetermined pIs) that facilitate their isolation and/or concentration.

The invention provides several advantages, as it permits simultaneous analysis of multiple analytes in a sample on a micro-scale with high sensitivity. When using IEF analysis of a moiety released from an analyte-bound probe, the method of the invention avoids the need for empirical determination of the pI of an analyte-probe complex for each analyte to be detected. Moieties having predetermined pIs can be attached to probes specific for any analyte of interest. Similarly, when isolating the moieties based on size or mass, moieties having predetermined sizes or masses are attached to specific probes. The method of the invention also requires only small sample and reagent volumes, and is rapid and readily adaptable to automation. In addition, amplification by physical concentration of a moiety (*e.g.*, by IEF or column chromatography) avoids background problems inherent in many detection methods employing enzymatic amplification (*e.g.*, PCR).

Unless otherwise defined, 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 methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, some preferred methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of a conflict, the present specification will control. In addition, the described materials, methods, and examples are illustrative only and are not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description and the claims.

It is to be understood that preferred features of the one aspect may apply as they apply for an appropriate corresponding aspect *mutatis mutandis.*

Brief Description of the Drawings

Fig. 1 is a schematic representation of a capture probe-analyte-detector probe complex, in which the capture probe contains the first member of a specific binding pair.

Fig. 2 is a schematic representation of a capture probe-analyte-detector probe complex, in which the capture probe is bound to a solid support.

Detailed Description

The invention provides methods for detecting the presence of a target analyte in a sample. The methods of the invention employ a detector probe labeled with a moiety that (1) contains a detectable label, and (2) has a property (*e.g.*, a predetermined pI) that facilitates its isolation and/or concentration. After the detector probe is bound to the target analyte to form a detector probe-analyte complex, and the complex is purified from sample components and unbound detector probe, the moiety is released from the complex and isolated and/or concentrated using methods appropriate for the specific moiety. For example, in the case of a moiety having a predetermined pI, the moiety is isolated and concentrated in a pH gradient by IEF. Detection of such a moiety at a position in the pH gradient corresponding to its pI is used as a measure of the presence and concentration of the target analyte in the sample.

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The methods of the invention are described below with reference to the use of a labeled moiety having a predetermined pI. However, the invention can also be carried out using a labeled moiety having other properties that facilitate its isolation and/or concentration. Methods for isolating and concentrating moieties based on other properties, including, *e.g.,* size, are well known in the art and include, *e.g.,* column chromatography. A molecular sieve can also be used to isolate and/or concentrate moieties having different molecular weights. For example, a stack of filters, having progressively smaller pore sizes, can be used. In addition, the moieties can contain paramagnetic particles, which can be isolated and concentrated by exposure to a magnetic field. In order to use such moieties in a multiplex assay, the moieties can be, *e.g.,* attached to their respective probes by different types of linkers, and thus released sequentially from the detector probes for isolation and concentration.

Hybridization Assays

Hybridization of a single probe to a target analyte to form an analyte-probe complex is carried out using standard methods. Alternatively, two probes, *e.g.*, in the context of a sandwich hybridization assay, can be used (see, *e.g.*, Ranki *et al.,* Gene 21:77-85, 1983; U.S. Patent No. 4,486,539 (1984)). As illustrated in Figs. 1 and 2, sandwich hybridization assays involve the use of a capture probe 10 and a detector probe 12, which are designed to bind concurrently to a target analyte 14. In the context of the present invention, the detector probe 12 used in a sandwich hybridization assay includes a moiety 16, which, as described herein, contains a detectable label and has a predetermined pI or other known characteristics that allow the moiety to be isolated and/or concentrated. The capture probe can, *e.g.*, (1) contain the first member of a specific binding pair 18 (Fig. 1), or (2) be immobilized on a solid support 20 (*e.g.*, on a dipstick or on a solid support in the lumen of a fractionation chamber, such as a capillary tube) (Fig. 2). In either of these cases, use of the capture probe facilitates purification of the analyte-probe complex 26 from sample components, as well as from unbound detector probes. The sample can be contacted with the detector and capture probes simultaneously, or in sequence.

As shown in Fig. 1, when a capture probe 10 containing the first member of a specific binding pair 18 is used in a sandwich hybridization assay, the detector probe-analyte-capture probe complex 26 can be purified by contact with the second member of the specific binding pair 22, bound to a solid support 24. For example, the second member of the specific binding pair can be immobilized on a bead, such as a glass or an agarose bead. Contact of the sample with the bead will immobilize the complex on the bead. As is known in the art, binding of the detector probe-analyte-capture probe complex to such beads can be promoted by, *e.g.*, continuous mixing of the sample and probes with the beads. The beads can then be washed to remove unbound probe and non-specifically bound sample components. The moiety 16 can then be released from the purified complexes, and applied to a fractionation chamber, such as a capillary tube, for concentration, *e.g.*, by IEF.

The second member of the specific binding pair 22 can also be immobilized on a solid support 24, such as a dipstick, or in the lumen of a fractionation chamber, for example, on the wall of the lumen of a capillary tube, or on a matrix, which can be made of any standard column-packing material (*e.g.*, glass microbeads, agarose beads, glass wool, or a gel, such as a polyacrylamide gel), contained within such a chamber. As an example, the specific binding pair can be a pair of complementary oligonucleotides, *e.g.*, poly-A and poly-T oligonucleotides. This configuration enables the use of a single capillary tube, for example, a capillary tube 24 containing immobilized poly-A 22, with any set of capture and detector probes, provided that the capture probe 10 contains a poly-T binding region 18.

Optimal lengths and levels of complementarity of oligonucleotides for use as specific binding pairs can be readily determined by one skilled in the art, and can vary depending on factors including sequence composition, sample complexity, and assay conditions (*e.g.*, ionic strength, the types of salts used, the presence of organic solvents, and/or hybridization temperature). In the case of homopolymeric oligonucleotides (*e.g.*, poly-A, poly-T, poly-G, and poly-C), for example, the first member of the specific binding pair, which is a component of the capture probe, can be, e.g., 8-100, 16-60, or 18-50 nucleotides in length. The second member of the specific binding pair, which is immobilized on the solid support (*e.g.*, on a dipstick or in the lumen of a fractionation chamber), should be at least 8 nucleotides in length, but preferably is much longer, for example, 16-50, 18-100, or up to several hundred or several thousand nucleotides in length. Such lengthy homopolymeric oligonucleotides are made using standard methods, for example, by the use of terminal transferase.

In the case of pairs of heteropolymeric oligonucleotides, the first and second members of the specific binding pair can be, *e.g.*, 8-100, 16-50, or 18-40 nucleotides in length. The second members of the specific binding pairs, for both homopolymers and heteropolymers, can be attached to the solid support by a linker, for example by a flexible carbon chain, such as a 3-glycidoxypropyltrimethoxysilane linker (see, *e.g.,* Maskos *et al.*, Nucl. Acids Res. 20(7):1679-1684, 1992).

The capture probe itself can also be immobilized in the lumen of a fractionation chamber (*e.g.*, a capillary tube), either on the wall of the lumen or on a matrix, which can be made of any standard column-packing material *(e.g.,* glass microbeads, agarose beads, glass wool, or a gel, such as a polyacrylamide gel) within the lumen. Methods for immo-

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bilizing probes (*e.g.*, nucleic acid or protein probes) onto surfaces, such as glass or plastic surfaces, *e.g.*, in the interior of a capillary tube, are well known in the art. For example, as mentioned above, oligonucleotides can be attached to a surface (*e.g.*, a smooth or porous surface made of, *e.g.*, glass, plastic (*e.g.*, polypropylene), silicon, gold, or platinum) by the use of a linker, such as a 3-glycidoxypropyltrimethoxysilane linker (see, *e.g.*, Maskos et *al., supra).*

The sample and the detector probe can be contacted with each other outside of such a fractionation chamber, then applied to the chamber as a mixture, or can be applied to the chamber individually, either simultaneously or sequentially. Formation of detector probe-analyte-capture probe complexes within the fractionation chamber can be facilitated by electrophoretic or physical *(e.g.,* mechanical pumping) transfer of the target analyte from one end of the chamber to the other, each time passing immobilized capture probes to which it can bind. Once the complexes are formed, non-specifically bound sample components and unbound detector probes can be removed from the chamber by application of an electric field to the chamber, by physical washing, or by a combination of these methods. The moiety can then be released from the immobilized detector probes and focused by IEF. As described further below, multiple capture probes, specific for multiple target analytes, can be immobilized at random positions in the lumen of a single capillary tube. Such configurations facilitate detection of multiple target analytes in a single assay, depending on the use of an isoelectrically distinct moiety for each detector probe.

In addition to the sandwich hybridization assays described above, other standard hybridization methods employing the use of, for example, dipsticks or a single, labeled probe can be used in the method of the invention. Dipsticks can be used, for example, by serving as the solid support to which multiple capture probes are bound. Detector probe-analyte-capture probe complexes can thus be formed on the dipstick, and moieties released from the purified complexes can be fractionated by IEF, as described below. The dipstick can also contain an immobilized member (*e.g.*, a poly-A tract) of a specific binding pair that binds to its cognate binding partner (*e.g.*, a poly-T tract), present on a capture probe (see above). This type of configuration permits the use of a dipstick containing only one species of immobilized probe to be used in conjunction with capture probes specific for multiple analytes.

Target Analytes

Target analytes that can be detected using the methods of the invention include, *e.g.*, nucleic acids, proteins, carbohydrates, lipids, metabolites, vitamins, and drugs. Detection of such molecules can be useful in fields such as medicine, forensics, agriculture, industry, food sciences, and veterinary medicine. For example, in the field of medicine, the methods of the invention can be used in the diagnosis of conditions (*e.g.*, cancer) characterized by the presence or absence of specific markers (*e.g.*, protein or nucleic acid markers) and/or altered levels of normally occurring proteins (*e.g.*, hormones, cytokines, lymphokines, antibodies, or enzymes) or nucleic acids. The methods of the invention can also be used to detect gene mutations, which can be characterized by, *e.g.*, single base pair changes, small or large deletions, insertions, or rearrangements (*e.g.*, chromosomal translocations as described below), and genetic polymorphisms. In addition, the methods of the invention can be used to detect the presence of an infectious pathogen (*e.g.*, a bacterium, virus, protozoan, parasite, or fungus) in a sample, *e.g.*, a sample from a patient. Samples that can be tested using the methods of the invention include, *e.g.*, biological fluids, such as blood, serum, plasma, urine, and saliva, as well as plant extracts, cell extracts, cell culture media, and fermentation mixtures. If necessary, protein and/or nucleic acid preparations can be prepared from the samples using standard methods, before application of the present methods. As discussed below, due to the sensitivity of the method, only small amounts of a sample are required for carrying out the methods of the invention.

Probes

The types of specific probes used in the invention depend on the particular type of target analyte to be detected, and are well known in the art. For example, in the case of a nucleic acid target (*e.g.*, a DNA or an RNA target), nucleic acid probes can be used. The nucleic acid probes can contain deoxyribonucleotides, ribonucleotides, or combinations or modifications thereof. The optimal sequences, lengths, and levels of complementarity with the target analyte, in order to achieve specific binding, are parameters that are readily determined by those skilled in the art. For example, the probes can contain at least 8, for example 16-100 or 18-40, consecutive nucleotides that are complementary to the target nucleic acid analyte. The design of such probes can be facilitated by reference to standard protocol manuals and publicly available computer programs (see, *e.g.,* Ausubel *et al.*, eds. *Current Protocols in Molecular Biology,* Wiley & Sons, New York, 1989). Synthesis of nucleic acid probes can be carried out using standard chemical or recombinant methods. Alternatively, nucleic acid probes can be purchased from commercial vendors. The nucleic acid probes can be single-stranded or can contain single-stranded and double-stranded regions. In addition to nucleic acid probes, nucleic acid target analytes can be detected using polypeptide probes, for example, probes that contain nucleotide sequence-specific nucleic acid binding domains.

In the case of protein targets (*e.g.*, antibodies, hormones, enzymes, pathogen proteins, cytokines, and lym-

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phokines), antibodies, such as monoclonal antibodies that specifically bind to the analyte, can be used in the invention. Techniques for producing antibodies are well known in the art (see, *e.g.*, Harlow *et al., Antibodies, A Laboratory Manual,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1988). Particularly useful antibody molecules include Fab fragments of immunoglobulins, as well as recombinantly made single chain antibodies (see, *e.g.*, Huston *et al., U.S.* Patent Numbers 5,091,513 (1992) and 5,132,405 (1992); and Ladner *et al.,* U.S. Patent Numbers 4,704,692 (1987) and 4,946,778 (1990)). In addition to antibodies, non-antibody proteins and nucleic acids that specifically bind to target protein analytes can be used.

As mentioned above, capture probes used in the method of the invention can, *e.g.,* (1) contain the first member of a specific binding pair, or (2) be immobilized on a solid support *(e.g.,* on a dipstick or on a solid support in the lumen of a fractionation chamber, such as a capillary tube). Binding pairs that can be used in conjunction with capture probes include, *e.g.*, streptavidin-biotin, as well as antibody-antigen, enzyme-substrate, receptor-ligand (*e.g.*, hormone), nucleic acid-nucleic acid binding protein, and nucleic acid-nucleic acid binding pairs, and other specific binding pairs known in the art. The second member of the binding pair is immobilized on a solid support, such as an agarose bead or a glass microbead, using standard methods, or such a complex can be purchased from a commercial vendor. Immobilization of capture probes to, *e.g.*, the lumen of a fractionation chamber or glass microbeads, which can be contained within the fractionation chamber, is described above. Detector probes used in the method of the invention contain moieties, which are described below.

Moieties

As mentioned above, detector probes used in the invention include a moiety that (1) contains a detectable label (*e.g.*, a fluorescent, phosphorescent, radioactive, luminescent, or colored label), and (2) has a property that allows its isolation and/or concentration, *e.g.,* a predetermined pI. The moiety can be a single molecule having both of these properties, or a complex of two or more molecules that together provide the moiety with these properties. The moiety can be a naturally occurring molecule or can be chemically synthesized. For example, the moiety can be a protein, peptide, protein complex, nucleic acid, carbohydrate, organic molecule, or a modification or combination thereof. Labels that can be used with the moiety include, *e.g.*, fluorescent labels *(e.g.*, fluorescein, rhodamine, or Texas Red), mixtures of fluorescent labels, chromophores, phosphorescent agents, and luminescent agents.

Specific examples of moieties that can be used in the invention include, *e.g.,* members of the phycobilliprotein family *(e.g.,* phycoerythrin and allophycocyanin), Starburst Dendrimers (Sigma Chemical, St. Louis, MO), an FITC coupled to a small amino acid chain (*e.g.*, an amino acid chain containing a proteolytic cleavage site), and fluorescent dextrans (Molecular Probes, Eugene, OR; *e.g.,* a dextran microparticle of several nanometers diameter covered with, *e.g.*, a fluorophore; the microparticle can be linked to a detector probe by a polystyrene spacer-arm containing a cleavable bridge, such as a disulfide bond). Other examples of suitable moieties and chemical linkages can be found, *e.g.*, in Haugland, "Handbook of Fluorescent Probes and Research Chemicals," Molecular Probes, Inc., Eugene, OR, 1992.

Streptavidin-phycoerythrin conjugates are extremely sensitive labels, as their fluorescence yield/molecule ratio is roughly equivalent to that of 100 rhodamine molecules or 30 fluorescein molecules. A commercially available streptavidin-phycoerythrin conjugate (Molecular Probes, Eugene, OR) focuses into multiple, discrete peaks having acidic pIs. Thus, streptavidin-phycoerythrin is a source for the isolation of numerous, distinct moieties having discrete pIs. The streptavidin-phycoerythrin can be fractionated by IEF in, *e.g.*, a narrow pH gradient, and peaks corresponding to species having discrete pIs can be pooled for use as separate moieties.

Moieties having discrete pIs can also be obtained by chemical treatment. For example, treatment of streptavidin-phycoerythrin with water soluble carbodiimide/N-hydroxysulfosuccinimide(NHS)/ethylene diamine (see below) results in the production of a multiplicity of streptavidin-phycoerythrin products containing ethylene diamine, which increases the pIs of the proteins. These products have pIs in the range of 6-9, and can be fractionated into distinct species for use as moieties. Modified versions of moieties, *e.g.*, phycoerythrin, can be made using these methods, or any other standard method in the art.

Moieties can be attached to detector probes using standard methods, which are selected based on the type of probe and moiety used. For example, molecules *(e.g.,* protein or nucleic acid molecules) containing primary amines can be linked to molecules containing biotin and a disulfide bridge, *e.g.*, as follows. The molecule containing the primary amine is reacted with an N-hydroxysulfosuccinimide (NHS) ester of biotin, that includes a disulfide bond in the spacer arm (*e.g.*, EZ-Link NHS-SS-Biotin, Pierce Chemical Co., Rockford, IL), at or above neutral pH, resulting in covalent linkage of the molecule with SS-biotin and release of N-hydroxysulfosuccinimide. As a specific example of the use of such a modified molecule, an analyte-specific nucleic acid probe linked to SS-biotin can be linked with avidinphycoerythrin to form a complete detector probe. Release of the moiety, after isolation of detector probe-analyte-capture probe complexes, can be achieved by contacting the complex with a reducing agent, such as dithiothreitol or β-mercaptoethanol.

Another well known coupling reaction involves reaction of a carboxyl group on one molecule with a primary amine

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on another molecule, in the presence of a carbodiimide coupling reagent, such as EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) (Biorad, Cambridge, MA). Reaction of these components results in a covalent linkage between the two molecules, accompanied by the release of urea. Moieties can also be introduced into detector probes as modified nucleotides during synthesis or after synthesis by chemical modification of nucleotides containing reactive groups, such as primary amines (see above).

In the case of a detector probe containing a nucleic acid segment between the analyte-binding region and the moiety (*e.g.*, a nucleic acid probe), a nuclease can be used to release the moiety from the detector probe-analyte-capture probe complex. For example, a DNAse can be used to release moieties from probes containing DNA, while an RNAse (*e.g.*, RNAse A or S1 nuclease) can be used to release moieties from probes containing RNA. Of course, such enzymes should not be used in cases where they adversely affect the integrity of the moiety.

In addition to enzymes such as DNAses and RNAses, enzymes having sequence specificity can be used to release moieties from detector probes. For example, the moiety can be linked to the probe by a single or double-stranded DNA sequence that contains a restriction endonuclease cleavage site. After isolation of the complex, the moiety can be released from the complex by incubation with the appropriate restriction endonuclease, using standard methods. With probes containing specific ribonucleotide sequences adjacent to the moiety, a ribozyme can be used to effect release.

Moieties can also be attached to detector probes *(e.g.,* nucleic acid and polypeptide detector probes) by peptide linkages containing amino acid sequences that can be cleaved by sequence specific enzymes. For example, the moiety can be attached to the probe by a peptide having a sequence recognized by an enzyme, such as pyroglutamyl peptidase II (see, *e.g.*, Wilk et *al.,* Neurochem. Int. 15(1):81-89, 1989; Wilk *et al.,* Neuropeptides 12:43-47, 1988; Wilk, Life Sciences 39:1487-1492, 1986; Charli *et al.,* Neuropeptides 9:373-378, 1987). Such polypeptide probes can be made, *e.g.,* using standard recombinant methods (see, *e.g.*, Ausubel *et al., supra).*

Additional methods for linking moieties to probes are well known in the art, and include, *e.g.,* the use of biodegradable ester linkers. For example, a palmitoyl residue can be linked to the 5' end of a nucleic acid probe by a bond that is cleavable by an esterase (see, *e.g.*, Polushin *et al.,* Nucleic Acids Res. 22:5492-5496, 1994). As is well known in the art, ester bridges can also be cleaved by increasing the pH from neutral to 9-10. Photochemical bridges that are cleaved by irradiation of specific wavelengths can also be used. Moieties can also be linked to detector probes by antibody-antigen and receptor-ligand interactions. For example, a labeled lectin that binds to a glycoconjugate, *e.g.*, concanavalin A, which binds to α-mannopyranosyl residues or α-glucopyranosyl residues, can be used. In addition, moieties can be cross-linked to probes, such as polypeptide probes.

Isoelectric Focusing

Moieties cleaved from purified detector probe-analyte-capture probe complexes can be concentrated by IEF in a pH gradient to facilitate subsequent detection. The pH gradient can be created by application of an electric field to a mixture of ampholytes, which are compounds (*e.g.*, low molecular weight polyamino-polycarboxylic acids or polymers of glycine, glycylglycine, amines, an epichlorohydrin) containing varying amounts of hydrogen-donating and hydrogen-accepting groups. In the presence of a charge gradient, the ampholytes migrate to their pIs, *i.e.*, to the positions between the positive and negative poles where their net charges are zero. The ampholytes can have closely spaced pI values and high conductivity, and thus can partition into smooth pH gradients upon application of an electric field.

Specific ampholytes can be selected, depending on the moiety or moieties to be fractionated. For example, shallow gradients, *i.e.*, gradients with relatively small changes in pH per unit length, often are useful as they permit resolution of moieties having similar pIs. For example, pH gradients which span, *e.g.*, less than 2.0 pH units can be used. Such gradients permit resolution among members of a family of closely related chemical structures, and can be readily prepared by, *e.g.*, fractionating conventional ampholyte mixtures to obtain preparations having a narrow pH range. It is often useful to prepare a gradient so that its ends have regions of constant pH that are fairly close to the predetermined pI of the moiety. For example, a gradient for detecting a moiety of pI 8.2 can extend from pH 7.7 to pH 8.7. This design can be used to eliminate charged species in the sample having pIs outside the range of the gradient. Such species rapidly migrate to the poles. Ampholytes are available from several commercial suppliers, such as Bio-Rad (Cambridge, MA) and Pharmacia Biotech. (Piscataway, NJ).

Capillary Electrophoresis

IEF for use in the invention can be carried out in an elongated channel, such as a capillary tube, which is made of glass, plastic, or other material, and has an internal diameter of, for example, less than about 1 millimeter, *e.g.,* less than 500 microns. For example, a capillary tube having an internal diameter of 100 microns and a length of 50 millimeters can be used. The surface area of such a capillary tube would allow the binding of on the order of 10^{13} capture probes. Higher surface to volume ratios can be achieved by using thinner and/or longer capillaries, thereby facilitating even more rapid hybridization kinetics. Methods for carrying out capillary IEF are well known in the art and are described,

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e.g., by Karger *et al.* (J. Chromatography, 492:585-614, 1989) and Novotny *et al. (supra).*

The use of a capillary tube, which has a high surface area to volume ratio, and thus a high capacity for dissipating heat, allows high voltages to be used in IEF methods, thus improving and accelerating the resolution of the moiety at its pI. In conventional IEF, electric fields on the order of 100 V/cm are generally used, while in capillary IEF, electric fields on the order of 200-500 V/cm can be used. Thus, IEF assays can be conducted rapidly, *e.g.*, in a few minutes or seconds. In addition, in the case where capillary tubes containing immobilized capture probes are used, the high surface area to volume ratio of the capillary tubes facilitates target analyte hybridization to the capture probes, as any target molecule that passes through the capillary is in close proximity to the capture probes.

Another consequence of the high surface area to volume ratio of capillary tubes with respect to the present invention is that only very small quantities of reagents and test samples are required. For example, since only a few hundred fluorophores are needed for detection by a charge-coupled device (CCD; see, *e.g.*, Mackay *et al.*, U.S. Patent No. 4,874,492 (1989)), after fluorescence induction by a laser, a single milliliter of blood, which contains approximately 10^6-10^7 white blood cells, should provide a sufficient amount of target for detection using the method of the invention. In the case where bacterial rRNAs are the target analytes, a single bacterium in a one milliliter sample should be detectable using the method of the invention.

Any of several standard capillary electrophoresis systems can be used to carry out the method of the invention. For example, the Beckman P/ACE System 2050 (Beckman Instruments, Columbia, MD) can be used. In addition, systems that facilitate simultaneous processing of several capillary tubes can be used (see, *e.g.*, Yeung *et al.*, U.S. Patent No. 5,324,401 (1994)).

In addition to capillary IEF, other IEF methods can be used in the invention. For example, IEF can be carried out in a groove etched into a plate, such as a glass or a plastic plate. In this method, each end of the groove is in contact with a well into one of which the sample is applied. Use of this type of system allows analysis of larger sample volumes. In addition, methods in which ampholytes are provided in a polyacrylamide gel support matrix, *e.g.*, in an elongated horizontal slab gel or in a tube gel, can be used.

Detection of Focused Moieties

The emission, absorbance, or other detectable signal of a focused moiety can be detected using any of several standard methods, depending on the nature of the moiety. For example, a spectrophotometer can be used for detecting ultraviolet or x-ray irradiation of a moiety. A fluorescent moiety can be detected with a CCD device. A standard mercury light source, or a laser beam, can be used for excitation of the moiety, and a CCD device, such as a linear CCD device, can be employed for detection. The light source (*e.g.*, a laser) can direct light perpendicularly to the moieties to be excited, or can be connected by a fiber optic cable in series with the capillary tube, so that the capillary tube itself is used as a light tunnel. In the case of an isotopically labeled moiety, a CCD device can be used to measure gamma irradiation directly. As will be apparent to one skilled in the art, appropriate filters, to remove scattered or reflected light, can be used with a detector device, such as a CCD device or a photomultiplier tube.

The detector can be positioned to specifically monitor the precise location in the pH gradient corresponding to the pI of the moiety. Alternatively, the detector can be moved along the pH gradient, or the chamber containing the gradient (*e.g.*, a capillary tube) can be moved past a detector. In another example, neither the capillary, nor the detector is moved. Rather, once the moiety is focused, it is transported within the capillary tube so that it moves past a detector. Such transport can be induced, *e.g.*, by pumping fluid through the capillary to convectively move the gradient. In any example where the plug or zone of complex is moved relative to the detector, the predetermined pI will remain constant, but the physical position of the focused moiety will shift. In a device where the whole gradient is moved, the detector can be programmed to take a reading only at a predetermined time after the start of flow, such as the time determined to coincide with passage of the moiety. Also, a linear CCD device, spanning the length of the fractionation chamber, can be used. Appropriate systems integrating signal induction (*e.g.*, lasers) and detection (*e.g.*, CCD devices) devices are well known in the art (see, *e.g.*, Fujimiya *et al.*, U.S. Patent No. 5,069,769 (1991); Pentoney, U.S. Patent No. 5,208,466 (1993)).

To conduct a quantitative analysis of a target analyte, a standard curve can first be prepared, using standard methods. For example, the amount of label detected using the method of the invention can be determined for several samples containing known quantities of analyte. A standard curve generated by these readings can then be used to determine the concentration of the analyte in a sample having an unknown analyte concentration, by comparing the level of the signal detected to the standard curve.

Detection of Multiple Analytes in a Sample

An advantage of the invention is that it facilitates detection of multiple analytes in a sample using a single pH gradient. For example, probes specific for several analytes, each containing isoelectrically distinct moieties, can be

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used together in the same assay. After hybridization, capture, moiety release, and IEF, the different moieties are detected at their unique positions in the pH gradient, as a measure of the presence of each analyte in the sample. In an assay for multiple analytes in a single pH gradient, it is necessary only that each moiety corresponding to a different target analyte has a unique and distinguishable pI in the gradient, *i.e.,* the detectable label can be the same on each otherwise distinct moiety. The number of simultaneous detections (*i.e.*, the number of different probes) that can be carried out is limited only by the resolution of the detection device and/or the ability to resolve the moieties during IEF.

Example I - Detection of Salmonella in a Biological Sample

The methods of the invention can be used to detect the presence of a pathogenic organism in a sample, for example, *Salmonella* in a food sample. Reagents and methods adaptable for carrying out this assay are provided in the GENE-TRAK *Salmonella* Assay Kit (GENE-TRAK Industrial Diagnostics, Hopkinton, MA). Briefly, a genosensor (a dipstick in the GENE-TRAK assay, but a capillary tube can also be used) coated with a poly-A probe is contacted with a sample, a capture probe, which contains a poly-T tail, and a detector probe. The detector probe is designed to contain a moiety of the invention, such as a streptavidin-phycoerythrin moiety. A specific example of a detector probe that can be used in this method is as follows: phycoerythrin-streptavidin-biotin-SS-AmT-AGCTCACAGCATATGCGCTTTTGT-GTAC-3' (SEQ ID NO:1), where AmT is an amino modifier, C_6dT, which is a thymidine that contains a primary amine attached via a 6-carbon linker. After washing to remove non-specifically bound material, including unbound detector probes, the dipstick is transferred to a tube containing a 1-2% solution of ampholytes and a releasing agent *(e.g.,* in the case of the specific probe described above, a reducing agent (*e.g.*, dithiothreitol or β-mercaptoethanol) is used to cleave the disulfide bond, resulting in the release of S-biotin-streptavidin-phycoerythrin). After removal of the moiety from the complex, the solution is transferred to an IEF tube, to which a current is applied to establish a pH gradient. The moiety is then detected at the position in the pH gradient corresponding to its pI, as described above.

Example II - Detection of Chromosomal Translocations

The invention can be used to detect chromosomal translocations. As a specific example, the method can be used to detect the so-called "Philadelphia" chromosome, the presence of which is the underlying cause of acute lymphocytic leukemia and chronic myelogenous leukemia. The Philadelphia chromosome is characterized by having a balanced translocation of the terminal region of chromosome 9 to the terminal region of chromosome 22. This results in a gene fusion between the *abl* oncogene on chromosome 9 and the *bcr* gene on chromosome 22. This gene fusion is transcribed into a message containing regions corresponding to both genes.

The present invention can be used to detect such translocations as follows. A capture probe can be designed to hybridize to the *bcr* region of the fusion (either the gene itself or an RNA (pre-mRNA or mRNA) transcript thereof), while a detector probe can be designed to hybridize to the *abl* region. Capture of the *abl* detector probe by the *bcr* capture probe indicates the presence of the Philadelphia translocation.

Other Embodiments

It is to be understood that, while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention. Other aspects, advantages, and modifications of the invention are within the scope of the following claims.

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SEQUENCE LISTING

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(i) APPLICANT: Müller, Uwe R.

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(v) COMPUTER READABLE FORM:

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(C) OPERATING SYSTEM: Windows for Workgroups
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(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AGCTCACAGC ATATGCGCTT TTGTGTAC 28

Claims

1. A method for detecting an analyte in a sample, comprising the steps of:

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contacting said sample with a detector probe and a capture probe to form a detector probe-analyte-capture probe complex, wherein said detector probe comprises a moiety comprising a detectable label;
separating said complex from any detector probe that is not bound in said complex;
releasing said moiety from said complex;
isolating said moiety released from said complex; and
detecting said moiety as a measure of the presence of said analyte in said sample.

2. A method as claimed in claim 1, wherein said isolating step includes concentrating said moiety.

3. A method as claimed in claim 2, wherein

said moiety has a predetermined pI;
said isolating and concentrating of said moiety released from said complex is carried out by isoelectric focusing; and
said detecting of said moiety as a measure of the presence of said analyte in said sample is carried out by detecting said concentrated moiety at a position in a pH gradient corresponding to said pI of said moiety.

4. A method as claimed in claim 2, wherein said isolating and concentrating is carried out by the use of column chromatography or a molecular sieve.

5. A method as claimed in any of claims 1 to 4, wherein said capture probe is immobilised in the lumen of a fractionation chamber and said isolating, releasing, and concentrating steps are carried out in said lumen.

6. A method as claimed in claim 5, wherein

(a) said sample is contacted with said detector probe prior to its contact with said immobilised capture probe in said lumen;
(b) said sample and said detector probe are independently contacted with said capture probe in said lumen; or
(c) said sample is contacted with said capture probe in said lumen prior to said detector probe.

7. A method as claimed in any of claims 1 to 4, wherein said capture probe comprises a first member of a specific binding pair and said separation step is carried out by contacting said complex with a second member of said specific binding pair, wherein said second member of said specific binding pair is immobilised on a solid support.

8. A method as claimed in claim 7, wherein said solid support is in the lumen of a fractionation chamber.

9. A method as claimed in claim 7 or 8, wherein

(a) said specific binding pair comprises a pair of complementary nucleic acids;
(b) said specific binding pair comprises a pair of polypeptides that specifically bind to one another; or
(c) said first member of said specific binding pair comprises avidin, and said second member of said specific binding pair comprises biotin.

10. A method as claimed in claim 7 or 8, wherein said first member of said specific binding pair comprises an antigen binding site of an antibody and said second member of said specific binding pair comprises an antigen to which said antigen binding site binds.

11. A method as claimed in any of claims 3 or 5 to 10, wherein said focusing step is carried out in the lumen of a capillary tube or in a groove in the surface of a plate.

12. A method as claimed in any of claims 1 to 11, wherein said analyte comprises a nucleic acid.

13. A method as claimed in any of claims 1 to 12, wherein said analyte comprises a chromosome comprising a translocation, or a pre-mRNA or mRNA transcript thereof.

14. A method as claimed in any of claims 1 to 13, wherein said detector probe and/or said capture probe comprises a nucleic acid.

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15. A method as claimed in any of claims 1 to 11 or 14, wherein said analyte comprises a polypeptide, a carbohydrate, lipid, metabolite, or drug.

16. A method as claimed in any of claims 1 to 13 or 15, wherein said detector probe comprises a polypeptide.

17. A method as claimed in any of claims 1 to 13, 15 or 16, wherein said capture probe comprises a polypeptide.

18. A method as claimed in claim 16 or 17, wherein said polypeptide comprises an antigen binding site of an antibody or a single chain antibody.

19. A method as claimed in any of claims 1 to 18, wherein said releasing step is carried out by chemical or enzymatic cleavage.

20. A method as claimed in any of claims 1 to 19, wherein said moiety is linked to said detector probe by a disulfide bond and said releasing step is carried out by reduction of said disulfide bond.

21. A method as claimed in any of the above claims, wherein said moiety comprises phycoerythrin, or a modified version thereof.

22. A kit comprising:

a detector probe comprising a moiety comprising a detectable label;
a capture probe capable of forming a complex with said detector probe and an analyte in a sample; and
a means for releasing said moiety from said complex.

23. A method for detecting the presence of a chromosome comprising a chromosomal translocation in a sample, wherein said chromosome comprises a portion of a first chromosome and a portion of a second chromosome, said method comprising the steps of:

contacting said sample with a capture probe and a detector probe to form a detector probe-analyte-capture probe complex, wherein

said capture probe binds to said portion of said first chromosome, or a corresponding RNA transcript thereof, and comprises the first member of a specific binding pair, and
said detector probe binds to said portion of said second chromosome, or a corresponding RNA transcript thereof, and comprises a detectable label;

isolating said complex from any detector probe that is not bound in said complex by contacting said complex with the second member of said specific binding pair, immobilized on a solid support; and
detecting said detectable label present in said complex as a measure of the presence of said chromosome containing said chromosomal translocation in said sample.

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FIG. 1

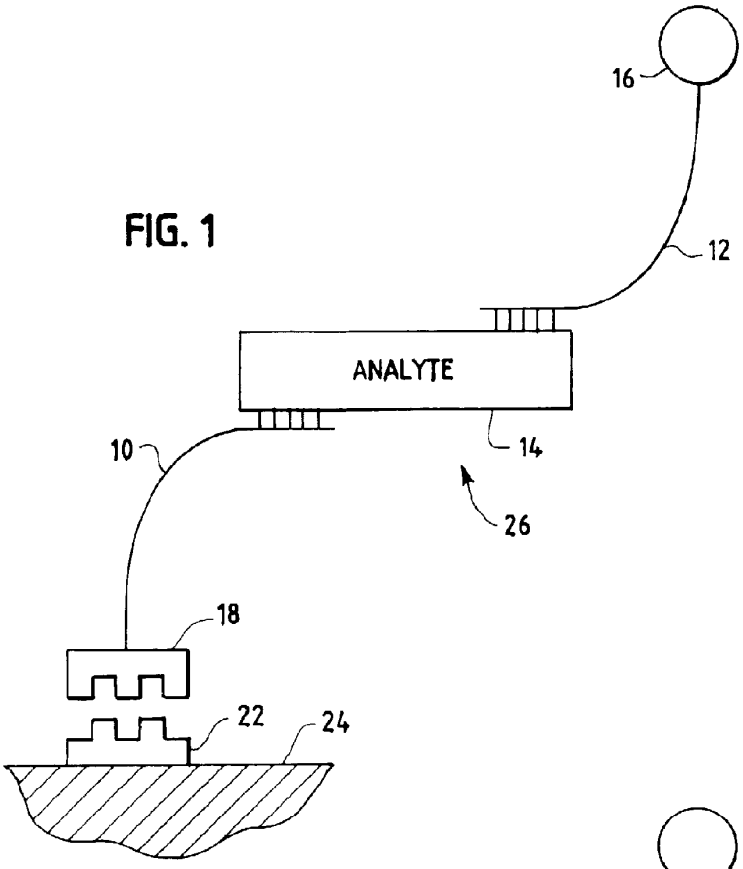
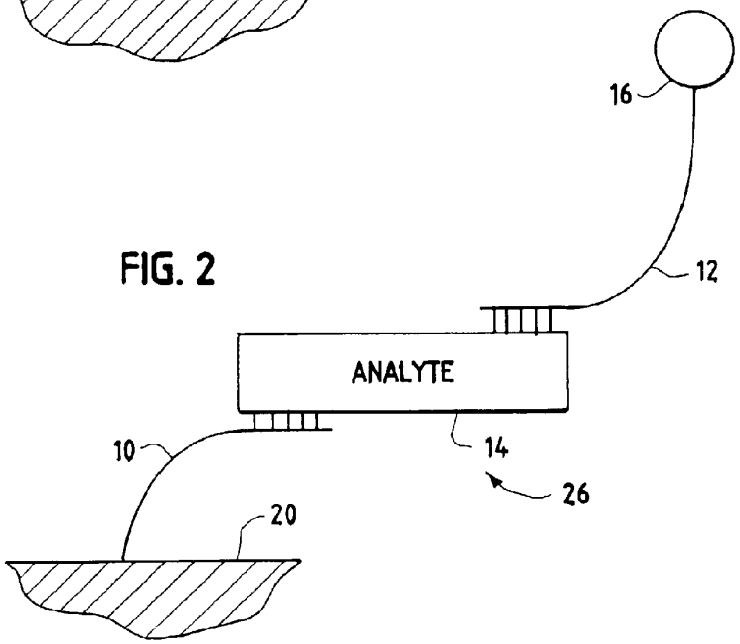


FIG. 2



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(54) A method for sensing fluorescent samples utilizing moment analysis

(57) The invention relates to a method for sensing fluorescent samples of cells, or soluble or solid supports in a medium, said method comprising the steps of:

(a) monitoring intensity of fluorescence emitted from said fluorescent samples by measuring with the help of one or more photon detectors numbers of photon counts **n** per counting time interval or digitized values **n** of fluorescence intensity per time interval in a plurality of sensed volumes,

(b) calculating a series of moments $\hat{M}_i$ of the measured photon count numbers or digitized values, defined as

$$\hat{M}_i = \sum_{\mathbf{n}} \mathbf{n}^i \hat{P}(\mathbf{n}) = average(\mathbf{n}^i) = average(n_1^{i_1} n_2^{i_2} \ldots),$$

where $\hat{P}(\mathbf{n})$ denotes a normalized histogram of photon count numbers or digitized values, and

(c) estimating characteristic parameters of the cells, or soluble or solid supports $\mathbf{a}_{cell}$ and of the medium $\mathbf{a}_{medium}$ from said series of moments $\hat{M}_i$ using a set of equations corresponding to different orders of the moment **i**, of a general form

$$\hat{M}_i = P_{cell} M_i^{(cell)}(\mathbf{a}_{cell}) + (1 - P_{cell}) M_i^{(medium)}(\mathbf{a}_{medium}),$$

where

P_{cell} denotes the probability that the sensed volume is situated in or on the cell, or soluble or solid support,
$M_i^{(cell)}(\mathbf{a}_{cell})$ denotes the expected **i**-th moment of the photon count number or digitized value provided the sensed volume is situated in or on the cell, or soluble or solid support, as a function of the parameters $\mathbf{a}_{cell}$ and
$M_i^{(medium)}(\mathbf{a}_{medium})$ denotes the expected **i**-th moment of the photon count number or digitized value

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provided the sensed volume is situated in the medium, as a function of the parameters $\mathbf{a}_{medium}$. An apparatus for working this method as well as different applications of the method are disclosed.

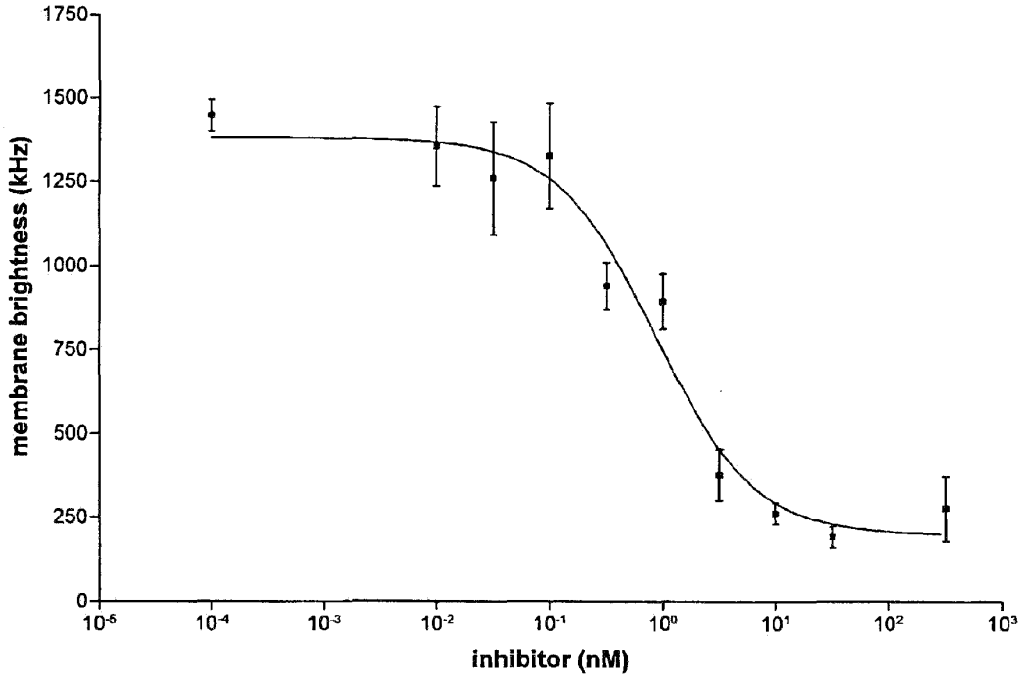


Fig. 3

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Description

[0001] Screening large numbers of chemical compounds against assays for macromolecular targets has become the major paradigm in drug discovery (Persidis A., Nat. Biotechnol., 16: 488 - 489, 1998). Whereas the widespread application of combinatorial chemistry techniques is already resulting in a quasi-exponential increase in the number of compounds available for testing, genome sequencing and analysis will inevitably feed even more targets into drug discovery. Rapid and cost-effective processing of these targets requires the development of novel assay formats and technologies. These not only have to allow screening at higher throughput in a miniaturized format thus reducing compound and reagent consumption, but also should overcome the waste-disposal problems associated with commonly used radioactive methods. In addition, they cannot depend on separation steps which hamper automation and miniaturisation.

[0002] G protein-coupled receptors (GPCR's) belong to a large superfamily of multi-spanning membrane proteins which probably represent the single most important class of drug targets. However, in comparison to other targets such as soluble proteins or enzymes, progress in the development of non-radioactive, homogeneous, miniaturized assays for GPCR's has so far been slow. In contrast, genome sequencing is continually leading to the identification of new GPCR targets (Uddhav, K. et al., Mol. Biol. Rep. 25: 27 - 43, 1998; Stadel et al.., Trends Pharmacol. Sci. 18: 430 - 437, 1997). In this situation, the development of suitable assays is often a critical factor in competitiveness for pharmaceutical companies.

[0003] It was therefore the object of the present invention to provide assay formats fulfilling the demands described above. These assay formats should also be suitable tools for analyzing solid support structures for target molecules, or for soluble support structures such as beads.

[0004] The object has been solved by the method according to the features of claim 1 and the apparatus according to the features of claim 16. The subclaims define preferred embodiments of the method and the apparatus of the present invention.

[0005] The present invention relates to a method for sensing fluorescent samples of cells, or soluble or solid supports in a medium, said method comprising the steps of:

(a) monitoring intensity of fluorescence emitted from said fluorescent samples by measuring with the help of one or more photon detectors numbers of photon counts **n** per counting time interval or digitized values **n** of fluorescence intensity per time interval in a plurality of sensed volumes,

(b) calculating a series of moments $\hat{M}_i$ of the measured photon count numbers or of the digitized values, defined as

$$\hat{M}_i = \sum_{\mathbf{n}} \mathbf{n}^i \hat{P}(\mathbf{n}) = average(\mathbf{n}^i) = average(n_1^{i_1} n_2^{i_2} \ldots),$$

where $\hat{P}(\mathbf{n})$ denotes a normalized histogram of photon count numbers or digitized values, and

(c) estimating characteristic parameters of the cells, or soluble or solid supports $\mathbf{a}_{cell}$ and of the medium $\mathbf{a}_{medium}$ from said series of moments $\hat{M}_i$ using a set of equations corresponding to different orders of the moment **i**, of a general form

$$\hat{M}_i = P_{cell} M_i^{(cell)}(\mathbf{a}_{cell}) + (1 - P_{cell}) M_i^{(medium)}(\mathbf{a}_{medium}),$$

where

P_{cell} denotes the probability that the sensed volume is situated in or on the cell, or soluble or solid support,
$M_i^{(cell)}(\mathbf{a}_{cell})$ denotes the expected **i**-th moment of the photon count number or digitized value provided the sensed volume is situated in or on the cell, or soluble or solid support, as a function of the parameters $\mathbf{a}_{cell}$ and
$M_i^{(medium)}(\mathbf{a}_{medium})$ denotes the expected **i**-th moment of the photon count number or digitized value provided the sensed volume is situated in the medium, as a function of the parameters $\mathbf{a}_{medium}$. The function $M_i^{(cell)}(\mathbf{a}_{cell})$ should be a given one.

[0006] Preferably, the medium is a liquid or gel. It preferably comprises fluorescent ligands capable of binding to said cells, or soluble or solid supports and $M_i^{(medium)}$ is calculated from cumulants $K_i = \mathbf{c}_{ligand}\mathbf{q}^i_{ligand}$,

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where $\mathbf{c}_{ligand}$ denotes the mean number of ligands per sensed volume,
and $\mathbf{q}_{ligand}$ denotes the mean number of photon counts detected per ligand by one or more photon detectors.

[0007] Preferably, values of those characteristic parameters of the cells, or soluble or solid supports which are expected to be constant through a series of samples are fixed to a single value while estimating further characteristic parameters from said series of moments $\hat{M}_i$.

[0008] In another embodiment, the method further comprises the application of an image analysis procedure to determine one or more of the parameters of P_{cell}, $\mathbf{a}_{cell}$, or $\mathbf{a}_{medium}$. It might additionally be preferred to monitor the intensity of emission scattered by said samples.

[0009] Said characteristic parameters of said cells, or soluble or solid supports $\mathbf{a}_{cell}$ are preferably chosen from the group consisting of concentration, specific brightness, width of a brightness distribution, and a function of intensities of different polarization components of emission or spectrally different components of emission.

[0010] In one embodiment, the number of photon counts is determined using at least two photon detectors monitoring emission of different wavelength and/or of different polarization and a series of at least two-dimensional moments $\hat{M}_{ij}$ of photon count numbers n_1 and n_2 is calculated, defined as

$$\hat{M}_{ij} = \sum_{n_1}\sum_{n_2} n_1^i n_2^j \hat{P}(n_1, n_2) = average(n_1^i n_2^j) .$$

[0011] A plurality of sensed volumes might be monitored sequentially in time by scanning a sensed volume through the sample. However, it is also possible to monitor a plurality of sensed volumes in parallel. Said plurality of sensed volumes can be used to form a two-or three-dimensional image.

[0012] A further embodiment of the invention relates to the use of non-photoncounting detectors. For example, many imaging detectors, such as CCD, CID, CMOS pixel arrays provide intensity values for each pixel, especially when combined with parallel confocal optics (e.g. applying a Nipkow disk embodiment; or a line scanner embodiment; or a Programmable Array Microscope embodiment (see e.g. EP 0 911 667 A1 "Programmable spatially light modulated microscope and microscopy method" and WO 98/286655 "Light modulated confocal optical instrument and method" the contents of which are herein incorporated by reference; or a Multiple Photon excitation embodiment, especially 2-photon excitation embodiment). Furthermore, traditional Laser Scanning Microscopes are usually equipped with photomultipliers which do not show photon counting resolution. All these detectors provide an analogue electric signal which is mainly proportional to the light intensity detected. The present invention can process also these signals if one further step is applied which is the digitization of the electric signal, especially by an analogue to digital converter. It is preferred to correct during this step for any nonlinear behavior of the detector or detectorarray. After the digitization of the signal, the method of the present invention can be applied, taking the digitized values of the electric signal instead of the photon counts n.

[0013] It is preferred to use a confocal optical device for monitoring intensity of emission. In a further embodiment, it is preferred to employ multiple photon excitation to stimulate fluorescence emission. Preferably, in each dimension X, Y and Z the diameter of said cells, or soluble or solid supports is greater than the diameter of the sensed volume. In another embodiment, the use of near field optics for exciting fluorescence and/or collecting the fluorescence photons is preferred. A feedback mechanism for adjusting the z-height of the nearfield optics tip is preferably employed in this embodiment to adapt for the nonplanar surface of cells or particles, especially beads.

[0014] The method is particularlay suited for use in diagnostics, high throughput drug screening, optimization of properties of molecules or identification of specific cell, or soluble or solid carrier populations.

[0015] In this respect, the invention also provides an assay for screening a single compound or preferably a plurality of compounds for inhibition between a ligand and a target molecule expressed by or attached to a cell, or soluble or solid support, said assay comprising the steps of:

- adding a liquid suspension of said target molecules expressed by or attached to said cell, or soluble or solid support to a plurality of containers;
- adding a plurality of compounds to be screened for said inhibition to said plurality of containers;
- adding fluorescently labelled ligand to said containers;
- incubating said target molecules expressed by or attached to said cell, or soluble or solid support, said compounds, and said fluorescently labelled ligand;
- individually measuring amounts of fluorescence associated with said cells, or soluble or solid supports; and
- determining the degree of inhibition by one or more of said compounds of binding of said ligand to said target molecule;

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wherein said amounts of fluorescence associated with said cells, or soluble or solid supports are estimated utilizing the moment analysis as outlined above.

[0016] The invention also provides an assay for testing a compound to determine the degree of binding of said compound to a target molecule expressed by or attached to a cell, or soluble or solid support, said assay comprising the steps of:

- adding a liquid suspension of said target molecule expressed by or attached to said cell, or soluble or solid support to a container;
- adding a fluorescent compound to be tested for its binding to said container;
- incubating said target molecule expressed by or attached to said cell, or soluble or solid support and said compound;
- measuring amounts of fluorescence associated with said cell, or soluble or solid support; and
- determining the degree of binding of said compound to said target molecule;

said amounts of fluorescence associated with said cell, or soluble or solid support is estimated utilizing the moment analysis as outlined above.

[0017] A further assay tests the influence of a compound on the degree of expression and/or secretion of an intrinsically fluorescent molecule or marker molecule, especially green fluorescenct proteine (GFP), said assay comprising the steps of:

- adding a liquid suspension of cells expressing said fluorescent target molecule to a container;
- adding a compound to said container;
- incubating said cells and said compound;
- measuring amounts of fluorescence associated with said cell, ; and
- determining the degree of expression and/or secretion of said target molecule;

wherein said amounts of fluorescence associated with said cell, is estimated utilizing the moment analysis as outlined above. However, this assay principle can also be applied without addition of a compound with the goal of estimating expression and/or secretion of fluorescent target molecules by a cell.

[0018] In one embodiment of the above disclosed assay principles, a plurality of different compounds and/or different targets is tested in a plurality of containers. Said container can e.g. be a micro- or nanoplate, preferably with a density of 96, 384, 768, 1536, 3456 or 9600 wells per plate. Other containers comprise microcapillaries or microfluidic networks, preferably with capillary electrophoretic or dielectrophoretic actuation of the liquid or hydrostatic pressure applied to the liquid.

[0019] In a further aspect, the invention provides an apparatus for working the above disclosed method and assay principles. The apparatus comprises:

- a stage for supporting a sample in an examination site, the sample comprising cells, or soluble or solid supports in a medium;
- a light source positioned to deliver light to the sample in the examination site;
- a detector positioned to receive fluorescence emitted from the sample in the examination site and constructed so that it determines numbers of photon counts **n** per counting time interval or digitized values **n** of fluorescence intensity per time interval in a plurality of sensed volumes and delivers the numbers **n** to an evaluation device;
- an evaluation device coupled to the detector for receiving and evaluating the numbers or values **n**; characterized in that
- the evaluation device is adapted to

(i) calculate a series of moments $\hat{M}_l$ of the measured photon count numbers or digitized values, defined as

$$\hat{M}_l = \sum_{\mathbf{n}} \mathbf{n}^l \hat{P}(\mathbf{n}) = average(\mathbf{n}^l) = average(n_1^{l_1} n_2^{l_2} \ldots),$$

where $\hat{P}(\mathbf{n})$ denotes a normalized histogram of photon count numbers or digitized values, and
(ii) estimate characteristic parameters of the cells, or soluble or solid supports $\mathbf{a}_{cell}$ and of the medium $\mathbf{a}_{medium}$ from said series of moments $\hat{M}_l$ using a set of equations corresponding to different orders of the moment l, of

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a general form

$$\hat{M}_i = P_{cell} M^{(cell)}_i(a_{cell}) + (1 - P_{cell}) M^{(medium)}_i (a_{medium}),$$

where

P_{cell} denotes the probability that the sensed volume is situated in or on the cell, or soluble or solid support, $M^{(cell)}_i(\mathbf{a}_{cell})$ denotes the expected **i**-th moment of the photon count number or digitized value provided the sensed volume is situated in or on the cell, or soluble or solid support, as a function of the parameters $\mathbf{a}_{cell}$ and $M^{(medium)}_i(\mathbf{a}_{medium})$ denotes the expected i-th moment of the photon count number or digitized value provided the sensed volume is situated in the medium, as a function of the parameters $\mathbf{a}_{medium}$. Preferably, the evaluation device comprises a computer unit for the above disclosed moment analysis.

[0020] The apparatus preferably comprises a confocal optical element located between the light source and the detector, the confocal optical element being capable of transmitting light substantially exclusively from a sensed volume of the sample. In one embodiment, the apparatus comprises an X, Y-axes adjustment device that automatically adjusts the relative positions of the sensed volume and the sample along the X and Y axes (wherein X, Y-axes are defined perpendicular to each other and parallel to the stage). The apparatus might further comprise a Z-axis adjustment device that automatically adjusts the relative positions of the sensed volume along the Z-axis (wherein a Z-axis is defined substantially perpendicular to the stage). The use of diffraction limited confocal optics results in a hightly focused laser beam which effectively decouples detection and assay volumes and makes the readout from these methods insensitive to miniaturization. In a further embodiment, the apparatus makes use of near field spectroscopy or is adapted for multiple photon excitation.

[0021] The accompanying figures illustrate the present invention. In the figures the following is shown:

Fig. 1 is a schematic drawing of a preferred embodiment of the principle of the assay performed according to example 1.

Fig. 2 depicts results of example 1 and shows the binding of fluorescently labelled ligand to the receptor by virtue of membrane brightness determined utilizing the method according to the present invention.

Fig. 3 depicts results of example 1 and shows the inhibition of binding by virtue of membrane brightness determined utilizing the method according to the present invention.

Example 1

[0022] The present example illustrates the determination of cells parameters from normalized photon count histograms recorded by confocal measurement. As an example, the determination of the membrane brightness of cells expressing the beta-2 adrenergic receptor in the presence of a flurorescently labelled ligand for this receptor, namely FL-CGP12177 (Molecular Probes) is described. Through determination of membrane brightness utilizing the moment analysis according to the invention, both the binding of ligand to receptor and inhibition thereof are followed.

[0023] The cells are incubated with different ligand concentrations for one hour at 37 °C and 5% CO_2. For the determination of the IC_{50}, different concentrations of non-fluorescent competitor CPG-12177 are added to a 2 nM solution of the bodipy-labelled ligand and incubated with the cells. Fluorescence is measured confocally utilizing either a confocal fluorescence reader equipped with a beamscanner and focussing means (see European patent application 98 124 314.0) at an excitation wavelength of 488 nm with a 535RDF45 emission filter or a Nipkow-device at an excitation wavelength of 488 without emission filter.

[0024] Photon counts are recorded for each of several sensed volumes within the sample. The fluorescence intensity in the different sensed volumes is recorded either sequentially or in parallel (Nipkow embodiment), and the frequency with which a particular photon count is observed is plotted. Some of the sensed volumes contain only the liquid surrounding the cells and therefore the fluorescence intensity measured in them reflects the ligand molecules not bound to the cells. Other sensed volumes reside on or in the cells and thus exhibit fluorescence orginating from the ligand that is bound to its receptor in the cell membrane. These different contributions to the photon count histogram are distinguished by recording a comparable measurement from a sample in which only the fluorescently labelled ligand, but no cells are present. Thus, the photon count distribution obtained from the latter describes the contribution of the free ligand and allows to calculate molecular brightness. The moments of this second distribution are weighted with

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1-P and subtracted from those of the first distribution (P denotes the propability of a particular sensed volume to reside in or on a cell). Thus, the histogram of the cell-containing sample is corrected for the contribution of free ligand molecules. The residuals reflect the brightness of cell membranes, which can then be calculated from the moments. Membrane brightness can be plotted as a function of either ligand or competitor concentration, which allows to determine by non-linear least squares fitting K_d and IC_{50} values, respectively. These parameters are generally used in biochemistry and pharmacology to characterize receptor-ligand interactions and their inhibition.

Claims

1. A method for sensing fluorescent samples of cells, or soluble or solid supports in a medium, said method comprising the steps of:

   (a) monitoring intensity of fluorescence emitted from said fluorescent samples by measuring with the help of one or more photon detectors numbers of photon counts **n** per counting time interval or digitized values **n** of fluorescence intensity per time interval in a plurality of sensed volumes,

   (b) calculating a series of moments $\hat{M}_i$ of the measured photon count numbers or digitized values, defined as

   $$\hat{M}_{\mathbf{i}} = \sum_{\mathbf{n}} \mathbf{n}^{\mathbf{i}} \hat{P}(\mathbf{n}) = average(\mathbf{n}^{\mathbf{i}}) = average(n_1^{i_1} n_2^{i_2} \ldots),$$

   where $\hat{P}(\mathbf{n})$ denotes a normalized histogram of photon count numbers or digitized values, and

   (c) estimating characteristic parameters of the cells, or soluble or solid supports $\mathbf{a}_{cell}$ and of the medium $\mathbf{a}_{medium}$ from said series of moments $\hat{M}_i$ using a set of equations corresponding to different orders of the moment **i**, of a general form

   $$\hat{M}_i = P_{cell} M_i^{(cell)}(\mathbf{a}_{cell}) + (1 - P_{cell}) M_i^{(medium)}(\mathbf{a}_{medium}),$$

   where

   P_{cell} denotes the probability that the sensed volume is situated in or on the cell, or soluble or solid support,
   $M_i^{(cell)}(\mathbf{a}_{cell})$ denotes the expected **i**-th moment of the photon count number or digitized value provided the sensed volume is situated in or on the cell, or soluble or solid support, as a function of the parameters $\mathbf{a}_{cell}$ and
   $M_i^{(medium)}(\mathbf{a}_{medium})$ denotes the expected **i**-th moment of the photon count number or digitized value provided the sensed volume is situated in the medium, as a function of the parameters $\mathbf{a}_{medium}$.

2. The method according to claim 1 wherein the medium is a liquid or gel.

3. The method according to claim 1 or 2 wherein the medium comprises fluorescent ligands capable of binding to said cells, or soluble or solid supports and wherein $M_i^{(medium)}$ is calculated from cumulants $K_i = c_{ligand} q_{ligand}^i$, where c_{ligand} denotes the mean number of ligands per sensed volume,
   and q_{ligand} denotes the mean number of photon counts detected per ligand by one or more photon detectors.

4. The method according to any of claims 1 or 3 wherein values of those characteristic parameters of the cells, or soluble or solid supports which are expected to be constant through a series of samples are fixed to a single value while estimating further characteristic parameters from said series of moments $\hat{M}_i$.

5. The method according to any of claims 1 to 4, further comprising:
   applying an image analysis procedure to determine one or more of the parameters of P_{cell}, $\mathbf{a}_{cell}$, or $\mathbf{a}_{medium}$.

6. The method according to any of claims 1 to 5, further comprising:
   monitoring intensity of emission scattered by said samples.

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7. The method according to any of claims 1 to 6 wherein said characteristic parameters of said cells, or soluble or solid supports $\mathbf{a}_{cell}$ are chosen from the group consisting of concentration, specific brightness, width of a brightness distribution, and a function of intensities of different polarization components of emission or spectrally different components of emission.

8. The method according to any of claims **1** to **7** wherein the number of photon counts is determined using at least two photon detectors monitoring emission of different wavelength and/or of different polarization and wherein a series of at least two-dimensional moments $\hat{M}_{ij}$ of photon count numbers n_1 and n_2 is calculated, defined as

$$\hat{M}_{ij} = \sum_{n_1}\sum_{n_2} n_1^i n_2^j \hat{P}(n_1, n_2) = average(n_1^i n_2^j).$$

9. The method according to any of claims 1 to 8 wherein said plurality of sensed volumes is monitored sequentially in time by scanning a sensed volume through the sample.

10. The method according to any of claims 1 to 9 wherein said plurality of sensed volumes is monitored in parallel.

11. The method according to claims 9 or 10 wherein said plurality of sensed volumes are used to form a two-or three-dimensional image.

12. The method according to any of claims 1 to 11 wherein a confocal optical device is used for monitoring intensity of emission.

13. The method according to any of claims 1 to 12 wherein multiple photon excitation is employed to stimulate fluorescence emission.

14. The method according to any of claims 1 to 13 wherein in each dimension X, Y and Z the diameter of said cells, or soluble or solid supports is greater than the diameter of the sensed volume.

15. The method according to any of claims 1 to 14 for use in diagnostics, high throughput drug screening, optimization of properties of molecules or identification of specific cell, or soluble or solid carrier populations.

16. An apparatus for working the method according to claims 1 to 15 comprising:

- a stage for supporting a sample in an examination site, the sample comprising cells, or soluble or solid supports in a medium;
- a light source positioned to deliver light to the sample in the examination site;
- a detector positioned to receive fluorescence emitted from the sample in the examination site and constructed so that it determines numbers of photon counts **n** per counting time interval or digitized values **n** of fluorescence intensity per time interval in a plurality of sensed volumes and delivers the numbers **n** to an evaluation device;
- an evaluation device coupled to the detector for receiving and evaluating the numbers or values **n**; **characterized in that**
- the evaluation device is adapted to

(i) calculate a series of moments $\hat{M}_i$ of the measured photon count numbers or digitized values, defined as

$$\hat{M}_{\mathbf{i}} = \sum_{\mathbf{n}} \mathbf{n}^{\mathbf{i}} \hat{P}(\mathbf{n}) = average(\mathbf{n}^{\mathbf{i}}) = average(n_1^{i_1} n_2^{i_2} \ldots),$$

where $\hat{P}(\mathbf{n})$ denotes a normalized histogram of photon count numbers or digitized values, and
(ii) estimate characteristic parameters of the cells, or soluble or solid supports $\mathbf{a}_{cell}$ and of the medium $\mathbf{a}_{medium}$ from said series of moments $\hat{M}_i$ using a set of equations corresponding to different orders of the

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moment **i**, of a general form

$$\hat{M}_i = P_{cell} M_i^{(cell)}(a_{cell}) + (1 - P_{cell}) M_i^{(medium)}(a_{medium}),$$

where

P_{cell} denotes the probability that the sensed volume is situated in or on the cell, or soluble or solid support,
$M_i^{(cell)}(\mathbf{a}_{cell})$ denotes the expected i-th moment of the photon count number or digitized value provided the sensed volume is situated in or on the cell, or soluble or solid support, as a function of the parameters $\mathbf{a}_{cell}$ and
$M_i^{(medium)}(\mathbf{a}_{medium})$ denotes the expected *i*-th moment of the photon count number or digitized value provided the sensed volume is situated in the medium, as a function of the parameters $\mathbf{a}_{medium}$.

17. The apparatus according to claim 16 further comprising a confocal optical element located between the light source and the detector, the confocal optical element being capable of transmitting light substantially exclusively from a sensed volume of the sample.

18. The apparatus according to claim 16 or 17 wherein X, Y-axes are defined perpendicular to each other and parallel to the stage, further comprising an X, Y-axes adjustment device that automatically adjusts the relative positions of the sensed volume and the sample along the X and Y axes.

19. The apparatus according to any of claims 16 to 18 wherein a Z-axis is defined substantially perpendicular to the stage, further comprising a Z-axis adjustment device that automatically adjusts the relative positions of the sensed volume along the Z-axis.

20. An assay for screening a plurality of compounds for inhibition between a ligand and a target molecule expressed by or attached to a cell, or soluble or solid support, said assay comprising the steps of:

- adding a liquid suspension of said target molecules expressed by or attached to said cell, or soluble or solid support to a plurality of containers;
- adding a plurality of compounds to be screened for said inhibition to said plurality of containers;
- adding fluorescently labelled ligand to said containers;
- incubating said target molecules expressed by or attached to said cell, or soluble or solid support, said compounds, and said fluorescently labelled ligand;
- individually measuring amounts of fluorescence associated with said cells, or soluble or solid supports; and
- determining the degree of inhibition by one or more of said compounds of binding of said ligand to said target molecule;

wherein said amounts of fluorescence associated with said cells, or soluble or solid supports is estimated according to any of claims 1 to 15.

21. An assay for testing a compound to determine the degree of binding of said compound to a target molecule expressed by or attached to a cell, or soluble or solid support, said assay comprising the steps of:

- adding a liquid suspension of said target molecule expressed by or attached to said cell, or soluble or solid support to a container;
- adding a fluorescent compound to be tested for its binding to said container;
- incubating said target molecule expressed by or attached to said cell, or soluble or solid support and said compound;
- measuring amounts of fluorescence associated with said cell, or soluble or solid support; and
- determining the degree of binding of said compound to said target molecule;

wherein said amounts of fluorescence associated with said cell, or soluble or solid support is estimated according to any of claims 1 to 15.

22. The assay according to claim 21 wherein a plurality of different compounds and/or different targets is tested in a

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plurality of containers.

23. The assay according to any of claims 19 to 21 wherein said container is a micro- or nanoplate, preferably with a density of 96, 384, 768, 1536, 3456 or 9600 wells per plate.

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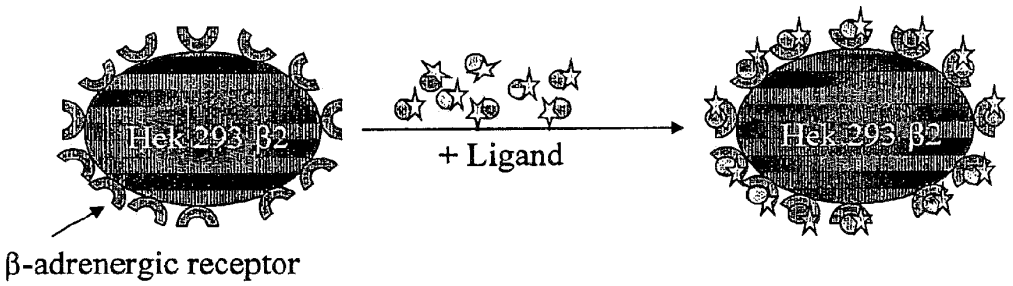


Fig. 1

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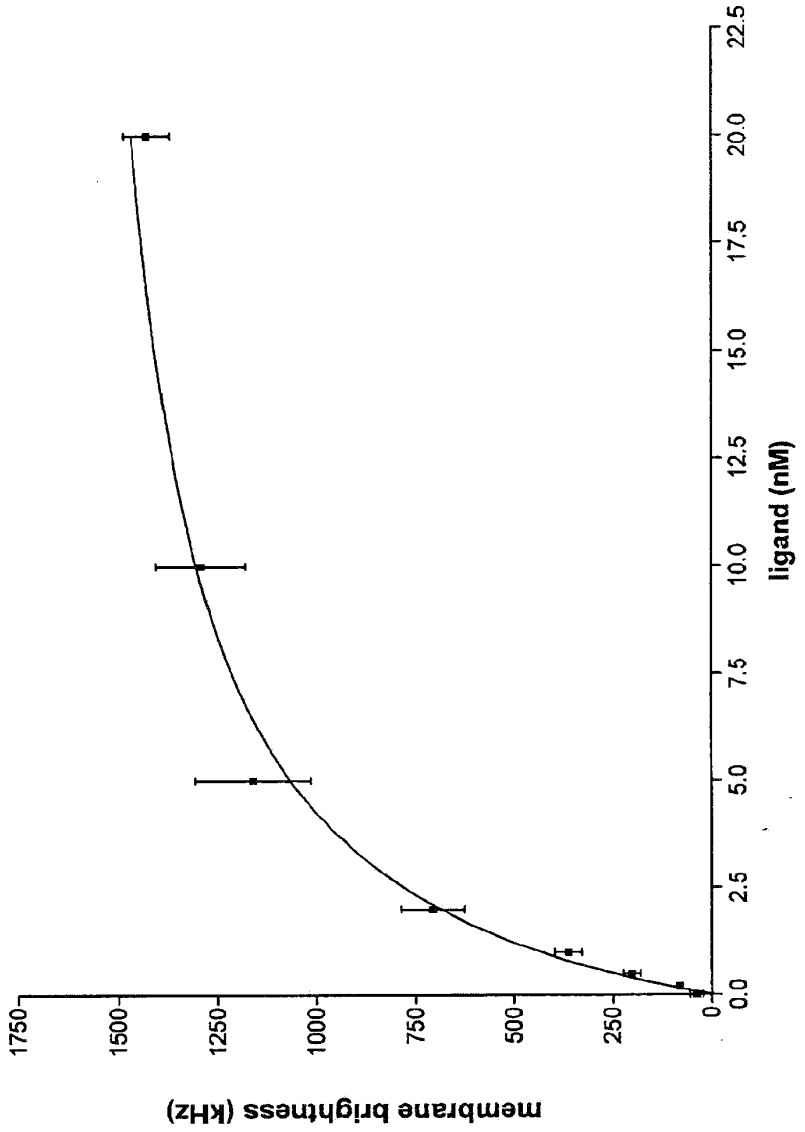


Fig. 2

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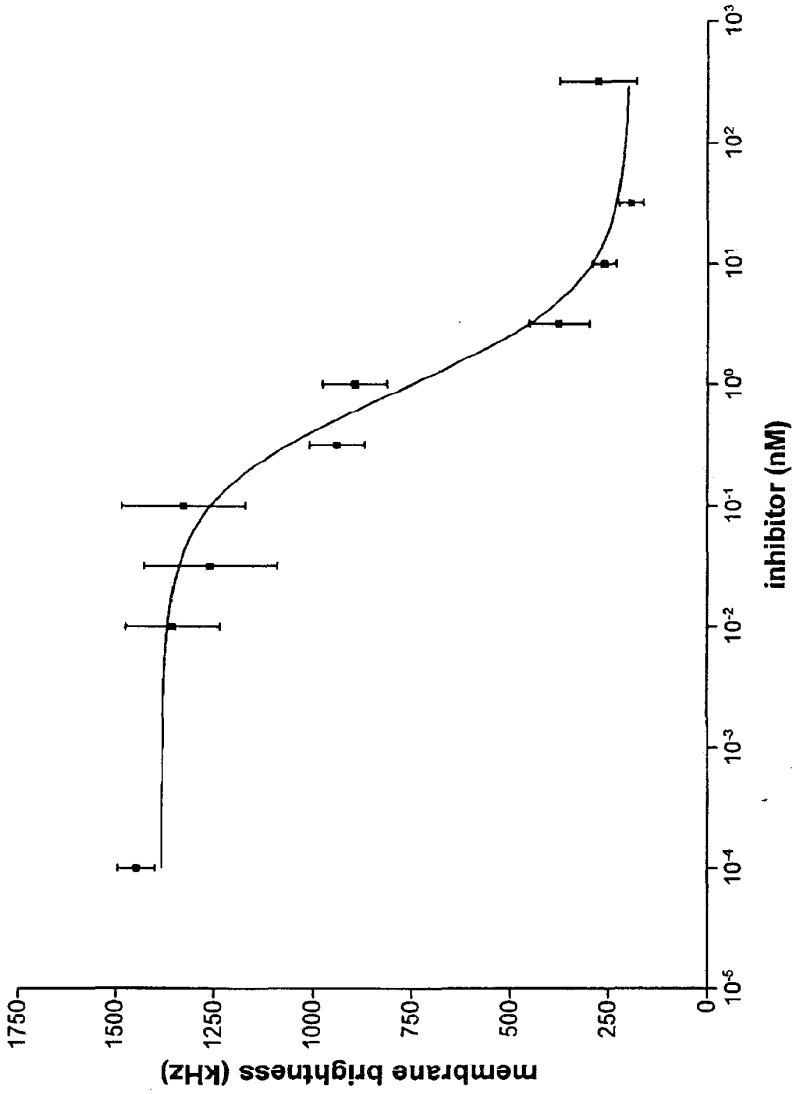


Fig. 3

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European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 00 11 7522

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Y	H. QIAN ET AL.: "Distribution of molecular aggregation by analysis of fluctuation moments" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 87, July 1990 (1990-07), pages 5479-5483, XP000647943 NATIONAL ACADEMY OF SCIENCE. WASHINGTON., US ISSN: 0027-8424 * page 5482, right-hand column, paragraph 1 - paragraph 3 * * figures 1,3; tables 2,3 *	1,2,5,7, 10,11,16	G01N21/64
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	19 January 2001	Thomas, R.M.

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X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
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T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
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Espacenet

Bibliographic data: EP1259810 (A2) — 2002-11-27

ANALYTICAL MEASURING AND EVALUATION METHOD FOR MOLECULAR INTERACTIONS

No documents available for this priority number.

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Application number: EP20010915204 20010207

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Abstract not available for EP1259810 (A2)
Abstract of corresponding document: WO0157520 (A2)

The invention relates to an analytical measuring and evaluation method for determining the interaction parameters between an analyte and a ligand, preferably in a biosensor. According to the inventive method, the concentration of the analyte is gradually changed at defined intervals ti and the initial association or dissociation rates or association and dissociation constants are determined. The invention further relates to a device for carrying out the inventive method.

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(54) **ANALYTISCHES MESS- UND AUSWERTEVERFAHREN FÜR MOLEKULARE INTERAKTIONEN**

ANALYTICAL MEASURING AND EVALUATION METHOD FOR MOLECULAR INTERACTIONS

PROCEDE ANALYTIQUE DE MESURE ET D'EVALUATION D'INTERACTIONS MOLECULAIRES

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Anmerkung: Innerhalb von neun Monaten nach der Bekanntmachung des Hinweises auf die Erteilung des europäischen Patents kann jedermann beim Europäischen Patentamt gegen das erteilte europäische Patent Einspruch einlegen. Der Einspruch ist schriftlich einzureichen und zu begründen. Er gilt erst als eingelegt, wenn die Einspruchsgebühr entrichtet worden ist. (Art. 99(1) Europäisches Patentübereinkommen).

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Beschreibung

1. Anwendungsgebiet

[0001] Die Erfindung bezieht sich auf ein Meß- und Auswerteverfahren zur Bestimmung von Interaktionsparametern zwischen einem Analyt und einem Ligand wie unter anderem Geschwindigkeitskonstanten oder Bindungspartner-Aktivitäten. Die Aufnahme der Meßwerte erfolgt z. B. mit Biosensoren, in denen ein i. allg. immobilisierter erster Bindungspartner (Ligand L) mit einem zweiten Bindungspartner (Analyt A) versetzt wird und die Bildung des Ligand-Analyt-Komplexes LA im zeitlichen Verlauf erfaßt wird.

2. Technischer Hintergrund

[0002] Sogenannte Biosensoren zur Erfassung der zeitlichen Verläufe der Bildung von Analyt-Ligand-Komplexen sind in den unterschiedlichsten Ausführungen bekannt, wobei auch andere Vorrichtungen wie u. a. Array-Systeme zur Erfassung derartiger Reaktionen verwendbar sind. Dabei werden sowohl die zeitlichen Verläufe der Assoziation des Analyten am Liganden erfaßt, wenn die Analytkonzentration durch die Zugabe einer Analytlösung bestimmter Konzentration erhöht wird, sowie die Dissoziation bei Zugabe einer geringer konzentrierten Lösung oder mit einer Konzentration von Null. I. allg. wird der zeitliche Verlauf der Komplexbildung mit der Funktion R(t) oder R_t beschrieben, wobei die Konzentration des Komplexes bzw. seine zeitliche Änderung mit c_t(LA) bzw. dc_t(LA)/dt bezeichnet wird. In erster Näherung wird für den zeitlichen Verlauf der Funktion R ein exponentieller Verlauf erster Ordnung mit dem Exponenten k_{on} bei der Assoziation und k_{off} bei der Dissoziation des Komplexes angenommen. Zur Erfassung dieser Werte ist es bisher üblich, die Reaktion, z. B. die Komplexbildung, teilweise oder bis zum Erreichen eines Gleichgewichtswertes R_{eq}, bei dem die Assoziation des Komplexes aus Analyt und Ligand im Gleichgewicht steht mit dessen Dissoziation, auszumessen. Es folgt eine Dissoziation des Komplexes und eine Regenerations- und Waschphase. Dies ist ein sehr zeitaufwendiges Verfahren, insbesondere wenn hintereinander mehrere Messungen mit unterschiedlichen Konzentrationsänderungen vorgenommen werden sollen. Eine weitere Auswertung der erhaltenen Exponenten führt zu den eigentlich interessierenden kinetischen Geschwindigkeitskonstanten, das Verfahren bedarf jedoch einer konstanten Analytkonzentration.

[0003] Bei den bisherigen Auswertungsverfahren der Meßwerte wird meistens davon ausgegangen, daß die Konzentration des zu dem Liganden zugegebenen Analyten trotz der Komplexbildung konstant ist. Dies wird z. B. dadurch angenähert, daß die Analytkonzentration vielfach höher ist als die Ligandkonzentration oder durch ständigen Austausch im Durchflußsystem. Tatsächlich jedoch tritt z. B. bei der Assoziation im Küvettensystem immer eine Verarmung des Analyten oder eine Konzentrationsänderung auf, die dazu führt, daß der Gleichgewichtswert R'_{eq}, der tatsächlich erreicht wird, sich von dem hypothetischen Wert R_{eq} bei konstanter Analytkonzentration unterscheidet. Eine Analyse der Meßwerte mit einer Näherungsfunktion zweiter Ordnung ergibt zwar einen genaueren Wert des Koeffizienten der Exponentialfunktion, ist aber aufwendig und bedarf der zusätzlichen Ermittlung bzw. Berücksichtigung einer Reihe von Randbedingungen des Tests.

[0004] Zur Konzentrationsbestimmung werden initiale Raten herangezogen. Die initialen Raten werden dadurch erhalten, daß in den Anfang der Assoziationskurve eine Ausgleichsgerade gelegt wird, deren Steigung die initiale Rate aber unterbewertet, da die Gerade die Krümmung der Kurve nicht berücksichtigt. Desweiteren ist es bekannt, daß beim Auftragen der initialen Raten in einem Diagramm über die Konzentration des Analyten c(A), bei mehrmaliger Messung mit jeweils unterschiedlichen Konzentrationen, theoretisch eine Gerade durch den Ursprung mit der Steigung $R_{max} * k_{ass}$ erhalten wird, wobei R_{max} die maximal mögliche Reaktion z. B. des Biosensors auf die Zugabe eines Analyten im Überschuß darstellt. Die Steigung dieser Geraden ist aber durch die o.g. Unterbewertung ebenfalls verfälscht, so daß diese Art von Diagramm nicht zur Bestimmung von k_{ass} herangezogen wird.

[0005] Zur Erfassung der Meßwerte werden u. a. Biosensoren nach dem Durchflußprinzip oder dem Küvettenprinzip verwendet, wobei die unterschiedlichsten Meßverfahren im Stand der Technik bekannt sind. Bei Durchflußsystemen wird eine Sensoroberfläche, auf der der Ligand immobilisiert ist, pro Analyse mit einem konstanten Strom einer Analytlösung beaufschlagt. Hierbei ist die Näherung einer vorgewählten konstanten Analytkonzentration im Prinzip gegeben. Bei Küvettensystemen wird eine Meßzelle mit einer Analytlösung gefüllt und die Reaktion mit dem Liganden an einer Sensoroberfläche erfaßt. Besonders hier tritt eine Verfälschung der Meßergebnisse auf, da bei tatsächlichen Reaktionen die Analytkonzentration verändert wird. Werden mehrere Meßvorgänge mit unterschiedlichen Konzentrationen hintereinander durchgeführt, so wird normalerweise die Küvette mit einer Pufferlösung gespült oder auf andere Weise regeneriert, und anschließend eine Lösung mit geänderter Konzentration eingebracht.

[0006] Bei mehrfachen Titrationen, d. h. Änderung der Konzentration, werden die Meßkurven bisher so lange aufgenommen, bis die Komplexbildung einen Gleichgewichtszustand erreicht hat. Es werden lediglich die Gleichgewichtszustände zur Bestimmung weiterer Größen, nicht jedoch der Kinetik oder initialer Raten, verwendet. Vielmehr wird bei solchen Mehrschritt-Titrationen ein Zugang zur Kinetik ausdrücklich ausgeschlossen.

[0007] So offenbaren z.B. Myszka D.G. et al., Anal Biochem. 265 (1998) 326-330, eine solche sukzessive Erhöhung

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der Analytkonzentration von außerhalb des Messgerätes (d.h. weit entfernt vom Messpunkt), die jedoch eine Gleichgewichtseinstellung nach jeder Konzentrationsänderung verlangt, was insgesamt bis zu mehr als 10 Stunden dauern kann und einen entsprechend hohen Probenverbrauch bedeutet. Zudem bewirkt der Wechsel einer Analyt-Lösung außerhalb des Gerätes eine deutliche Vermischung mit der vorauslaufenden Lösung auf dem Weg zum Messpunkt und damit eine lange andauernde Verzerrung der zeitlichen Messkurven. Daher können lediglich die finalen Gleichgewichtswerte hinsichtlich der allgemeinen Affinität bewertet, nicht jedoch die Messkurven bezüglich der gewünschten kinetischen Konstanten mittels eines kinetischen Fittings ausgewertet werden.

[0008] Für Durchfluß-Systeme sind sequentiell bzw. hintereinander angeordnete sogenannte Probenschleifen bekannt, die nacheinander befüllt und in die Meßkammer ausgespült werden können. Jedoch weisen die Probenschleifen jeweils ein produktionstechnisch aufwendiges exakt definiertes Volumen und zwischen ihnen ein undefiniertes Extravolumen, das auch als Totvolumen bezeichnet wird, auf. Im Totvolumen kann es zu unerwünschten Durchmischungen verschiedener Lösungen kommen. Die Schleifen sind weder von ihrer Auslaß-Seite her noch teilweise und auch nicht unabhängig voneinander befüllbar; und sie sind nicht unabhängig voneinander in die Meßkammer spülbar.

3. Darstellung der Erfindung

a) Technische Aufgabe

[0009] Ausgehend vom Stand der Technik hat sich die Erfindung die Aufgabe gestellt, ein Verfahren anzugeben, mit dem die Meßzeiten bei der Erfassung der Meßwerte bei der Komplexbildung verkürzbar sind, die tatsächlichen Verhältnisse bei der Komplexbildung, d. h. Änderung der Analytkonzentration, berücksichtigt werden, und die Durchführung mehrerer Messungen hintereinander ohne aufwendige Spülvorgänge z. B. in einer Küvette möglich ist, sowie es möglich ist, die kinetischen Konstanten aus den initialen Raten, und unter idealen Verhältnissen auch aus den Kurvenexponenten zu ermitteln.

b) Lösung der Aufgabe

[0010] Erfindungsgemäß wird diese Aufgabe durch die Verfahrensmerkmale des Anspruchs 1 und die Vorrichtungsmerkmale des Anspruchs 16 gelöst.

[0011] Vorteilhafte Ausgestaltungen der Erfindung sind jeweils Gegenstand von Unteransprüchen.

[0012] Der Kemgedanke der Erfindung besteht darin, daß die Änderung der Analytkonzentration sowohl bei der Assoziation als auch der Dissoziation des Komplexes berücksichtigt wird. Dadurch nimmt die Funktion R einen Gleichgewichtswert R'_{eq} an, der sich vom Wert R_{eq} unterscheidet. Bei der Assoziation, d.h. Zugabe eines Analyten erhöhter Konzentration zu dem Liganden, tritt während der Assoziation eine Verarmung des Analyten auf, die dazu führt, daß R'_{eq} kleiner ist als der theoretische Wert R_{eq}. Im ersten Moment der Reaktion jedoch, d. h. wenn die Analytlösung z. B. in die Küvette eines Biosensors eingebracht wird, ist die initiale Rate der Funktion R unabhängig davon, ob im späteren Verlauf eine Analytverarmung durch Bindung des Analyten an den Liganden auftritt oder konstante Analytkonzentration angenommen wird. Zum Zeitpunkt t = 0 hat noch keine Reaktion zwischen Analyt und Ligand stattgefunden. Durch die Berücksichtigung des tatsächlichen Reaktionsverlaufs ändert sich auch der Exponentialkoeffizient von k_{on} in k'_{on}. Tatsächlich jedoch bleibt die Beziehung zwischen den einzelnen Größen der Exponentialfunktion erhalten, d. h.:

$$k'_{on} * R'_{eq} = \frac{dR_{ass}(t=0)}{dt} \qquad (1)$$

und

$$k'_{off} * \Delta R' = \frac{dR_{diss}(t=0)}{dt} \qquad (2)$$

wobei $\Delta R'$ die Differenz zwischen dem Gleichgewichtswert der Dissoziation und dem Startwert R_{st} der Funktion R ist.

[0013] Das bedeutet, daß aus den tatsächlichen Meßwerten z. B. die initiale Rate ermittelbar ist. Dies gilt ebenso, wenn die Exponentialfunktion zwar nach 1. Ordung, aber mit zwei Koeffizienten $k'_{on(1)}$ und $k'_{on(2)}$ verläuft, wobei sich die beiden korrespondierenden initialen Raten zu einer Gesamtrate addieren.

[0014] Dabei ist es möglich, daß die Meßkurve der Funktion R bis zum Erreichen des Gleichgewichtswertes R'_{eq} aufgenommen wird, um z. B. mit Hilfe eines mathematischen Programms den Exponenten k'_{on} oder k'_{off} zu ermitteln

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und daraus die initialen Raten der Assoziation oder Dissoziation. Im Rahmen der Erfindung wird lediglich ein Teil der Kurve erfaßt, d.h. die Erfassung der Funktion R wird zu einem früheren Zeitpunkt abgebrochen, um aus einer Extrapolation des Kurvenabschnitts die Gleichgewichtslage zu ermitteln. Der Exponent k wird dabei mit einer non-linearen Näherung erhalten, kann aber auch über die Lineare Regression der Ableitung der Funktion R bestimmt werden.

[0015] Aus den initialen Raten können dann die jeweiligen Assoziations- und Dissoziationsgeschwindigkeitskonstanten k_{ass} und k_{diss} ermittelt werden gemäß:

$$\frac{dR_{ass}(t=0)}{dt} = k_{ass} * R_{max} * c_0(A) \qquad (3)$$

im Falle der Komplexassoziation mit der Startkonzentration des Analyten $c_0(A)$ bzw.:

$$\frac{dR_{diss}(t=0)}{dt} = k_{diss} * (-R_{st}) \qquad (4)$$

im Falle der Komplexdissoziation, wobei die Größe R_{st} den Startwert der Funktion R bei der Dissoziation angibt. Die Startkonzentration $c_0(A)$ ist i. allg. durch den Versuch von außen vorgegeben.

[0016] Der Vorteil der Erfindung besteht darin, daß mehrmals hintereinander, z. B. in einer Küvette, eine Messung mit jeweils schrittweise veränderter Analytkonzentration durchführbar ist, wobei es nicht notwendig ist, daß jede Messung bis zum Erreichen des Gleichgewichtswertes zu Ende geführt wird, sondern bereits vorher abgebrochen werden kann bzw. die Konzentration des Analyten erhöht oder erniedrigt werden kann. Auch die Durchführung von Meßserien mit mehrmaliger auch abwechselnd aufeinanderfolgender Analyterhöhung und -verminderung zur Erfassung von Assoziations- und Dissoziationskurven ist möglich.

[0017] Obschon sich die Start-Raten von multiplen, i-fachen Assoziationsschritten aus der Summe der vorangegangenen und der neuen Assoziation bilden, lassen sich die Netto-Start-Raten der jeweiligen Assoziationsschritte basierend auf den eingangs formulierten Gleichungen (1) und (3) für die Assoziation ermitteln gemäß:

$$k'_{on,i} * (R'_{eq,ass,i} - R'_{st,ass,i}) = (dR_{ass,i}/dt)_{t=0} \qquad (5)$$

und:

$$(dR_{ass,i}/dt)_{t=0} - (dR_{ass,i-1}/dt)_{t=st,ass,i} = (dR_{ass,i,net}/dt)_{t=0} \qquad (6)$$

und auswerten gemäß:

$$(dR_{ass,i,net}/dt)_{t=0} / c_{0,i,net}(A) = -k_{ass} * R_{st,ass,i} + k_{ass} * R_{max} \qquad (7)$$

was einer Geradengleichung entspricht und sich entsprechend auftragen lässt.

Der Quotient aus initialer Netto-Assoziationsrate und Analyt-Netto-Startkonzentration, aufgetragen gegen das Assoziations-Startsignal $R_{st,ass,i}$, liefert linear gefittet eine Gerade mit der Steigung = $-k_{ass}$ und einen x-Achsenabschnitt R_{max}. Die einzelnen Phasen multipler Dissoziationsschritte aus Küvettensystemen können direkt nach den Gleichungen (2) und (4) für die Dissoziation ausgewertet werden.

[0018] Die Berechnung von sogenannten Fit-Kurven, mit denen die erhaltenen Meßergebnisse angenähert oder im Falle des vorzeitigen Abbruchs der Messung extrapoliert werden, erfolgt mit mathematischen Annäherungsprogrammen, die eine Exponentialfunktion, auch eine Doppelt-Exponentialfunktion, erster oder höherer Ordnung ermitteln, die die Meßergebnisse mit größtmöglicher Genauigkeit annähert.

[0019] Die Berechnung einer derartigen Kurve kann durch die Auswertungseinheit z. B. eines Biosensors selbsttätig während der Messung erfolgen.

[0020] Die schrittweise Änderung der Konzentration in der Meßkammer kann zu beliebigen Zeitpunkten während der

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Aufnahme der Meßergebnisse erfolgen. Insbesondere wird jedoch die Halbwertszeit der Funktion R vorgeschlagen, zu der die Funktion, beispielsweise bei einer Assoziation die Hälfte ihres Endwertes R_{eq} erreicht hat.

[0021] Wird die Ableitung der Funktion R(t) in einem Diagramm gegen die Funktion R(t) aufgetragen, so wird, wenn die aufgetragenen Werte z. B. mit einer linearen Regression genähert werden, eine Gerade erhalten, deren y-Achsenabschnitt gerade der initialen Rate entspricht, die sich auch, und in Übereinstimmung mit der Gleichung (1), aus dem absoluten Wert der Geradensteigung (-k'on) und dem x-Achsenabschnitt R'_{eq} ergibt.

[0022] Zur Vereinfachung der Auswertung wird angenommen, daß die Reaktionen unter idealen Bedingungen ablaufen, d. h., daß insbeondere keine Analytverarmung auftritt. Dies kann z. B. bei Biosensoren mit Durchflußsystemen angenähert werden, indem ein stetiger Durchsatz der Lösung in der Meßkammer erfolgt. damit vereinfachen sich die Näherungsfunktionen, da z. B. eine Exponentialfunktion erster Ordnung für den Verlauf der Funktion R angenommen werden kann.

[0023] Gemäß obenstehender Formel (3) zur Bestimmung der Assoziationsgeschwindigkeitskonstante kann, wenn die anderen Größen bekannt sind, auch die Anfangskonzentration $c_0(A)$ einer neu in eine Meßkammer eingebrachten Lösung bestimmt werden. Dazu kann z. B. in einer vorhergehenden Versuchsreihe mit bekannten $c_0(A)$-Werten aus den ermittelten initialen Raten eine Kalibrationsgerade erstellt werden gemäß initiale Rate = $f(c_0(A))$; der unbekannte $c_0(A)$-Wert einer Probe kann dann mit der aus den Meßwerten ermittelten initialen Rate aus der Kalibrationsgeraden berechnet werden.

[0024] Ebenso ist es, besonders unter Verwendung von Küvettensystemen, möglich, daß aus den aus einem Assoziationsvorgang erhaltenen Werten die Dissoziationsgeschwindigkeitskonstante erhalten wird und umgekehrt. Dies beruht darauf, daß z. B. bei einer Assoziation im ersten Moment, zu dem die Reaktion beginnt, noch keine Dissoziation erfolgt. Da die Funktion R aber einen Gleichgewichtswert annimmt, bei dem Assoziation und Dissoziation im Gleichgewicht stehen, enthält dieser Wert also auch Informationen über $k_{diss.}$ Die Umkehrung dieses Ansatzes führt zu einem analogen Ergebnis für die Dissoziation. Die entsprechenden Formeln lauten:

$$k_{ass} = \frac{\left(dR_{diss}/dt\right)_{t=0}}{\left(\frac{c_{eq'\_diss}(A)}{R_{eq'\_diss}}\right)_{c_{eq'\_diss}=0} \cdot R_{max}} \qquad (8)$$

wobei ΔReq-st_diss der Differenz zwischen dem Endwert, der idealerweise 0 ist, und den Anfangswerten von R bei der Dissoziation entspricht, $C_{eq'\_diss}$ (A) die Konzentration von A nach der Dissoziation im Gleichgewicht ist und $R_{eq'\_diss}$ der Gleichgewichtswert von R nach der Dissoziation ist, und in den Nenner der Grenzwert für $c_{eq'\_diss} = 0$ eingesetzt wird, und:

$$k_{diss} = \frac{\left(dR_{ass}/dt\right)_{t=0}}{\left(\frac{R_{eq'\_ass}}{c_{eq'\_ass}(A)}\right)_{R_{eq'\_ass}=0} \cdot c_0(A)} \qquad (9)$$

mit den entsprechenden Werten für die Assoziation.

[0025] Ebenso kann das Verfahren zur Auswertung bei anderen Affinitätsbiosensoren oder Array-Systemen in Lösung angewendet werden, sowie bei zeitaufgelösten ELISA- oder RIA-Techniken oder anderen festphasengestützten Systemen, die dem Stand der Technik entnehmbar sind.

[0026] Zur Realisierung einer ständig die Meßkammer durchströmenden Lösung wird in einem Küvettensystem die Konzentration der Lösung durch ständige Zugabe und Entnahme eines Teils der Lösung konstant gehalten. Dazu sind z. B. zwei Kanülen mit Lösungen vorhanden, mit denen entweder ständig frische Lösung in der Meßkammer umgewälzt wird, oder mit denen ein Teil der Lösung ständig entnommen und wieder zugeführt wird, um ein Durchflußsystem mit konstanter Konzentration zu simulieren.

[0027] In diesem Fall, wie auch in idealen Durchfluß-Systemen mit konstanter Konzentration der Lösung in der Meßzelle, wird die Funktion R durch eine Exponentialfunktion erster Ordnung angenähert und es gilt folgende Beziehung:

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$$k'_{on,i} = k_{ass} * c_{0,i}(A) + k_{diss} \quad (10)$$

wobei der Index i die i-te schrittweise Änderung der Konzentration in einem Mehrschritt-Experiment angibt. Nach diesem Verfahren lassen sich beide Geschwindigkeitskonstanten aus derselben Beziehung ermitteln.

[0028] Für ideale Durchfluß-Systeme mit konstant gehaltener Analyt-Startkonzentration während einer Assoziationsphase ist als bekannt gezeigt worden, daß die Startkonzentration genau dann gleich der Dissoziations-Gleichgewichtskonstante $K_D = k_{diss}/k_{ass}$ ist, wenn mit dieser Konzentration die Hälfte der immobilisierten Ligandenmoleküle mit Analytmolekülen gebunden werden, d.h. ½ R_{max} erreicht wird. Für diesen Fall läßt sich darüber hinaus aber zeigen, daß die Assoziationsphase bis zur Erreichung des Gleichgewichtswertes gerade halb so lange dauert wie die sich anschließende Dissoziationsphase, um wieder auf den Ausgangswert Null zurückzukehren. Bei höheren Analyt-Startkonzentrationen verläuft die Assoziation schneller. Unter dieser Voraussetzung schneidet die Dissoziationskurve, wenn sie mit der vorausgelaufenen Assoziationskurve überlagert wird, diese Assoziationskurve exakt im Goldenen Schnitt des Assoziations-Gleichgewichtswertes, was dem Wert 0,618...* R_{eq_ass} entspricht. D.h. durch die Applikation verschiedener Analyt-Startkonzentrationen und eine darauf folgende Regression auf den oben beschriebenen Schnittmeßwert läßt sich der K_D-Wert extrapolieren. Umgekehrt muß eine Analyt-Startkonzentrationen, numerisch im K_D-Wert appliziert, den oben beschriebenen Schnittmeßwert ergeben; andernfalls verläuft die aufgezeichnete Interaktion nicht ideal. Prinzipiell erlaubt die Multi-Schritt-Kinetik einen analogen Ansatz.

[0029] Generell, insbesonders für Küvettensysteme mit variabler Analytkonzentration in der Meßzelle während eines Meßschrittes, gelten desweiteren die folgenden Beziehungen:

$$k'_{on} = k_{ass} * c'_{eqass}(A) + k'_{offmax} \quad (11)$$

mit der Geradensteigung k_{ass}, wobei $c'_{eqass}(A)$ die Analytgleichgewichtskonzentration nach der Assoziation und k'_{offmax} der Maximalwert des Koeffizienten der Exponentialfunktion bei der Dissoziation ist; und

$$k'_{off} = k_{diss} * (-R_{stdiss})/(R'_{eq} - R_{stdiss}) \quad (12)$$

mit der Geradensteigung k_{diss}, wobei der Index stdiss den Startwert bei der Dissoziation bedeutet.

c) Ausführungsbeispiele des Biosensors

[0030] Zur Durchführung des Verfahrens wird ein an sich bekannter Biosensor verwendet, der mit einer Sensoroberfläche ausgestattet ist, an der die Liganden immobilisiert d. h. gebunden sind. Diese Bindung kann z. B. wie im Stand der Technik bekannt über eine chemische Bindung des Liganden oder mit einem auf den Liganden abgestimmten Rezeptor erfolgen. Über der Sensoroberfläche ist eine Meßkammer oder Küvette ausgebildet, die mit einer Lösung des Analyten beaufschlagbar ist. Zur Erfassung der Komplexbildung, d. h. der Bindung des Analyten am Liganden, sowie deren zeitlicher Verlauf können verschiedene Meßverfahren verwendet werden, z. B. die Erfassung des Reflexionsvermögens der Rückseite des Sensors, das sich mit dem Grad der Komplexbildung c(LA) ändert. Zum Einbringen der Lösung in die Meßkammer dient eine Kanüle, die mit der Meßkammer verbunden ist und entweder die Lösung zugibt und/oder absaugt. Die Meßkammer kann auch mit zwei getrennten Kanülen zur Zugabe/Herausnahme ausgestattet sein. Auch die Beaufschlagung mit einer Spülflüssigkeit über eine der Kanülen ist möglich. Zur Durchmischung der Lösung in der Meßkammer, um Konzentrationsgradienten in der Lösung während der Reaktion zu vermeiden, ist eine Vorrichtung zur Durchmischung der Lösung vorhanden, dies kann z. B. ein Vibrationsrührer sein. Die Ausstattung des Biosensors mit einer vorzugsweise elektronischen Steuerung sowie geeigneten Meßgeräten nebst Datenerfassungssoftware ist dem Stand der Technik entnehmbar.

[0031] Erfindungsgemäß ist der Biosensor derart ausgebildet, daß die Lösung in der Meßkammer zumindest teilweise instantan mehrmals mit Lösungen unterschiedlicher Analytkonzentration austauschbar ist, wobei das Flüssigkeitsvolumen konstant gehalten wird. Das bedeutet, daß zumindest ein Teil der Lösung in der Meßkammer, oder die gesamte Lösung, entnommen und gleichzeitig eine neue Lösung mit einer anderen Konzentration zugegeben wird. Durch den Austausch zumindest eines Teils der Lösung durch eine andere Lösung mit gewünschter Konzentration wird die Gesamtkonzentration in der Meßkammer in definierter Weise verändert. Die Art des Austauschens der Flüssigkeit kann im Rahmen der Erfindung beliebig ausgeführt oder wie im folgenden dargelegt vorgenommen werden. In einem Durch-

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flußsystem wird die komplette Lösung in der Meßkammer ausgetauscht, bei einem Küvettensystem zumindest ein Teil davon. Mit dem mehrmaligen Austauschen der Lösung wird eine schrittweise Änderung der Konzentration in der Meßkammer ermöglicht, um das erfindungsgemäße Verfahren durchführen zu können.

[0032] Der Vorteil des erfindungsgemäßen Biosensors besteht darin, daß z.B. bei Küvettensystemen das Volumen der Lösung konstant gehalten ist und damit die Anzahl der Parameter bei der mathematischen Auswertung der Meßergebnisse verringert wird. Der instantane Austausch der Lösungen führt zudem besonders bei Durchflußsystemen zu scharfen Übergängen zwischen einzelnen Assoziations- bzw. Dissoziationsphasen, was für die Bestimmung der initialen Raten notwendig ist.

[0033] Vorzugsweise erfolgt der Austausch zumindest eines Teils der Lösung in der Meßkammer über eine oder über mehrere Kanülen gleichzeitig. Insbesondere wird die Lösung mit einer Kanüle entnommen und mit einer anderen Kanüle eine neue Lösung mit einer beliebig wählbaren Konzentration zugegeben.

[0034] Werden die Kanülen getrennt voneinander an der Meßkammer angeordnet, z. B. einander gegenüberliegend, kann die Entnahme und Zuführung auch gleichzeitig erfolgen, wobei zum vollständigen Austausch der Lösung in einem Küvettensystem die Entnahme bzw. Zuführung derart erfolgt, daß die Meßkammer durchgespült wird, um das Verbleiben unerwünschter Rückstände zu vermeiden. Die Kanülen können auch koaxial angeordnet werden, d. h. eine verläuft in der anderen, wobei die innere entweder die Lösung entnimmt oder zuführt.

[0035] Zur Förderung der Lösungen durch die Kanülen in die Meßkammer hinein oder heraus sind Pumpen vorgesehen, mit denen die Menge der jeweils geförderten Lösung dosierbar ist. Dazu werden sogenannte Mikropumpen verwendet, die eine Dosierung mit der gewünschten Genauigkeit erlauben, um wohl definierte Mengen an Lösung der Meßkammer zu entnehmen oder ihr zuzuführen. Diese Pumpen sind unter anderem als Schlauchpumpen, Spritzenpumpen oder piezoelektrische Pumpen auslegbar, wobei die Betätigung mit Stellmotoren, elektrischen Antrieben etc. erfolgt und sie ebenfalls elektronisch angesteuert sind.

[0036] Die Durchmischung der Lösung in der Meßkammer kann auch dadurch erfolgen, daß mit einer oder mehreren Kanülen ein Teil der Lösung angesaugt und sofort wieder zurückgepumpt wird, so daß die Lösung ständig umgewälzt und durchmischt wird. Vorzugsweise erfolgt eine Umwälzung mit mindestens 1 µl/min, um eine homogene Konzentration der Lösung in der Meßkammer während der Komplexbildung zu gewährleisten.

[0037] Eine weitere vorteilhafte Ausgestaltung der Erfindung besteht darin, daß an der Meßkammer in bestimmter Weise zwei oder mehr sogenannte Probenschleifen beliebigen Volumens vorhanden sind, die manuell und/oder mittels eines Probengebers automatisch befüllt werden können. Vorzugsweise sind die Probenschleifen parallel angeordnet zwischen zwei Mehrfach-Wahlventilen, wobei diese gesamte Anordnung über ein Schaltventil in den Durchfluß einschaltbar ist. Die Probenschleifen können so außerhalb des Durchflusses einerseits über die Wahlventile unabhängig voneinander und andererseits über das Schaltventil von ihrem Ausflußende her ganz oder nur teilweise befüllt werden, um z. B. Analytlösung zu sparen. Nach der Befüllung steht an der Schnittstelle zum Durchfluß am Schaltventil ohne andere Zwischenvolumina, d.h. ohne sogenanntes Totvolumen, die Lösung der zuletzt befüllten Probenschleife an. Das bedeutet, daß die Meßkammer mit mehreren unterschiedlichen Lösungen aus den einzelnen Probenschleifen über die Wahlventile nacheinander, unabhängig voneinander und totvolumenfrei im Durchfluß beaufschlagbar ist, und ohne daß eine beliebige Probenschleife vollständig ausgespült bzw. der Fluß durch diese in eine andere Schleife geleitet werden muß. Damit ist eine variable, instantane, praktisch durchmischungsfreie und probensparende schrittweise Konzentrationsänderung gewährleistet. Zudem kann jeweils die Konzentration des Analyten in der Lösung in der Meßkammer in erster Näherung konstant gehalten werden, um eine Verarmung zu verhindern. Vorzugsweise ist in jeder Probenschleife eine Lösung unterschiedlicher Konzentration vorhanden, um Messungen mit unterschiedlichen Konzentrationen auch unmittelbar hintereinander ausführen zu können oder durch die gezielte Ansteuerung von Probenschleifen eine Zwischenkonzentration zwischen den Werten in den einzelnen Probenschleifen zu erhalten. Die Zahl der Probenschleifen ist praktisch zwar nicht begrenzt; zwecks höherer Flexibilität kann jedoch die o.g. gesamte Anordnung mitsamt des Schaltventils z.B. zwischen zwei im Durchfluß positionierten Zweifach-Wahlventilen ohne weiteres dupliziert werden. So kann der eine Probenschleifensatz abgearbeitet werden, während der andere Satz befüllt wird und praktisch totvolumenfrei in den Fluß geschaltet werden kann, noch bevor die letzte Schleife des ersten Satzes vollständig ausgespült worden ist.

[0038] Eine Ausgestaltungsmöglichkeit der Probenaufgabe z.B. in einem Durchfluß-Meßsystem ist in Figur 1 wiedergegeben, die im folgenden beschrieben ist.

[0039] Mit Ausnahme der Pumpe 30, die z.B. eine Spritzenpumpe oder eine peristaltische Pumpe sein kann, symbolisieren alle übrigen Kreise Ventile, die wie hier beschrieben ausgelegt sein können. Die Ventile 1 und 11 sind Schaltventile, mit denen je nach Schaltung der beiden Schaltpositionen die entsprechenden Ein- bzw. Auslässe der Ventile verbunden werden; wobei in Figur 1 die aktuellen Verbindungen fett gezeichnet dargestellt sind und die alternativen Verbindungen gestrichelt. Die Ventile 4, 8, 14, 18, 28 und wenn gewünscht auch 21, 22, 33 und 39 sind Wahlventile, mit denen ein zentraler Anschluß, der mit normaler Linienbreite dargestellt ist, mit einem von beliebig vielen anderen Anschlüssen verbunden werden kann. Die Ventile 21, 22, 33 und 39 sind hier beispielhaft als Zweifach-Wahlventile gezeigt. Alle Ventilpositionen können manuell oder automatisch und auch vorprogrammiert mit z.B. elektrischem oder

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Druck-Antrieb gestellt werden. Alle Ventile sind vorzugsweise im Typ der in der Chromatographie verwendeten Drehventile ausgelegt, z.B. in volumenarmer Mikroausführung, können aber auch z.B. als Magnetventile ausgelegt werden. 36 ist eine beliebig dimensionierbare Temperierungs-Kapillare, 37 ist die Interaktions-Detektionseinheit, kurz Detektor, z.B. vom Typ eines Affinitäts-Biosensors. Alle mit normaler durchgezogener Linie angezeigten Verbindungen bestehen aus Kapillaren, gefertigt z.B. aus Tefzel, PE, PEEK oder Edelstahl, mit beliebigen Dimensionen bezüglich Innendurchmesser / Außendurchmesser / Länge, z.B. 1 mm /$^{1}/_{16}$ " / 0,5 m für die Verbindung 31. Eine beliebige Lösung, aus Vorräten 25 bis 27 oder folgenden n ausgewählt, wird mittels der Pumpe 30 über Ventil 33 entweder für Waschzwecke in den Abfall 43 oder durch den Detektor 37 gepumpt und über Ventil 39 in den Abfall 43 geleitet. Aus dem Detektor 37 ausgespülte Lösungen von weiterem Interesse können über das Ventil 39 im Sammler 41 gesammelt, in mehreren Gefäßen n fraktioniert aufgefangen oder mit anderen Techniken, z.B. Massenspektrometrie, direkt gekoppelt werden. Die in C enthaltenen Komponenten, mit Strich-Punkt umrahmt, können gemeinsam temperiert werden, z.B. mittels eines hier nicht gezeigten Peltier-Elementes.

[0040] Das erfindungsgemäß Besondere dieses Teils ist die Art der Probenaufgabe, die für eine repetitive Probenaufgabe und besonders für die Multischritt-Kinetik von Vorteil ist. Die Komponenten 1 bis 10 sind als Probenaufgabe A zusammengefaßt und über Ventil 1 entweder direkt in Verbindung 31 oder beliebig oft dupliziert über die Ventile 21 und 22 in die Verbindungen 31/32 einschaltbar; dargestellt ist hier eine Duplizierung von A, mit analoger Probenaufgabe B zusammengefaßt. In A befindet sich Ventil 1 in der Position Ladung für die Probenschleifen 5 bis 7, die über Probeneinlaß 2 entweder manuell oder mittels eines Probengebers automatisch mit beliebigen Lösungen, d.h. auch mit Analytlösungen jeweils fallender Konzentration, befüllt werden können, wobei die verdrängten Lösungen in Abfall 10 gespült werden. Minimal-Volumina, z.B. für Mikro- oder Test-Analysen, lassen sich realisieren, indem lediglich Verbindung 3 teilweise befüllt wird. Theoretisch unbegrenzte Maximal-Volumina einer Lösung, z.B. für sogenannte Präparationen mit besonders großen Sensoroberflächen, lassen sich realisieren, indem alle Probenschleifen mit derselben Lösung befüllt werden.

[0041] Nach jeweils synchronem Schalten der Ventile 4 und 8 können die Probenschleifen 5 bis 7 nacheinander und unabhängig voneinander sowie jeweils vollständig oder zwecks Proben-Ersparnis nur teilweise befüllt werden, und zwar in jedem Fall "rückwärts", d.h. von ihrer späteren Auslaßseite her. Damit ist gewährleistet, daß, nach dem Schalten von Ventil 1 in die Position Fluß, an die Verbindung 24a totvolumenfrei die Lösung der letzten Befüllung ansteht und sich daran nach zeitlich beliebigem, schrittweisen und jeweils synchronen Schalten der Ventile 4 und 8 übergangslos und unabhängig voneinander die Lösungen der anderen Probenschleifen auf den Detektor 37 beaufschlagen lassen. Diese Beaufschlagung ist in B gezeigt, wo sich die Komponenten11 bis 20 spiegelbildlich zu A in Position Fluß befinden. Durch bevorzugt synchrones Schalten der Ventile 1, 11, 21 und 22 können A und B wechselseitig in Position Fluß bzw. Ladung umgeschaltet werden, so daß sich A im Fluß befindet, während B befüllt wird bzw. umgekehrt. Alle Verbindungskapillaren und Schleifen... 3, 9, 13, 19, 23a,b, 24a,b, 29, 31, 32, 34, 35, 38, 40, 42 und 5, 6, 7, 15, 16, 17 können je nach Anwendung in ihren Dimensionen besonders optimiert ausgelegt werden. In 24a und/oder 24b befindet sich normalerweise Spül- oder sogenannter Lauf-Puffer vom letzten Spül- oder Analysengang. Bei Bedarf können diese Verbindungskapillaren auch höhervolumiger ausgestaltet werden, um nach der Beaufschlagung der Lösungen aus B bzw. A und der gleichzeitigen Umschaltung der Lösungen aus A bzw. B in die Position Fluß zunächst eine Wasch- oder Dissoziationsphase in dem Detektor 37 durchzuführen, deren Dauer durch das Volumen von 24a bzw. 24b und der Flußrate bestimmt wird, was je nach Anwendung von Interesse sein kann. Die Anordnung ist darüber hinaus offen für die Positionierung weiterer Ventile und/oder Pumpen zwecks Waschung / Spülung beliebiger Abschnitte oder zwecks Einbringung weiterer Lösungen. So können z.B. B und/oder weitere Duplikate von B über Ventil 11 wahlweise z.B. in die Verbindungen 3, 5, 6, 7 oder 32 positioniert werden.

[0042] Desweiteren wird durch ein häufiges Schalten bestimmter, je nach Anwendung unterschiedlicher Ventile, eine Mischung von Lösungen ermöglicht, bei Bedarf auch durch Installation eines Flußteilers z.B. in Verbindung 31 anstelle von oder zusätzlich zu Ventil 21 und/oder einer sogenannten Mikro-Mischkammer z.B. in Verbindung 32 anstelle von oder zusätzlich zu Ventil 22. Diese Anordnung ergibt insgesamt eine größtmögliche Flexibilität der Lösungs-Beaufschlagung auf die Meßzelle. Ein Mischen der Vorräte 25 bis 27 oder mehr kann auch durch häufiges Schalten von Ventil 28 erreicht werden. Für Lösungen, die temperatursensitiv sind, können alle oder nur Teile der in C enthaltenen Komponenten, exklusive z.B. der in D enthaltenen mit Strich-Punkt-Punkt umrahmten Komponenten, gesondert temperiert werden, während die z.B. in D enthaltenen Komponenten ihrerseits gesondert auf eine Arbeitstemperatur temperiert werden.

Index-Liste

[0043]

ass	Assoziation
diss	Dissoziation
i	i-ter Schritt
st	jeweiliger Startwert einer Assoziation oder Dissoziation

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eq jeweiliger Gleichgewichtswert einer Assoziation oder Dissoziation
net Netto
k_{on} Exponentialkoeffizient bei Assoziation
k_{off} Exponentialkoeffizient bei Dissoziation

Patentansprüche

1. Analytisches Mess- und Auswerteverfahren für molekulare Interaktionen von chemischen Gleichgewichts-Bindungspaaren oder -Bindungssystemen, wobei ein erster Bindungspartner ("Ligand" L) mit einem in Lösung befindlichen zweiten Bindungspartner ("Analyt" A), der mit dem Liganden reversibel unter Komplexbildung reagiert, versetzt wird, Assoziations- und/oder Dissoziations-Kurven aufgenommen werden und die zeitliche Entwicklung der Komplexbildung $c_t(LA)$ sowie deren Änderung $\frac{dc_t(LA)}{dt}$ erfasst werden,

   dadurch gekennzeichnet, dass
   zu bestimmten Zeitpunkten t_i die Konzentration $c_{ti}(A)$ einmal oder mehrmals schrittweise erhöht oder emiedrigt wird, wobei die Erhöhung bzw. Erniedrigung der Konzentration $c_{tj}(A)$ jeweils zu einem beliebigen Zeitpunkt vor dem Erreichen der jeweiligen Gleichgewichtswerte der einzelnen Schritte durchgeführt wird, und der Reaktionsverlauf mittels eines mathematischen Annäherungsprogramms in 1. Näherung jeweils mit einer exponentiell verlaufenden Funktion R(t), die jeweils auf einen Gleichgewichtswert $R'_{eq,i}$ zuläuft, mit wenigstens einem Exponenten $k'_{on,i}$ oder $k'_{off,i}$ beschrieben wird, wobei $\frac{dR(t)}{dt}$ die Assoziations- oder Dissoziationsrate des Analyten am Liganden angibt, um hieraus die Assoziations-Geschwindigkeitskonstante k_{ass} und die Dissoziations-Geschwindigkeitskonstante k_{diss} zu ermitteln.

2. Verfahren nach Anspruch 1,
   dadurch gekennzeichnet, dass
   aus den jeweiligen Differenzen zwischen den Assoziationsgleichgewichtswerten $R'_{eq,ass,i}$ und Assoziationsstartwerten $R'_{st,ass,i}$ oder den jeweiligen Differenzen zwischen den Dissoziationsgleichgewichtswerten $R'_{eq,diss,i}$ und Dissoziationsstartwerten $R'_{st,diss,i}$ und den jeweiligen Exponenten $k'_{on,i}$ oder $k'_{off,i}$ die jeweiligen initialen Assoziations- oder Dissoziationsraten $\left(\frac{dR_{ass,i}(t)}{dt}\right)_{t=0}$ bzw. $\left(\frac{dR_{diss,i}(t)}{dt}\right)_{t=0}$ und aus diesen Raten die Assoziations- oder Dissoziationsgeschwindigkeitskonstante k_{ass} bzw. k_{diss} ermittelt werden.

3. Verfahren nach Anspruch 1,
   dadurch gekennzeichnet, dass
   die Dissoziationsgeschwindigkeitskonstante k_{diss} aus den initialen Assoziationsraten $\left(\frac{dR_{ass,i}(t)}{dt}\right)_{t=0}$ und den jeweiligen Assoziationsgleichgewichtswerten $R'_{eq,ass,i}$ der Funktion R(t) und die Assoziationsgeschwindigkeitskonstante k_{ass} aus den initialen Dissoziationsraten $\left(\frac{dR_{diss,i}(t)}{dt}\right)_{t=0}$ und den jeweiligen Dissoziationsgleichgewichtswerten $R'_{eq,diss,i}$ der Funktion R(t) ermittelt wird.

4. Verfahren nach Anspruch 1,
   dadurch gekennzeichnet, dass
   im Falle von jeweils konstanter Analytstartkonzentration $c_0(A)$ bei Mehr-Schritt-Analysen die Werte für k_{ass} und k_{diss} aus der Beziehung zwischen k'_{on} und der Analytstartkonzentration $c_0(A)$ ermittelt werden.

5. Verfahren nach Anspruch 1,

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dadurch gekennzeichnet, dass
die Assoziationsgeschwindigkeitskonstante k_{ass} aus der Beziehung zwischen k'_{on} und der Analytgleichgewichtskonzentration $c'_{eq,ass,i}(A)$ und die Dissoziationsgeschwindigkeitskonstante k_{diss} aus der Beziehung zwischen k'_{off} und dem Gleichgewichtswert $R'_{eq,diss,i}$ der Funktion R(t) bei der Dissoziation ermittelt wird.

6. Verfahren nach einem der vorangehenden Ansprüche,
dadurch gekennzeichnet, dass
die Änderung der Analytkonzentration zur Halbwertszeit der Funktion $R_i(t)$ erfolgt.

7. Verfahren nach einem der vorangehenden Ansprüche,
dadurch gekennzeichnet, dass
die initiale Assoziations- oder Dissoziationsrate $\left(\frac{dR_{ass,i}(t)}{dt}\right)_{t=0}$ bzw. $\left(\frac{dR_{diss,i}(t)}{dt}\right)_{t=0}$ $(dR_{diss,i}(t)\ dt)_{t=0}$ einer linearen Beziehung zwischen $\frac{dR(t)}{dt}$ und R(t) oder $(R(t) - R'_{st,diss,i})$ ermittelt wird.

8. Verfahren nach einem der vorangehenden Ansprüche,
dadurch gekennzeichnet, dass
die Ermittlung der initialen Raten $\left(\frac{dR_{ass,i}(t)}{dt}\right)_{t=0}$ bzw. $\left(\frac{dR_{diss,i}(t)}{dt}\right)_{t=0}$ unter der Annahme idealer Bedingungen erfolgt.

9. Verfahren nach Anspruch 8,
dadurch gekennzeichnet, dass
die Ermittlung der initialen Raten $\left(\frac{dR_{ass,i}(t)}{dt}\right)_{t=0}$ bzw. $\left(\frac{dR_{diss,i}(t)}{dt}\right)_{t=0}$ unter der Annahme erfolgt, dass bei der Assoziation keine Analytverarmung und bei der Dissoziation keine Analytanreicherung erfolgt.

10. Verfahren nach einem der vorangehenden Ansprüche,
dadurch gekennzeichnet, dass
die unbekannte Analytstartkonzentration $c_0(A)$ zu Beginn der Reaktion ermittelt wird.

11. Verfahren nach einem der vorangehenden Ansprüche,
dadurch gekennzeichnet, dass
das Verfahren mit bekannten Affinitätsbiosensoren und/oder Array-Systemen in Lösung und/oder zeitaufgelösten ELISA- oder RIA-Techniken oder anderen festphasengestützten Systemen durchgeführt wird.

12. Verfahren nach einem der vorangehenden Ansprüche,
dadurch gekennzeichnet, dass
in einem Küvettensystem die Analytkonzentration c(A) der Lösung in der Messkammer durch kontinuierliche Entnahme eines Teils der Lösung und Zugabe einer Lösung mit einer entsprechenden Analytkonzentration c(A) über eine oder mehrere Kanülen während des Assoziations- oder Dissoziationsvorgangs konstant gehalten wird.

13. Verfahren nach einem der vorangehenden Ansprüche,
dadurch gekennzeichnet, dass
bei konstanter Konzentration der Lösung in der Messkammer derjenige $c_0(A)$-Wert bestimmt wird, bei dem sich die Assoziationskurve oder ein Abschnitt davon mit der Dissoziationskurve oder einem Abschnitt davon in einem Punkt schneidet, dessen Wert 0,618 des gesamten oder des jeweiligen Teilwerts des Assoziations-Gleichgewichtswertes entspricht.

14. Verfahren nach Anspruch 13,
dadurch gekennzeichnet, dass

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die Konzentration in der Messkammer mehrmals schrittweise geändert wird und die jeweiligen Startkonzentrationen des Analyten $c_{0,i}(A)$ ermittelt werden.

15. Verfahren nach einem der vorangehenden Ansprüche,
dadurch gekennzeichnet, dass
in einem idealen Durchflusssystem bei jeweils konstant gehaltener Analytstartkonzentration $c_{0,i}(A)$ während einer Assoziationsphase die Dissoziations-Gleichgewichtskonstante $K_D = k_{diss}/k_{ass}$ als diejenige Analytstartkonzentration $c_{0,i}(A)$ ermittelt wird, mit der der ermittelte Gleichgewichtswert gleich der Hälfte der maximal möglichen Reaktion R_{max} auf die Zugabe des Analyten im Überschuss wird.

16. Vorrichtung zur Durchführung des Verfahrens nach einem der vorhergehenden Ansprüche, mit einer Sensoroberfläche zur Bindung des in Lösung befindlichen Analyten am Liganden, mit einer Meßkammer auf der Sensoroberfläche, die mit dem Analyt in Lösung befüllt ist, mit jeweils einer Kanüle zur Zugabe und/oder Herausnahme der Lösung und/oder einer Spülflüssigkeit, mit einer Vorrichtung zur Durchmischung der Lösung in der Meßkammer
dadurch gekennzeichnet, dass
die Lösungen in der Meßkammer zumindest teilweise in vorbestimmter Menge instantan mehrmals gegen Lösungen anderer Konzentration austauschbar sind, wobei das Volumen der Lösung konstant gehalten ist und wobei mehrere Probenschleifen vorhanden sind, deren Inhalt entgegen seiner Befüllrichtung und unabhängig voneinander in die Meßkammer einbringbar ist, so dass die Messkammer mit mehreren unterschiedlichen Lösungen aus den einzelnen Probenschleifen nacheinander, unabhängig voneinander und totvolumenfrei im Durchfluss beaufschlagbar ist.

17. Vorrichtung nach Anspruch 16,
dadurch gekennzeichnet, dass
der Austausch der Lösungen in der Meßkammer über eine oder mehrere Kanülen erfolgt.

18. Vorrichtung nach Anspruch 16 oder 17,
dadurch gekennzeichnet, dass
die Kanülen getrennt an der Meßkammer angeordnet sind oder die eine koaxial innerhalb der anderen.

19. Vorrichtung nach einem der Ansprüche 16 bis 18,
dadurch gekennzeichnet, dass
eine oder mehrere Pumpen zur Dosierung der Menge der auszutauschenden Lösungen an den Kanülen vorgesehen sind.

20. Vorrichtung nach einem der Ansprüche 16 bis 19,
dadurch gekennzeichnet, dass
über mehrere Kanülen die Lösung in der Meßkammer durch zumindest teilweise Absaugung und wieder Zugabe in die Meßkammer durchmischt wird.

Claims

1. An analytical measuring and evaluation process for molecular interactions of chemical equilibrium binding couples and binding systems, wherein a first binding partner ("ligand" L) reversibly reacts with a second binding partner ("analyte" A) mixed in a solution, forming a complex compound, wherein the association and/or disassociation curves are being recorded and the temporal development of the complex forming $c_t(LA)$ and its derivative $\frac{dc_t(LA)}{dt}$ are recorded,
characterized in that
at certain points in time t_i the concentration c_{ti} (A) is increased or decreased in increments once or several times, wherein the increase or decrease of the concentration $c_{ti}(A)$ is performed at any time before reaching the respective equilibrium values of the particular steps and the reaction process is described with a mathematical approximation program, in a first approximation with an exponential function R(t), which approaches an equilibrium value $R'_{eq,i}$ with at least one exponent $k'_{on,i}$ or $k'_{off,i}$, wherein $\frac{dR(t)}{dt}$ provides the association or disassociation rate of the

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analyte at the ligand, in order to determine the association velocity constant K_{ass} and the disassociation constant k_{diss}.

2. A process according to claim 1,
characterized in that
from the respective differentials between the association equilibrium values $R'_{eq,ass,i}$ and the association start values $R'_{st,ass,i}$ or the respective differentials between the disassociation equilibrium values $R'_{eq,diss,i}$ and the disassociation start values $R'_{st,diss,i}$ and the respective exponents $K'_{on,i}$ or $k_{off,i}$ the respective initial association or disassociation rates $\left(\frac{dR_{ass,i}(t)}{dt}\right)_{t=0}$ or $\left(\frac{dR_{diss,i}(t)}{dt}\right)_{t=0}$ and from these rates the association or disassociation velocity constant k_{ass} or k_{diss} are determined.

3. A process according to claim 1,
characterized in that
the disassociation velocity constant k_{diss}, is determined from the initial association rates $\left(\frac{dR_{ass,i}(t)}{dt}\right)_{t=0}$ and the respective association equilibrium values $R'_{eq,ass,i}$ of the function R(t) and the association velocity constant k_{ass} from the initial disassociation rates $\left(\frac{dR_{diss,i}(t)}{dt}\right)_{t=0}$ and the respective disassociation equilibrium values $R'_{eq,diss,i}$ of the function R(t).

4. A process according to claim 1,
characterized in that
in case of a respectively constant initial analyte concentration $c_0(A)$ within multiple step analyses the values k_{ass} and k_{diss} are determined from the ratio of k'_{on} and the analyte start concentration $c_0(A)$.

5. A process according to claim 1,
characterized in that
the association velocity constant k_{ass} is determined from the ratio of k'_{on} and the analyte equilibrium concentration $c_{,eq,ass,i}$ (A) and the disassociation velocity constant k_{diss} from the ratio between k'_{off} and the equilibrium value $R'_{eq,diss,i}$ of the function R(t) during the disassociation.

6. A process according to one of the preceding claims,
characterized in that
the change of the analyte concentration happens at the half life of the function $R_i(t)$.

7. A process according to one of the preceding claims,
characterized in that
the initial association or disassociation rate $\left(\frac{dR_{ass,i}(t)}{dt}\right)_{t=0}$ or $\left(\frac{dR_{diss,i}(t)}{dt}\right)_{t=0}$ is determined from a linear relationship between $\frac{dR(t)}{dt}$ and R(t) or (R(t) - $R'_{st,diss,i}$).

8. A process according to one of the preceding claims,
characterized in that
the determination of the initial rates $\left(\frac{dR_{ass,i}(t)}{dt}\right)_{t=0}$ or $\left(\frac{dR_{diss,i}(t)}{dt}\right)_{t=0}$ is performed under the assumption of ideal conditions.

9. A process according to claim 8, **characterized in that**

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the determination of the initial rates $\left(\frac{dR_{ass,i}(t)}{dt}\right)_{t=0}$ or $\left(\frac{dR_{diss,i}(t)}{dt}\right)_{t=0}$ is performed under the assumption, that during the association no analyte reduction and during the disassociation no analyte increase occurs.

10. A process according to one of the preceding claims,
characterized in that
the unknown analyte concentration $c_0(A)$ is determined at the beginning of the reaction.

11. A process according to one of the preceding claims,
characterized in that
the process is performed with known affinity biosensors and/or array systems in a solution and/or time based ELISA or RIA techniques or other solid phase based systems.

12. A process according to one of the preceding claims,
characterized in that
in a cuvette system the analyte concentration c(A) of the solution in the measuring chamber is kept constant through continuous subtraction of a part of the solution and addition of a solution with the corresponding analyte concentration c(A) through one or more canules during the association or disassociation process.

13. A process according to one of the preceding claims,
characterized in that
at a constant concentration of the solution in the measuring chamber the $c_0(A)$ value is determined, at which the association curve or a section thereof intersects with the disassociation curve or a section thereof in a point whose value 0.618 is equivalent to the whole or respective partial value of the association equilibrium value.

14. A process according to claim 13,
characterized in that
the concentration in the measuring chamber is changed incrementally several times and the respective start concentration of the analyte $c_{0,i}(A)$ is determined.

15. A process according to one of the preceding claims,
characterized in that
in an ideal flow through system with the analyte concentration $c_{0,i}(A)$ kept constant during an association phase, the disassociation equilibrium constant $K_D = k_{diss}/k_{ass}$ is determined as the analyte start concentration $c_{0,i}(A)$, through which the determined equilibrium value equals half of the maximum possible reaction R_{max} with respect to the excessive addition of the analyte.

16. A device for performing the process according to one of the preceding claims with a sensor surface for binding the analyte in solution at the ligand, with a measuring chamber on the sensor surface which is filled with the analyte in solution, with a respective canule for adding and/or subtracting solution and/or a flushing liquid, with a device for mixing the solution in the measuring chamber,
characterized in that
the solutions in the measuring chamber are instantaneously exchangeable at least partially in a predetermined quantity, with solutions with other concentrations, wherein the volume of the solution is kept constant and wherein several sample loops are present, whose contents can be independently induced into the measuring chamber against their filling direction, so that the measuring chamber can be filled with several different solutions from the particular sample loops in sequence, independent from each other and without dead volume.

17. A device according to claim 16,
characterized in that
the replacement of the solutions in the measuring chamber is performed through several canules.

18. A device according to claim 16 or 17,
characterized in that
the canules are separately located at the measuring chamber or one is coaxial inside the other.

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19. A device according to one of the claims 16-18
characterized in that
one or several pumps for dosing the quantity of solution to be replaced are provided at the canules.

20. A device according to one of the claims 16-19
characterized in that
the solution in the measuring chamber is mixed through several canules through at least partial subtraction and addition in the measuring chamber.

Revendications

1. Procédé analytique de mesure et d'évaluation pour des interactions moléculaires de paires de liaison chimiques ou systèmes de liaison équilibrés, un premier partenaire de liaison ("ligand" L) mélangé à un second partenaire de liaison se trouvant dans une solution ("Analyte" A), qui réagit avec le ligand de manière réversible en formant un complexe, des courbes d'association et /ou de dissociation étant relevées et l'évolution temporelle de la formation du complexe $c_t(LA)$ ainsi que sa modification $\frac{dc_i(LA)}{dt}$ étant enregistrées,

caractérisé
en ce qu' à des moments déterminés t_i, la concentration $c_{ti}(A)$ est augmentée ou abaissée une fois ou plusieurs fois, l'augmentation respectivement l'abaissement de la concentration $c_{ti}(A)$ étant effectués respectivement à n'importe quel moment avant d'atteindre les valeurs d'équilibre respectives de chacune des étapes et le déroulement de la réaction est décrit au moyen d'un programme mathématique d'approximation dans une première approximation avec une fonction R(t) évoluant exponentiellement, qui aboutit respectivement à une valeur d'équilibre $R'_{eq,i}$ avec au moins un exposant $k'_{on,i}$ ou $k'_{off,i}$, $\frac{dR(t)}{dt}$ indiquant les taux d'association et de dissociation de l'analyte sur le ligand, pour en déterminer les constantes d'équilibre d'association k_{ass} et les constantes d'équilibre de dissociation k_{diss}.

2. Procédé selon la revendication 1,
caractérisé
en ce que les débits respectifs d'association ou de dissociation initiaux $\left(\frac{dR_{ass,i}(t)}{dt}\right)_{t=0}$ respectivement $\left(\frac{dR_{diss,i}(t)}{dt}\right)_{t=0}$ sont déterminés à partir des différences respectives entre les valeurs d'équilibre d'association $R'_{eq,ass,i}$ et les valeurs initiales d'association $R'_{st,ass,i}$ ou à partir des différences entre les valeurs d'équilibre d'association $R'_{eq,aiss,i}$ et les valeurs initiales d'association $R'_{st,diss,i}$ et les exposants respectifs $k'_{on,i}$ ou $k'_{off,i}$, et les constantes d'équilibre d'association k_{ass} respectivement de dissociation k_{diss} sont déterminées à partir de ces taux.

3. Procédé selon la revendication 1,
caractérisé
en ce que la constante de vitesse de dissociation k_{diss} est déterminée à partir des taux initiaux d' association $\left(\frac{dR_{ass,i}(t)}{dt}\right)_{t=0}$ et des valeurs respectives d'équilibre d'association $R'_{eq,ass,i}$ de la fonction R(t) et la constante de vitesse d'association k_{ass} est déterminée á partir des taux initiaux d'association $\left(\frac{dR_{diss,i}(t)}{dt}\right)_{t=0}$ et des valeurs respectives d'équilibre de dissociation $R'_{eq,diss,i}$ de la fonction R(t).

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4. Procédé selon la revendication 1,
caractérisé en ce que
dans le cas d'une concentration de départ d'analyte respectivement constante $c_0(A)$ pour des analyses en plusieurs étapes, les valeurs pour k_{ass} et k_{diss} sont déduites de la relation entre k'_{on} et de la concentration de départ d'analyte $c_0(A)$.

5. Procédé selon la revendication 1,
caractérisé en ce que
la constante de vitesse d'association k_{ass} résulte du rapport entre k'_{on} et la concentration d'équilibre d'association $c'_{eq,ass,i}(A)$ et la constante de vitesse de dissociation k_{diss} résulte du rapport entre k'_{off} et la valeur d'équilibre $R'_{eq,diss,i}$ de la fonction R(t) lors de la dissociation.

6. Procédé selon l'une des revendications précédentes,
caractérisé en ce que
la modification de la concentration d'analyte s'effectue à la période de demi-valeur de la fonction $R_i(t)$.

7. Procédé selon l'une des revendications précédentes,
caractérisé en ce que
les taux initiaux d'association et de dissociation $\left(\frac{dR_{ass,i}(t)}{dt}\right)_{t=0}$ respectivement $\left(\frac{dR_{diss,i}(t)}{dt}\right)_{t=0}$ sont déterminés à partir d'un rapport linéaire entre $\frac{dR(t)}{dt}$ et R(t) ou $(R(t) - R'_{st,diss,i})$.

8. Procédé selon l'une des revendications précédentes,
caractérisé en ce que
la détermination des taux initiaux $\left(\frac{dR_{ass,i}(t)}{dt}\right)_{t=0}$ respectivement $\left(\frac{dR_{diss,i}(t)}{dt}\right)_{t=0}$ s'effectue dans l'hypothèse de conditions idéales.

9. Procédé selon la revendication 8,
caractérisé en ce que
la détermination des taux initiaux $\left(\frac{dR_{ass,i}(t)}{dt}\right)_{t=0}$ respectivement $\left(\frac{dR_{diss,i}(t)}{dt}\right)_{t=0}$ s'effectue dans l'hypothèse que lors de l'association, il ne se produit aucun appauvrissement et lors de la dissociation aucun enrichissement d'analyte

10. Procédé selon l'une des revendications précédentes,
caractérisé en ce que
la concentration d'analyte inconnue $c_0(A)$ est déterminée au début de la réaction.

11. Procédé selon l'une des revendications précédentes,
caractérisé en ce que
le procédé est effectué avec des biocapteurs connus d'affinité à résolution temporelle et/ou des systèmes de matrices (Array) en solution et/ou par des techniques d'immunodosage telles que les dosages radio-immunologiques (**RIA**) et immuno-enzymatiques (**ELISA**) ou autres systèmes en phase solide.

12. Procédé selon l'une des revendications précédentes,
caractérisé en ce que
dans un système de cuvette, la concentration d'analyte c(A) de la solution dans la chambre de mesure est maintenue constante par prélèvement continu d'une partie de la solution et ajout d'une solution contenant une concentration correspondante d'analyte c(A) par une ou plusieurs canules pendant l'opération d'association ou de dissociation.

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13. Procédé selon l'une des revendications précédentes,
caractérisé en ce
qu'en présence d'une concentration constante de la solution dans la chambre de mesure, il est déterminé la valeur $c_0(A)$, à laquelle la courbe d'association ou une section se recoupe avec la courbe de dissociation ou une section en un point d'intersection, dont la valeur correspond à 0,618 de l'ensemble ou de la valeur partielle respective de la valeur d'équilibre.

14. Procédé selon la revendication 13,
caractérisé en ce que
la concentration dans la chambre de mesure est modifiée plusieurs fois progressivement et les concentration respective de départ de l'analyte $c_{0,i}(A)$ sont déterminées.

15. Procédé selon l'une des revendications précédentes, **caractérisé en ce que** dans un système idéal d'écoulement en présence d'une concentration de départ d'analyte maintenue constante $c_{0,i}(A)$ pendant une phase d'association, la constante d'équilibre de dissociation $KD = k_{diss}/k_{ass}$ est déterminée en tant que concentration de départ d'analyte $c_{0,i}(A)$ à laquelle la valeur déterminée d'équilibre égale à la moitié de la réaction maximale possible Rmax à l'ajout de l'analyte devient excédentaire.

16. Dispositif de mise en oeuvre du procédé selon l'une des revendications précédentes comprenant une surface de capteur pour la liaison de l'analyte, se trouvant en solution, au ligand, comprenant sur la surface de capteur une chambre de mesure qui est remplie de l'analyte en solution, comprenant respectivement une canule pour l'ajout ou le prélèvement de la solution et/ou d'un liquide de rinçage, comprenant un dispositif pour le mélange de la solution dans la chambre de mesure,
caractérisé en ce
les solutions sont échangeables dans au moins dans la chambre de mesure partiellement en quantité prédéterminée instantanément plusieurs fois contre des solutions d'une autre concentration, le volume de la solution étant maintenu constant et plusieurs boucles d'échantillon étant présentes, dont le contenu peut être introduit dans la chambre de mesure dans le sens contraire à la direction de remplissage et indépendamment les uns des autres de sorte que la chambre de mesure peut être alimentée de plusieurs solutions provenant des différentes boucles d'échantillon les unes après les autres indépendamment les uns des autres et sans volume mort dans le débit.

17. Dispositif selon la revendication 16, **caractérisé en ce que** l'échange des solutions dans la chambre de mesure s'effectue par une ou plusieurs canules.

18. Dispositif selon la revendication 16 ou 17, **caractérisé en ce que** les canules sont disposées séparément sur la chambre de mesure ou coaxialement l'une dans l'autre.

19. Dispositif selon l'une des revendications 16 à 18,
caractérisé en ce qu'
une ou plusieurs pompes sont prévues sur les canules pour le dosage de la quantité des solutions à échanger.

20. Dispositif selon l'une des revendications 16 à 19,
caractérisé en ce que
la solution est mélangée par plusieurs canules dans la chambre de mesure par au moins aspiration partielle et réintroduction dans la chambre de mesure.

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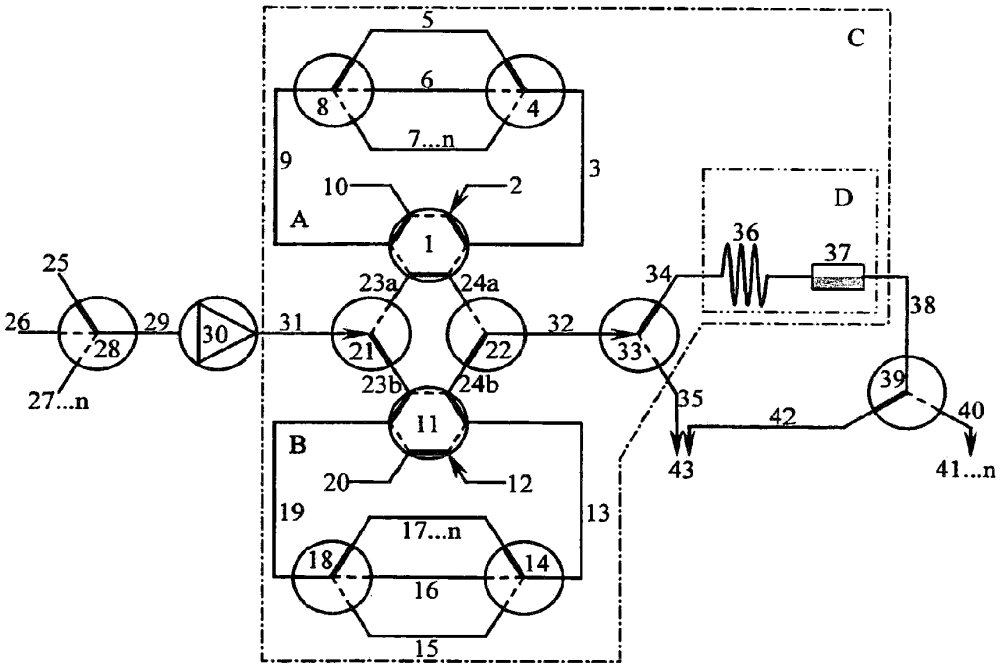


Fig. 1

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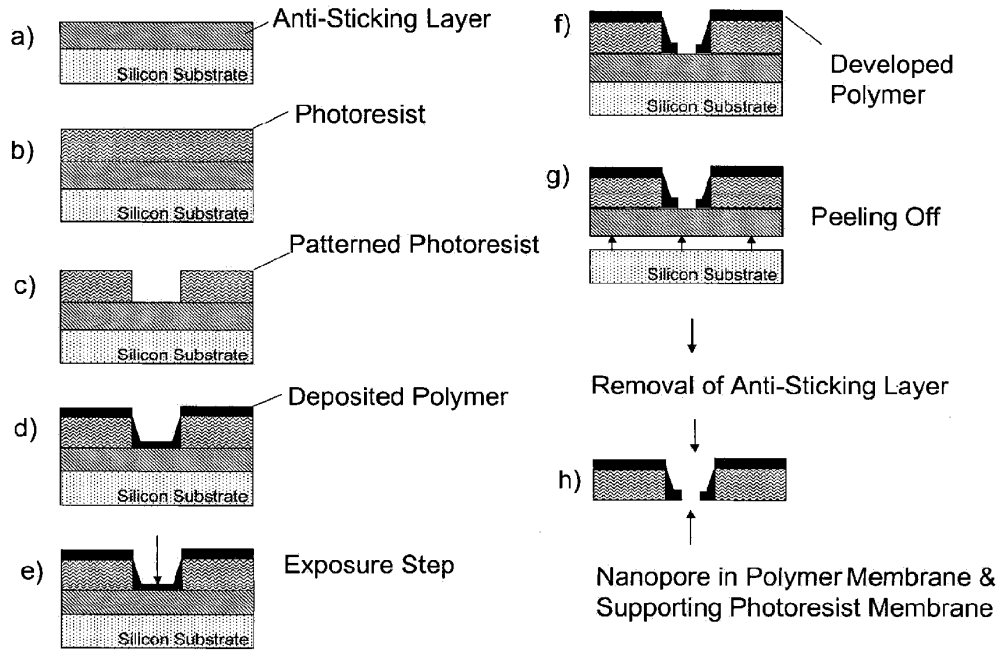
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(54) A method of fabricating a polymeric membrane having at least one pore

(57) The present invention relates to a method of fabricating a polymer membrane having at least one pore, to polymeric membranes fabricated by such method, and to uses of such polymeric membranes. The pores thus formed are in the nanometer range and therefore make such porous membranes amenable for use in devices for single molecule detection.

Figure 1



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Description

[0001] The present invention relates to a method of fabricating a polymer membrane having at least one pore, to polymeric membranes fabricated by such method, and to uses of such polymeric membranes. The pores thus formed are in the nanometer range and therefore make such porous membranes amenable for use in devices for single molecule detection.

[0002] Reasons for an increasing interest in artificial membranes with incorporated nanopores with diameters in the range of 1.5-100 nm are potential applications in the area of single molecule detection. Recently, studies of direct DNA sequencing methods concentrated onto using a single pore - mainly biological pores in form of membrane proteins - for detecting the translocation event of DNA molecules through such a local constriction. The target DNA molecules to be analysed were driven by a DC field, and the presence of DNA inside the pore can be detected by a partial blocking of the ionic current that flows through the pore. The idea of not only detecting the presence of target DNA in the pore, but also the detection of the base sequence (=direct sequencing) at relatively high speed exceeding 1 base pair reading per ms was stated several years ago.[i] However, it was clear that extremely high-resolution measurement techniques would be required. Up to date, direct sequencing at single base resolution level was not yet achieved.

[0003] In the earliest work in 1996 J. J. Kasianowicz et al. showed that DNA molecules can be detected during their translocation through membrane channels which are formed by an alpha-hemolysin pore protein.[i] They reported on the characterization of individual polynucleotide molecules using a 2.6 nm membrane channel. They demonstrated that the blockade lifetime of the ionic current could be utilised to derive the molecule length.

[0004] Akeson et al. demonstrated discrimination of polyC and poly A segments within a single RNA molecule by using an alpha-hemolysin channel.[ii] [iii]

[0005] Later, Meller et al. reported successful discrimination of polynucleotides of the same length that only differed in their sequence. In their work also an alpha-hemolysin channel and unlabeled DNA molecules were used.[iv] However, a direct read-out of the sequence was not possible.

[0006] Howorka et al. reported on target DNA detection through an alpha-hemolysin nanopore at single-base resolution by covalently modifying the nanopore with a single stranded DNA molecule (probe DNA = DNA molecule which senses the target DNA molecule).[v] The target DNA that was transported through the nanopore interacted with the tethered strand in different way, depending on whether a complete mismatch, a partial match or a complete match was present. The interacting was resolved at the time scale by measuring the ionic blocking current.

[0007] A different approach which uses hairpin DNA molecules was published by Vercoutere et al.[vi] The specific form of hairpin DNA and the resulting ionic current signature was utilised to rapidly discriminate at single-nucleotide resolution. The fact that the hairpin DNA could not pass the nanopore at once, but after the hydrogen bonds between bases were dissociated, slowed down the translocation velocity of the DNA molecule through the pore, making electrical reading easier.

With the advancing nanotechnology, recently also nanopore sequencing concepts based on artificial nanopores came up. The majority of artificial inorganic membranes that embed the nanopore are based on thin SiO_2 or Si_3N_4 layers, known from various applications in the area of micro engineered mechanical systems (MEMS).

[0008] Li et al. published data about nanopores that were fabricated into silicon nitride membranes by using ion beam etching.[vii] A pre-defined cavity (made by either wet-chemical etching methods or focussed ion beam (FIB) etching) was un-covered by applying a focussed Ar-ion beam, which removes material layer by layer (ion sculpting). Finally, the cavity broke through which resulted in a nanopore within the thin membrane. The size adjustment was achieved by using a feedback loop in form of an ion detector that was located behind the etched membrane: as soon as the nanopore opens up, ions were detected on the backside and the sculpting process was stopped. They showed that the pore diameter could be further controlled by adjusting the ion dose of the sculpting process and the background temperature: even after opening the pore, the pore diameter could be reduced by ion-induced material transport towards the pore.[viii] They demonstrated the fabrication of 3-5 nm pores. Using these single nanopores 500 to 10k base DNA molecules could be detected in solution.[ix]

[0009] More recently, artificial pores of diameters down to 3 nm were fabricated in a SiO_2 membrane by utilising electron beam lithography, wet chemical etching and a high energy, focussed electron beam for pore diameter reduction.[x] Storm et al. reported that the high-energy electron beam of a transmission electron microscope can be utilized to shrink the size of a fabricated nanopore through fluidization of the SiO_2 surface. A resulting increase in surface tension leads to a decrease of pore size. This method of diameter-control basically relies on the visual feedback through the TEM.

[0010] Another approach for controlled diameter reduction that was reported recently is the deposition of thin layers of Al_2O_3 by using atomic layer deposition techniques.[xi] FIB-fabricated nanopores in Si_3N_4 membranes with an initial diameter of about 20 nm had been shrunk to almost 2nm by depositing atomic layers of Al_2O_3. At the same time, the thickness of the membrane increases because the material is deposited isotropically. Another important aspect of this method is that surface defects that were introduced by the FIB process could be covered by the Al_2O_3 layer. A clear reduction of 1/f noise could be observed. Also, the surface charge of Al_2O_3 is different from that of the negatively charged SiO_2 and Si_3N_4 surfaces in KCl buffers: Al_2O_3 has its isoelectric point at

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pH~9 and therefore, at pH~8 DNA should be not repelled from the surface.[xi]

[0011] In contrast to these oxide-based membranes, membranes based on polymer materials were studied as well. Nanopores were for example introduced by shooting high-energy ions through foils of poly(ethylene terephthalate).[xii] The required accelerator technology makes this approach less attractive.

[0012] Saleh et al. report on successful DNA detection by using an artificial, PDMS based nanopore.[xiii] Because of the relatively large dimensions of their pore of the order of 200 nm in width and more than a μm in length, no high-resolution detection could be achieved.

[0013] So far there does not exist a simple fabrication method for polymeric membranes having pores in the nanometer range. The artificial membranes known from the prior art which are based on oxide materials like SiO_2 and Si_3N_4 require a number of lithographic steps and processing steps. Furthermore, the adjustment of the pore size of such artificial membranes appears to be rather complicated. Moreover, artificial membranes based on oxide materials having pores which were fabricated by using focussed ion beams can suffer from elevated 1/f noise levels because of the presence of introduced defects and trap centres. Additionally, membranes based on oxide materials like SiO_2 are negatively charged in buffer solution which makes such membranes unsuitable for use in the detection of negatively charged biopolymers like nucleic acids.

[0014] Accordingly, it was an object of the present invention to provide for a method of fabrication of a membrane having pores with a diameter in the nanometer range, which method is easy to perform. Furthermore it was an object of the present invention to provide for a method that allows the fabrication of membranes with pores wherein the formation of the pores can be easily monitored. Moreover, it was an object of the present invention to provide for a method of fabrication of membranes having pores in the nanometer range, wherein the size of the pores can be easily adjusted. It was furthermore an object of the present invention to provide a fabrication method of membranes having pores in the nanometer range that is simple and does not produce negatively charged membranes when placed into buffer solution. It was also an object of the present invention to provide for a method of fabrication of membranes having pores in the nanometer range wherein no harsh etching steps and subsequently the application of masks that withstand such etching steps, are required.

[0015] All these objects are solved by a method of fabricating a polymeric membrane having at least one pore with a diameter in the range below 500 nm, comprising the steps:

a) providing a substrate,
b) depositing a polymeric membrane on said substrate,
c) subjecting said polymeric membrane to a lithography step, thus introducing at least one pore with a diameter in the range below500 nm into said polymeric membrane, optionally an array of such pores,
d) lifting-off said membrane from said substrate.

[0016] In one embodiment step a) comprises the following substeps:

aa) providing a substrate having a surface, preferably a plane surface which substrate is made of a material selected from the group comprising oxides, metals and plastics,
ab) depositing on said surface of said substrate an anti-sticking layer, which anti-sticking layer has little adhesion to said surface of said substrate,
ac) depositing on said anti-sticking layer a carrier membrane, to which carrier membrane said anti-sticking layer has a greater adhesion than to said substrate,
ad) patterning said carrier membrane by introducing at least one recessinto it by a lithography step, said recesshaving a diameter in the range of from 1 μm to 500 μm into it

[0017] The term "plane surface", as used herein, is meant to characterize a surface which does not have substantial irregularities in it, such as elevations or cavities, that would interfere with the formation of a nanopore.

[0018] Preferably, step b) comprises the following substeps:

ba) depositing a polymeric membrane on said carrier membrane having said at least one recess, by a procedure selected from spin-coating and evaporating, such that said polymeric membrane covers said carrier membrane and, at the site of said at least one recess, forms a lining within said at least one recess,

and wherein step c) comprises the substep:

ca) performing said lithography step at said lining within said at least one recess.

[0019] In one embodiment said carrier membrane is made of an electrically insulating material selected from the group comprising polymers and oxides, wherein, preferably, it is made of a photoresist material. Typical examples of materials useful for producing said carrier membrane are chemically amplified resists and non-chemically amplified resists, that are sensitive to either light (IR, visible, UV) exposure, electron exposure, ion exposure or X-ray exposure; more specifically photoresists, like AZ resists, ZEP resists and others, deep-UV resists like UV 6/UV 5 and others, electron beam resists like poly(methyl(methacrylate)) (PMMA) and others; and resists used for nanoimprint lithography.

[0020] Preferably, said polymeric membrane is made

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of an electrically insulating material selected from the group comprising polymers and oxides, wherein, preferably, it is made of a resist material selected from photoresist materials and electron beam resist materials. Typical examples of materials useful for producing said polymeric membrane include polyethylene (PE), polyethylene terephthalate (PET), polyvinyl chloride (PVC), polycarbonate, pentacene and other plastic materials. In some embodiments, said polymeric membrane is a bilayer comprising a polymeric layer of one of the aforementioned materials and a layer of inorganic material, such as SiO_2, Si_xN_y, oxides, insulators and metals, or it is a multilayer of polymeric layers and inorganic layers in between. For example, a polymeric membrane according to the present invention may comprise a first polymeric layer, an inorganic layer on top thereof, and second polymeric layer on top of said inorganic layer, i.e. a sandwich structure. Possibly this structure may be repeated several times leading to a multiple sandwich structure of the layer sequence $P_1I_1P_2I_2P_3I_3P_4I_4P_5$ etc., with P_x and I_x denoting the x-th polymeric layer and inorganic layer respectively.

[0021] In one embodiment step d) occurs by application of force, such as suction, or by application onto said polymeric membrane of an adhesive tape having a hole (holes) so as not to cover said pore(s), and by subsequently lifting-off said polymeric membrane from said substrate.

[0022] In one embodiment said anti-sticking layer has a thickness in the range of from about 1 nm to about 100 nm, preferably about 5 nm to about 75 nm, more preferably about 10 nm to about 60 nm and most preferably about 20 nm to about 50 nm.

[0023] In one embodiment said anti-sticking layer is made of a material selected from the group comprising polymers, oxides, silanes, carbon (graphite), sputtered SiO_2 , Si_xN_y; preferably these materials are provided as nanoparticles. In one embodiment the anti-sticking layer is made of electrically conducting materials, in another embodiment it is made of electrically insulating material (s), in yet another embodiment it is made of a mixture of such material(s).

[0024] In a preferred embodiment said method comprises the additional step:

> e) dissolving said anti-sticking layer in a suitable solvent, wherein, preferably, said anti-sticking layer is made of a material selected from the group comprising metals, oxides and plastics, and wherein, preferably, said suitable solvent is an aqueous basic solution (e.g. for metals), an aqueous KI/I_2-solution (e.g. for metals), water (e.g. for water soluble oxides), and an organic solvent (e.g. for plastics), such as acetone, alcohols, organic acids, wherein, preferably, steps d) and e) occur concomitantly.

[0025] In one embodiment said lithography step in step c) is selected from the group comprising optical lithography, electron beam lithography and atomic force microscope (AFM) lithography, and includes a developing step.

[0026] In one embodiment said lithography step in step ad) is selected from the group comprising optical lithography and electron beam lithography, and includes a developing step.

[0027] Preferably, steps ab) and ac) occur by a procedure selected from thermal evaporation electron-gun deposition, spin-coating, dip-coating, sputtering and vapour-phase deposition.

[0028] In one embodiment said developing step in ad) and ca) occurs by applying a developing solution to said carrier membrane and said polymeric membrane, respectively, preferably at the site where said recessand said pore is to be introduced, respectively, wherein, preferably, said anti-sticking layer is made of an electrically conducting material, preferably a metal, such as gold or aluminium, and wherein, more preferably, said introduction of said at least one pore having a diameter in the range below500 nm into said polymeric membrane in step c) is monitored by connecting an electrode with the developing solution and by connecting a counter-electrode with the anti-sticking layer, and wherein voltage and/or current variations are measured between said electrodes.

[0029] In one embodiment a constant DC voltage or current is applied via said electrodes and the current or voltage is monitored over time, wherein an increase in current and/or a decrease in voltage is indicative of completion of said introduction of the pore.

[0030] In another embodiment, AC current and/or voltage measurements are performed via said electrodes, using an impedance analyzer or lock-in-amplifier wherein such measurements afford a real and imaginary part of an impedance versus time-curve, and wherein the in-phase signal corresponds to the real part of the impedance and wherein an increase in current and/or a decrease in voltage is indicative of completion of said introduction of the pore, and wherein, furthermore the out-of-phase-signal corresponds to the imaginary part of the impedance versus time-curve and wherein, after completion of said introduction of the pore, the in-phase-signal and out-of-phase-signal reveal information about the size of the pore, said in-phase signal of the impedance reflecting the ohmic resistance of said pore in solution, thus reflecting the depth and the area size of said pore, said out-of-phase-signal of the impedance reflecting the capacitance of said pore.

[0031] The objects of the present invention are also solved by a membrane structure comprising a polymeric membrane on a carrier membrane, said polymeric membrane having at least one pore with a diameter in the range of below-500 nm, produced by the method according to the present invention.

[0032] Preferably, said pore has a diameter in the range of from 0.1 nm to 100 nm, preferably from 1 nm to 75 nm, more preferably 1 nm to 50 nm, and most pref-

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erably 1 nm to 10 nm.

[0033] In one embodiment, said polymeric membrane has a thickness in the range of from about 0.1 nm to about 500 nm, preferably from about 1 nm to about 250 nm.

[0034] Preferably, said carrier membrane has a thickness in the range of from about 1 μm to about 100 μm, preferably about 1 μm to about 50 μm, more preferably about 1 μm to about 20 μm, and most preferably about 10 μm.

[0035] In one embodiment, an array of n-times m pores has been introduced into a polymeric membrane using the method according to the present invention, n and m being positive integers, n and m may be the same or different positive integers. In one embodiment, a film heater is integrated into the polymeric membrane. This will allow for more accurate control during pore formation and also for further processing of the pore(s) after their/its formation.

[0036] The objects of the present invention are also solved by a use of a membrane structure according to the present invention in an electronic device, preferably for determining the size and/or sequence of a biopolymer, preferably of a protein or a nucleic acid, and/or as a counter for nanoparticles, proteins, nucleic acids or biological macromolecules.

[0037] The present inventors use a membrane that is based on a polymer material that can, for example, be spin-coated or evaporated or another comparable deposition method. Such method of spin-coating or evaporation is well known to someone skilled in the art. The polymeric material is deposited onto a flat surface, such as a silicon oxide surface of a silicon wafer which has a thin anti-sticking layer on top. Furthermore, on top of this anti-sticking layer, there may be another stabilizing polymeric layer, a so called "carrier layer" which serves the purpose of stabilizing the polymeric membrane. Onto the polymeric carrier layer, the desired polymeric layer is deposited, into which, subsequently pores in the nanometer range are introduced.

[0038] Such introduction of pores may be by way of lithography, preferably electron beam lithography, AFM lithography or optical lithography. After the pore(s) has (have) been introduced, the membrane structure, comprising the anti-sticking layer, the carrier layer and the polymeric membrane, can be lifted from the substrate simply by peeling it off using for example an adhesive tape with a centre hole, so as to avoid coverage of the pore. The pores according to the present invention within artificial polymeric membranes do not require any hard etching techniques, such as KOH etching which are otherwise required for the pores in SiO_2 or Si_3N_4-membranes. Consequently, no hard masks having the capability to withstand KOH etching are required. Because such masks would have to be applied on both sides of the substrate, no high quality deposition methods are required in the method of the present invention. The pores according to the present invention are based on polymeric materials and therefore enable simple engineering approaches to control the final pore size. For example the pore can be manipulated by treatment with oxygen plasma, heating or cross-linking. The surface charge of the polymers can be varied within certain ranges and be easily made neutral which is a clear advantage over the negatively charged surface of oxide membranes, such as SiO_2 and Si_3N_4-membranes. Surface charges are one of the sources of noise and the reason for a blocking of the pores. Moreover, polymer membranes are flexible and are therefore easy to integrate into a mechanical microfluidic environment. The present inventors' approach of using an anti-sticking layer and a polymeric carrier layer, preferably made of a photoresist, enables the integration of a large number of different membrane materials that can be deposited by evaporation, sputtering, spin-coating etc. Moreover, the feedback mechanism by monitoring the pore formation/pore introduction using two electrodes is straightforward and simple to realize. Hence, the present inventors do not need to rely on transmission electron microscopy or ion detection methods that were used in the prior art for such monitoring. The present inventors' approach allows the formation of very thin polymeric membranes based on a wide range of polymeric materials amenable to spin coating, evaporating or depositing from the vapour phase. Furthermore the membranes according to the present invention can be patterned by standard lithography methods. Also, the fact that membranes are fabricated on a substrate, with one layer, e.g. the anti-sticking layer on the substrate possibly functioning as an "integrated" back electrode enables to engineer the nanopore size for example through substrate heating and to monitor formation of the nanopore at the same time.

[0039] As used herein, a "pore with a diameter in the range below500 nm" is also sometimes referred to as a "nanopore". Preferably, such term "nanopore" more specifically refers to a pore having a diameter in the range of from 0.1 nm to 100 nm, more preferably from 1 nm to 75 nm, even more preferably from 1 nm to 50 nm and most preferably from 1 nm to 10 nm. The term "anti-sticking layer" as used herein is meant to describe a layer which, because of its low adhesion to a substrate, allows the peeling off of said anti-sticking layer, together with any additional layer on top of it, from said substrate. It is clear from the foregoing that said anti-sticking layer should only weakly adhere to the flat surface of a substrate, but it should have better adhesion properties to a layer attached to the anti-sticking layer on the other side. The exact choice of material of the anti-sticking layer therefore depends on the material of the substrate and can be determined by someone skilled in the art using his knowledge without undue experimentation. For example, someone skilled in the art knows that a layer of gold has very little adhesion to a silicon oxide surface. In general, the anti-sticking layer is made of a material selected from the group comprising metals, oxides, plastics or other organic components which show a weak adhe-

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sion to the material of which the substrate may be made. Useful examples of materials for the anti-sticking layer are gold, mica, carbon and fluorosilanes. In some embodiments, an anti-sticking effect is achieved by providing the material of the anti-sticking layer as nanoparticles. The substrate according to the present invention is made of a material selected from the group comprising oxides, metals, plastics or other organic components.

[0040] In a preferred embodiment of the method according to the present invention, a carrier membrane based on a suitable polymer, preferably a resist that can be patterned by optical lithography or electron beam lithography, is deposited on the anti-sticking layer (which itself is on a substrate). The patterning by optical or electron beam lithography results in a hole or recess within the carrier membrane, which hole or recess has a diameter in the micrometer range (1 μm - 500 μm). Onto such carrier membrane, the desired polymeric membrane is deposited, preferably by spin-coating or evaporating. Such deposition results in a thin polymeric layer on top of the carrier layer and in a lining of the aforementioned hole or recess within said carrier layer, thus effectively creating a thin polymeric layer also within the hole or recess, i.e. at the bottom and at the walls of the hole or recess. Thereafter, a further patterning occurs, namely the actual introduction of the nanopore into the polymeric membrane, preferably at the site, where the polymeric membrane forms a lining of the aforementioned hole or recess. Such patterning preferably occurs by optical lithography, electron beam lithography, AFM lithography or other methods known from the prior art, such as ion-beam lithography, x-ray lithography, and scanning tunnelling lithography, but also etching techniques if the polymeric membrane includes inorganic layers. Both the carrier membrane as well as the polymeric membrane should preferably be electrically insulating. The material of the carrier membrane is selected from the group comprising polymers and oxides, more preferably it is chosen from the group comprising photoresists. In a preferred embodiment, the material of the polymeric membrane is selected from the group comprising polymers and oxides in a more preferred embodiment, the material of the polymeric membrane is selected from the group comprising electron beam resists and photo resists. If the polymeric membrane comprises both polymeric layers and layers of inorganic material, it is clear to someone skilled in the art that for introducing the nanopore into the inorganic layer, one may have to use a different technique than for the polymeric layers. Whereas the aforementioned lithography steps are used for the polymeric layers, the inorganic layers may require etching techniques, such as ion etching, reactive ion etching, wet etching, and O_2-plasma etching.

[0041] The entire structure, comprising the anti-sticking layer, the carrier membrane and the polymeric membrane can be easily lifted of the substrate, for example by using an adhesive tape. After lifting/peeling, the anti-sticking layer can be removed by dissolution in an appropriate solvent. It is clear to someone skilled in the art which solvent to use, depending on the choice of materials that is used for the anti-sticking layer. For example, someone skilled in the art knows, that metals, like gold and aluminium can be dissolved in aqueous KI/I_2-solution (gold) or basic solutions (aluminium). Water-soluble oxides that are used for the anti-sticking layer require water. An organic anti-sticking layer may be removed by using an appropriate organic solvent, such as acetone, ethanol, DMF etc.

[0042] The entire structure, comprising anti-sticking layer, carrier membrane and polymeric membrane has a thickness of the anti-sticking layer in the range of from about 1 nm to about 100 nm, preferably about 5 nm to about 75 nm, more preferably about 10 nm to about 60 nm and even more preferably about 20 nm to about 50 nm. The thickness of the carrier membrane, preferably, is in the range of from about 1 μm to about 100 μm, preferably about 1 μm to about 50 μm, more preferably about 1 μm to about 20 μm and most preferably about 10 μm. The thickness of the polymeric membraneis in the range of from about 0.1 nm to about 500 nm, preferably from about 1 nm to about 250 nm. In some embodiments, the polymeric membrane may only have a thickness in the range of from 1 nm to 5 nm, preferably about 2 nm. Furthermore, the thickness of both the carrier membrane and the polymeric membrane can be varied, depending on the deposition time. In the case of spin-coating, parameters like rotational rate and the duration of spinning can be used to that extent. Furthermore post-processing steps may be used after the deposition steps, for example the use of oxygen plasma in order to remove layers of the polymeric membrane and/or the carrier membrane. In the case where the membranes are made of photoresist materials, the development time may be varied, as a result of which the membranes are developed to a different extent. For example, the "dark removal rate" is related to the parasitic development of resist areas that were not exposed.

[0043] In order to influence the diameter of the pores, various parameters may be influenced, such as exposure dose in the lithographic steps, subsequent processing steps such as a baking step after exposure, and the duration of the subsequent development. Furthermore, thermal annealing may be used to vary the pore diameter by heating the polymeric membrane to and above the glass transition temperature of the polymer. Additionally, the pore diameter may be varied by performing chemical and/or UV-induced crosslinking steps and/or by performing a plasma treatment step. In addition, the surface properties of the polymeric membrane can be changed by performing further treatments, such as thermal treatment, chemical treatment, light treatment, which vary the surface charge and the wettability of the polymer surface.

[0044] In addition thereto, the present invention provides for a feedback mechanism for monitoring, controlling and adjusting the diameter of the nanopores. The present inventors' idea is to measure the electrical re-

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sistance between a developer bath, i.e. a developing solution on one side of the pore, and the anti-sticking layer. Most developers solutions contain ions that can be used for electric transport measurements. Even developers based on organic solvents show a residual resistance or can be made conductive by the addition of a small amount of ions. Examples of developers are: tetramethylammoniumhydroxide (TMAH) for UV photoresists for optical lithography, methyl isobutyl ketone (MIBK) for poly(methyl methacrylate) (PMMA).

[0045] The schematic arrangement of one embodiment of the feedback mechanism for monitoring is shown in figure 2a. An electrode, preferably a chemically inert electrode is inserted into the developer solution, and a counter electrode is connected to the anti-sticking layer. The anti-sticking layer is electrically conducting in this case. Example are layers from gold, aluminium and others that can be wet-chemically etched. The electrical resistance between the developers solution and the conducting anti-sticking layer depends on the progress of developing. The reduction of the thickness of the polymeric membrane and the final break through (figure 2b and 2c, respectively) lead to a detectable electrical signal. Two types of measurements are possible:

1.) DC measurement of leakage current and break-through current
A constant bias (either voltage or current) leads to a current or voltage variation, once the nanopore structure is developed (break through current and break through voltage drop, respectively). The derived electrical resistance will basically drop as the nanopore opens. Before the break-though, the reduction of the polymeric layer thickness might be detectable if a significant leakage current can flow. After the break-through, the level of the flowing current or voltage drop reveals information about the size of the nanopore.

2.) AC measurement to determine layer thickness reduction and break-through
AC current and voltage measurements can be performed by using an impedance analyser or lock-in-amplifier. Such methods provide the real and imaginary part of the impedance versus time. The in-phase signal corresponds to the real part of the impedance and the information content is similar to that mentioned under 1.). The polymer membrane thickness reduction also results in a change of the capacitance between developer bath and the anti-sticking layer. This signal is measurable as the out-of-phase signal component. After the break-through, the in-phase and out-of-phase component reveal information about the size of the nanopore. The development time could be extended in order to reach a larger desired nanopore diameter.

[0046] This feedback approach leads to a direct control of the nanopore diameter during the developing process. This feedback approach can be also used to adjust the nanopore diameter as a post-development-adjustment after the developing step has been completed. Some methods were already listedabove. Shrinkage of the diameter through thermal annealing or other methods or diameter increase through oxygen plasma treatment or other methods can be monitored by measuring the current though or the voltage drop across the nanopore, using the anti-sticking layer as counter electrode. The fluidic medium does not need to be a developer in this case.

[0047] In the following, reference is made to the figures, wherein

Figure 1 shows an embodiment of a process flow for fabricating nanopores using an anti-sticking layer and a supporting carrier membrane,

Figure 2 shows an example of the setup and time-dependent monitoring of the developing process in order to open a nanopore in a resist membrane in a controlled way,

Figure 3a shows an embodiment of a process flow for fabricating a nanopore using a gold anti-sticking layer, a 10 μm thick SU-8 supporting membrane and a UV6.02 electron beam resist membrane,

Figure 3b shows an embodiment of a process flow for fabricating a nanopore using a polymeric membrane that is a bilayer or multilayer of polymer materials and inorganic materials, such as SiO_2, Six Ny,

Figure 4 shows a SEM image of SU8/UV6.02 membrane system. The inset shows a 45 nm nanopore that was introduced into the UV6.02 resist by electron beam exposure.

Figure 5 shows the in-phase-current vs. time for the developing process of pores for different exposure times of the UV6-membrane and for different developer concentrations (TMAH = tetramethyl ammonium hydroxide.) The curves show a distinct rise of in-phase-current at the "break-through" of the pores with the in-phase-current being represented in arbitrary units with "μ" signifying $x10^{-6}$.

Figure 6 shows a SEM image of a poly(methyl methacrylate) system (molecular weight of 600 000 with a nanopore of a diameter of 10 nm. The present inventors envisage that nanopores of a diameter in the 1 nm range or even below (down to 0.1 nm) can be fabricated.

[0048] Furthermore, in the following reference is made to the following example 1, which is given to illustrate, not to limit the present invention.

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[0049] This example describes the fabrication of single nanopores in a membrane based on 200 nm thick UV6.02 (commercially available from Shipley Inc., USA) electron beam resist. The supporting membrane was based on 10 μm thick SU-8 photoresist (commercially available from Microresist GmbH, Berlin).

1. The anti-sticking layer was made of 50 nm thick gold that was sputter deposited onto a polished SiO_2 surface. **(Figure 3a).**
2. SU-8 photoresist was spin-coated onto the gold layer at 3000 rpm. The resulting thickness was about 10 μm. The resist was soft-baked at 65°C for 2 minutes and 95°C for 5 minutes. **(Figure 3b)**
3. A 60 μm wide hole or recess was exposed into the SU-8 resist layer at 360 nm for 20 seconds. The sample was postbaked at 65°C for 1 minute and 95°C for 2 minutes, and then developed in SU-8 developer for 2 minutes and rinsed in propanol. **(Figure 3c)**
4. UV6.02 (Shipley Inc.), was spin-coated onto the developed SU-8 sample at 4000 rpm. The resulting thickness of the resist layer was about 200 nm. The layer was soft-baked at 130°C for 60 seconds. (**Figure 3d**)
5. The UV6.02 resist layer was exposed with a single shot electron beam exposure at 10 kV, 18pA, and a dose of 125 $\mu C/cm^2$. (**Figure 3e**) The exposed sample was post baked at 130°C for 90 seconds and developed in MIF 726 metal-ion free developer for 45 seconds. A final rinse in DI (= de-ionised) water was applied. (**Figure 3f**)
6. The membrane layers were removed from the supporting substrate by peeling it off using an adhesive tape which had a hole in its center in order not to cover the nanopore. Peeling of the sputtered gold works very well because of the weak adhesion property of gold on silicon oxide. (**Figure 3g**)
7. The 50nm thick anti-sticking gold-layer was removed in aqueous KI/I_2 solution within 2 minutes. (**Figure 3h**)

Figure 4 shows an SEM image of the 60 μm hole or recess that was patterned into the SU-8 resist layer. The center of the hole holds the UV6.02 membrane and the nanopore. The inset in **Figure 4** displays a 45 nm nanopore introduced into the UV6.02 membrane by electron beam exposure. It is envisaged that nanopores in the single nanometer range or even sub-nanometer range (down to 0.1 nm) can thus be easily produced.

Figure 5 shows the in-phase-current vs. time during pore formation according to the present invention.

Figure 6 shows a 10 nm diameter pore in a PMMA system according to the present invention.

[0050] In one embodiment, the polymer membrane mayalso consist of a bilayer or a multilayer of polymer materials and inorganic materials, e.g. SiO_2, Si_xN_y, any oxides, other insulators, and metals. The advantage of such structures is a larger mechanical stability of the membranes and the realisation of ultra-thin inorganic membranes with integrated nanopores. Figure 3b) shows possible sandwich structures.

[0051] The process flow on the left side of Figure 3b) describes how a polymer mask can be used to pattern a thin inorganic membrane layer. The inorganic membrane material canbe deposited by standard deposition methods like vacuum evaporation, sputtering, or spray/spin-coating if applicable (Fig.3 b, a)). The thickness of the inorganic membrane layer should be similar to the thickness of the above-disclosed polymer membrane layers. The applied polymer layer (Fig. 3b, b)) can be patterned by a lithography step (Fig. 3b, c)), for example electron beam (ebeam) lithography,and depending on the inorganic material, the pattern (nanopore) can be extended into the inorganic material by for example reactive ion etching, ion etching, or wet etching (Fig. 3b, d)). In order to reduce the access resistance to the nanopore, 02-plasma etching can be applied to widen the access hole to the nanopore as shown in Fig. 3b, e'). Otherwise, the membrane structure is just completed by removing the anti-sticking layer as described above (Fig. 3b, e) und f')).

[0052] The process flow on the right side of Figure 3b) describes a slight variation of the just described process. In this case, an inorganic material layer is completely embedded between two polymer layers. Advantage of this approach might be a better protection of the thin inorganic material layer against chemical degradation, e.g. during the removal of the anti-sticking layer. Fig. 3b,1)) shows that the first polymer layer is deposited onto the anti-sticking layer. Then, the inorganic material layer is added (Fig. 3b, 2)), and finally, the second polymer layer is deposited (Fig 3b, 3)). Lithographic patterning is applied to the 2nd polymer layer (Fig 3b, 4)). The pattern (e.g. nanopore) is then introduced into the inorganic material layer by means of reactive ion etching, ion etching, wet etching (Fig 3b, 5)). The pattern transfer into the 1st polymer layer can be either performed by additional developing, or by 02 plasma etching (Fig 3b, 5)). Finally, the anti-sticking layer is removed to complete the membrane.

[0053] The features of the present invention disclosed in the specification, the claims and/or in the accompanying drawings, may, both separately, and in any combination thereof, be material for realising the invention in various forms thereof.

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Claims

1. A method of fabricating a polymeric membrane having at least one pore with a diameter in the range below 500 nm, comprising the steps:

   a) providing a substrate,
   b) depositing a polymeric membrane on said substrate,
   c) subjecting said polymeric membrane to a lithography step, thus introducing at least one pore with a diameter in the range below 500 nm into said polymeric membrane, optionally an array of such pores,
   d) lifting-off said membrane from said substrate.

2. The method according to claim 1, wherein step a) comprises the following substeps:

   aa) providing a substrate having a a surface, preferably a plane surface which substrate is made of a material selected from the group comprising oxides, metals and plastics,
   ab) depositing on said surface of said substrate an anti-sticking layer, which anti-sticking layer has little adhesion to said surface of said substrate,
   ac) depositing on said anti-sticking layer a carrier membrane, to which carrier membrane said anti-sticking layer has a greater adhesion than to said substrate,
   ad) patterning said carrier membrane by introducing at least one recess into it by a lithography step, said recess having a diameter in the range of from 1 μm to 500 μm into it.

3. The method according to claim 2, wherein step b) comprises the following substeps:

   ba) depositing a polymeric membrane on said carrier membrane having said at least one recess, by a procedure selected from spin-coating and evaporating, such that said polymeric membrane covers said carrier membrane and, at the site of said at least one recess, forms a lining within said at least one recess,

   and wherein step c) comprises the substep:

   ca) performing said lithography step at said lining within said at least one recess.

4. The method according to any of claims 2 - 3, wherein said carrier membrane is made of an electrically insulating material selected from the group comprising polymers and oxides, wherein, preferably, it is made of a photoresist material.

5. The method according to any of the foregoing claims, wherein said polymeric membrane is made of an electrically insulating material selected from the group comprising polymers and oxides, wherein, preferably, it is made of a resist material selected from photoresist materials and electron beam resist materials.

6. The method according to any of the foregoing claims, wherein step d) occurs by application of force, such as suction, or by application onto said polymeric membrane of an adhesive tape having a hole (holes) so as not to cover said pore(s), and by subsequently lifting-off said polymeric membrane from said substrate.

7. The method according to any of claims 2 - 6, wherein said anti-sticking layer has a thickness in the range of from about 1 nm to about 100 nm, preferably about 5 nm to about 75 nm, more preferably about 10 nm to about 60 nm and most preferably about 20 nm to about 50 nm.

8. The method according to any of the foregoing claims, wherein said polymeric membrane has a thickness in the range of from about 0.1 nm to about 500 nm, preferably from about 1 nm to about 250 nm.

9. The method according to any of claims 2-8, wherein said carrier membrane has a thickness in the range of from about 1 μm to about 100 μm, preferably about 1 μm to about 50 μm, more preferably about 1 μm to about 20 μm, and most preferably about 10 μm.

10. The method according to any of the foregoing claims, wherein said method comprises the additional step:

    e) dissolving said anti-sticking layer in a suitable solvent, wherein, preferably, said anti-sticking layer is made of a material selected from the

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group comprising metals, oxides and plastics, and wherein, preferably, said suitable solvent is an aqueous basic solution (e.g. for metals), an aqueous KI/I_2-solution (e.g. for metals), water (e.g. for water soluble oxides), and an organic solvent (e.g. for plastics), such as acetone, alcohols, organic acids.

11. The method according to claim 10, wherein steps d) and e) occur concomitantly.

12. The method according to any of the foregoing claims, wherein said lithography step in step c) is selected from the group comprising optical lithography, electron beam lithography and atomic force microscope (AFM) lithography, and includes a developing step.

13. The method according to any of claims 2-12, wherein said lithography step in step ad) is selected from the group comprising optical lithography and electron beam lithography, and includes a developing step.

14. The method according to any of claims 2-13, wherein steps ab) and ac) occur by a procedure selected from thermal evaporation, electron-gun deposition, spin-coating, dip-coating, sputtering and vapour-phase deposition.

15. The method according to any of claims 12-14, wherein said developing step in ad) and ca) occurs by applying a developing solution to said carrier membrane and said polymeric membrane, respectively, preferably at the site where said recess and said pore is to be introduced, respectively.

16. The method according to claim 15, wherein said anti-sticking layer is made of an electrically conducting material, preferably a metal, such as gold or aluminium.

17. The method according to claim 16, wherein said introduction of said at least one pore having a diameter in the range below 500 nm into said polymeric membrane in step c) is monitored by connecting an electrode with the developing solution and by connecting a counter-electrode with the anti-sticking layer, and wherein voltage and/or current variations are measured between said electrodes.

18. The method according to claim 17, wherein a constant DC voltage or current is applied via said electrodes and the current or voltage is monitored over time, wherein an increase in current and/or a decrease in voltage is indicative of completion of said introduction of the pore.

19. The method according to claim 17, wherein, via said electrodes, AC current and/or voltage measurements are performed using an impedance analyzer or lock-in-amplifier wherein such measurements afford a real and imaginary part of an impedance versus time-curve, and wherein the in-phase signal corresponds to the real part of the impedance and wherein an increase in current and/or a decrease in voltage is indicative of completion of said introduction of the pore, and wherein, furthermore the out-of-phase-signal corresponds to the imaginary part of the impedance versus time-curve and wherein, after completion of said introduction of the pore, the in-phase-signal and out-of-phase-signal reveal information about the size of the pore, said in-phase signal of the impedance reflecting the ohmic resistance of said pore in solution, thus reflecting the depth and the area size of said pore, said out-of-phase signal of the impedance reflecting the capacitance of said pore.

20. A membrane structure comprising a polymeric membrane on a carrier membrane, said polymeric membrane having at least one pore with a diameter below 500 nm, produced by the method according to any of the foregoing claims.

21. The membrane structure according to claim 20, wherein said pore has a diameter in the range of from 0.1 nm to 100 nm, preferably from 1 nm to 75 nm, more preferably 1 nm to 50 nm, and most preferably 1 nm to 10 nm.

22. The membrane structure according to any of claims 20 - 21, wherein said polymeric membrane has a thickness in the range of from about 0.1 nm to about 500 nm, preferably from about 1 nm to about 250 nm.

23. The membrane structure according to any of claims 20-22, wherein said carrier membrane has a thickness in the range of from about 1 μm to about 100 μm, preferably about 1 μm to about 50 μm, more preferably about 1 μm to about 20 μm, and most preferably about 10 μm.

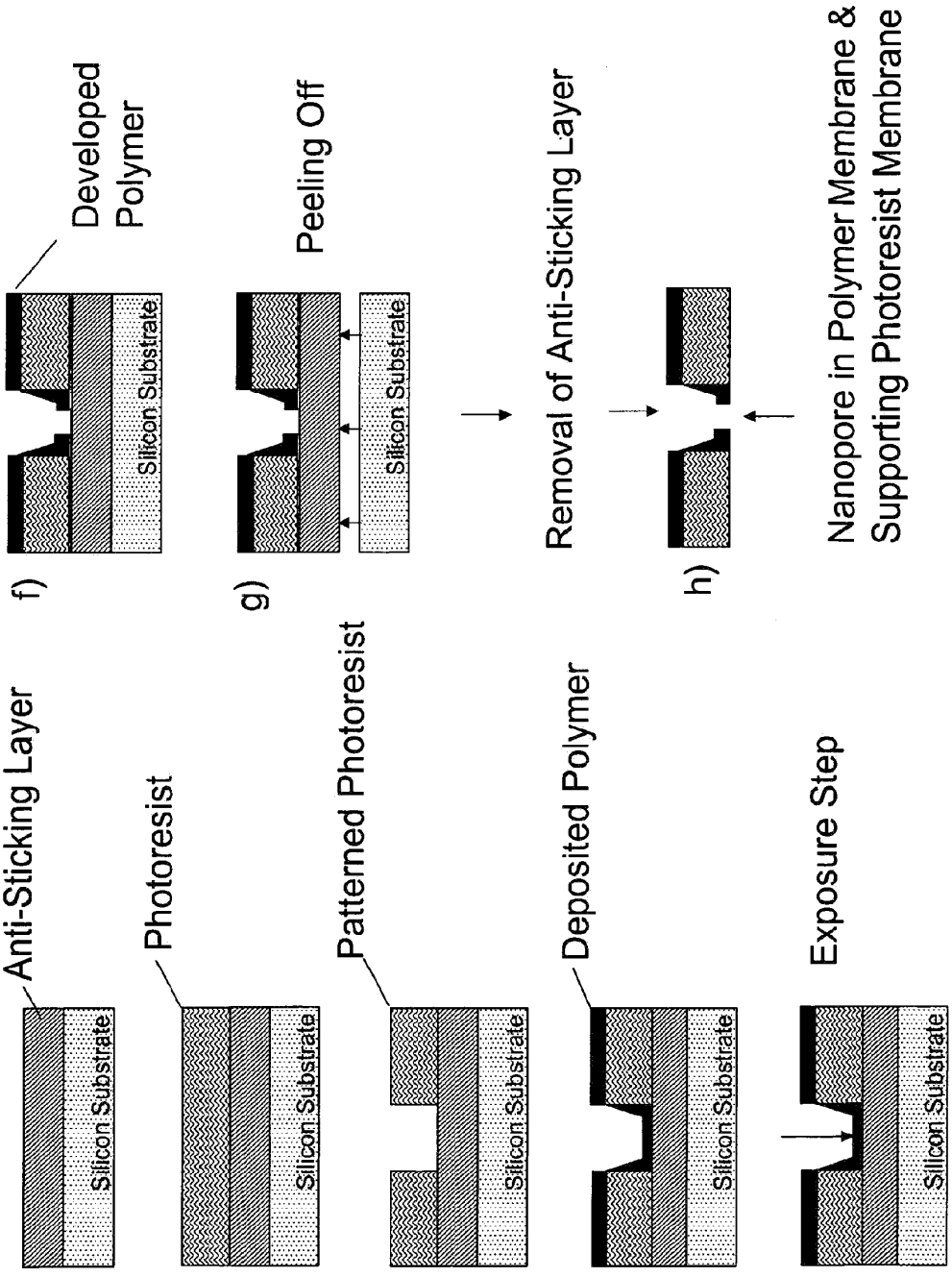
24. The membrane structure according to any of claims 20 - 23, wherein an array of n times m pores has been introduced by the method according to any of claims 1 - 19, n and m being positive integers.

25. The membrane structure according to any of claims 20 - 24, into which additionally a film heater is integrated.

26. Use of a membrane structure according to any of claims 20 - 25 in an electronic device, preferably for determining the size and/or sequence of a biopolymer, preferably of a protein or a nucleic acid, and/or as a counter for nanoparticles, proteins, nucleic acids or biological macromolecules.

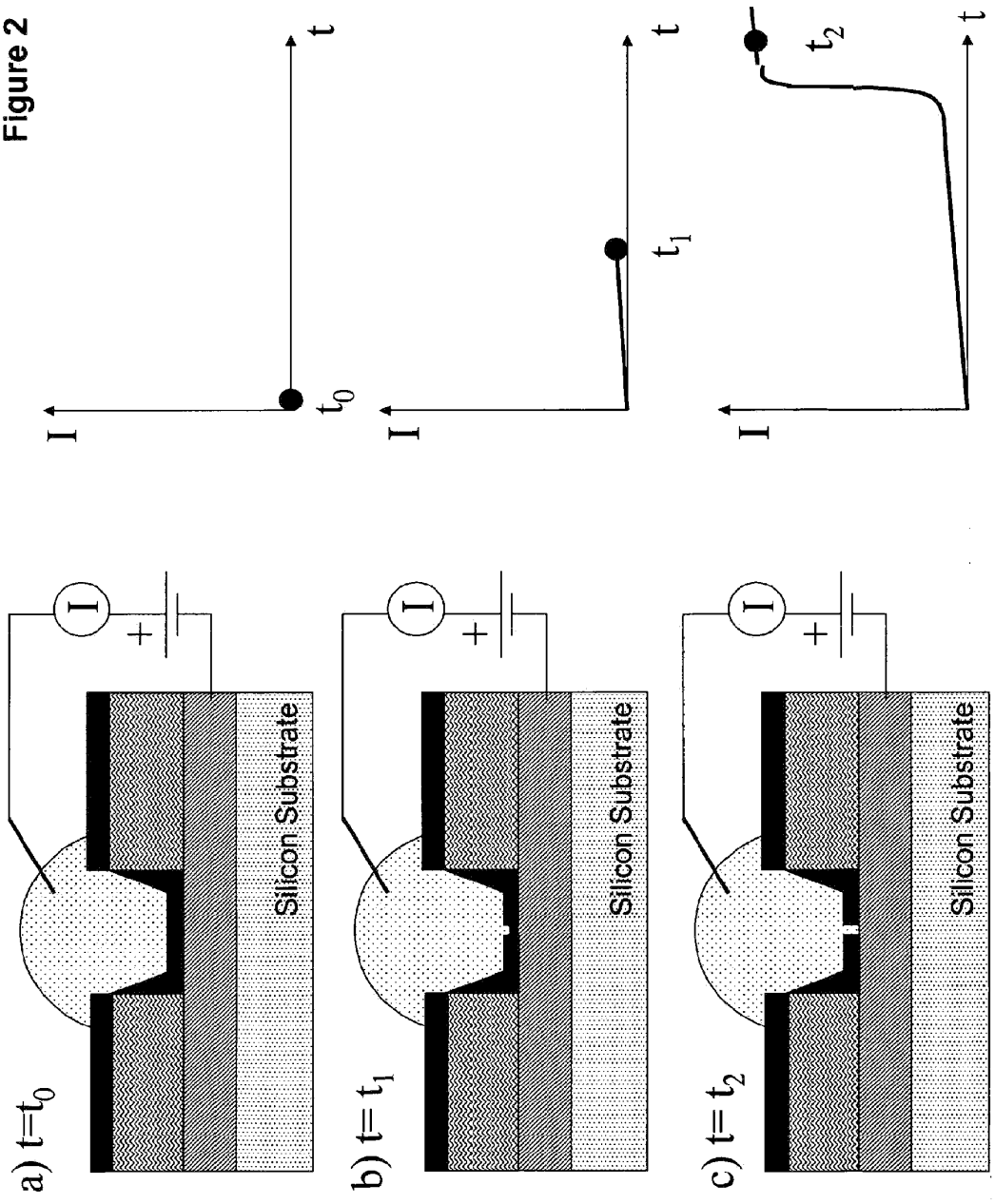
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Figure 1



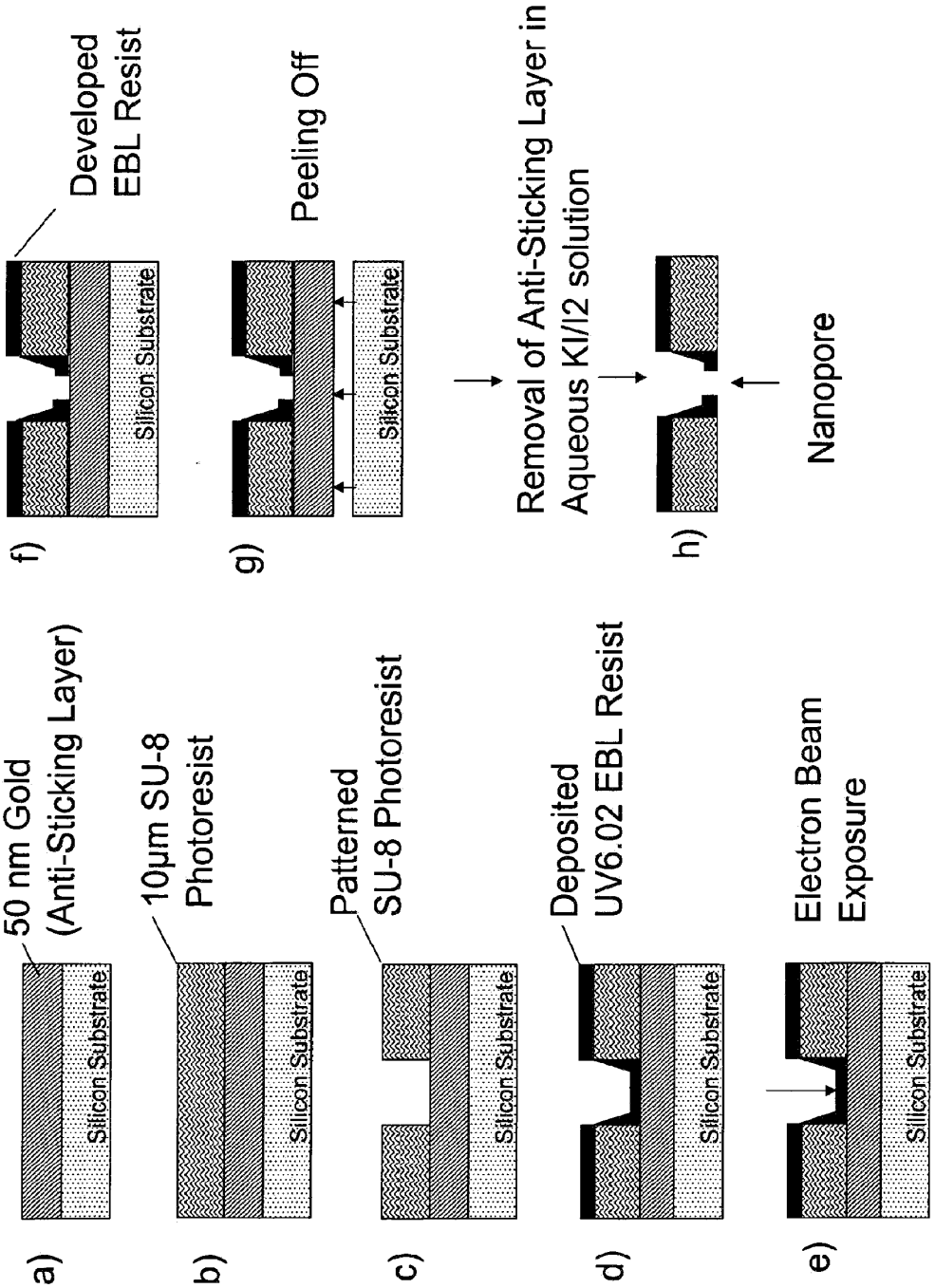
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Figure 2



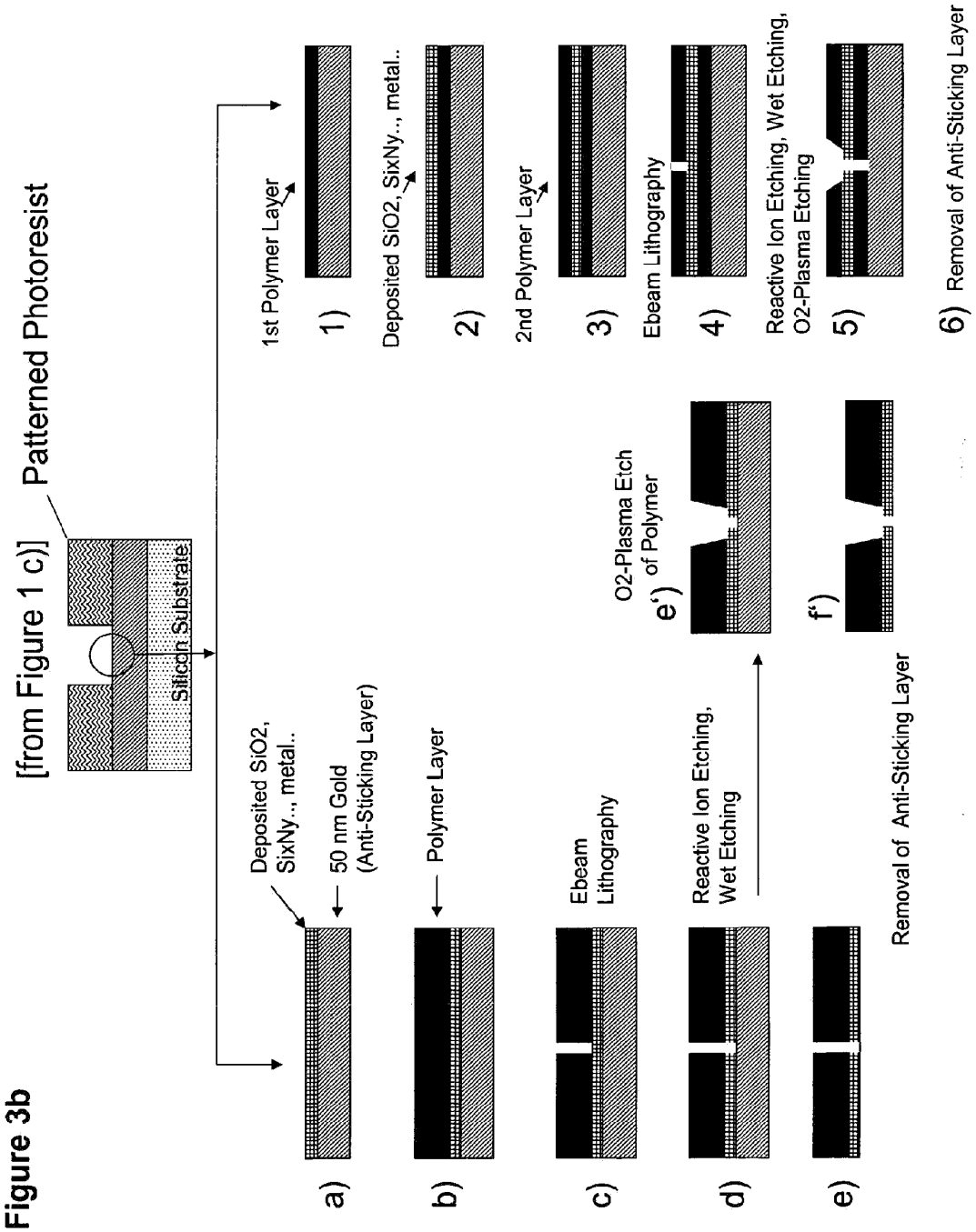
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Figure 3a



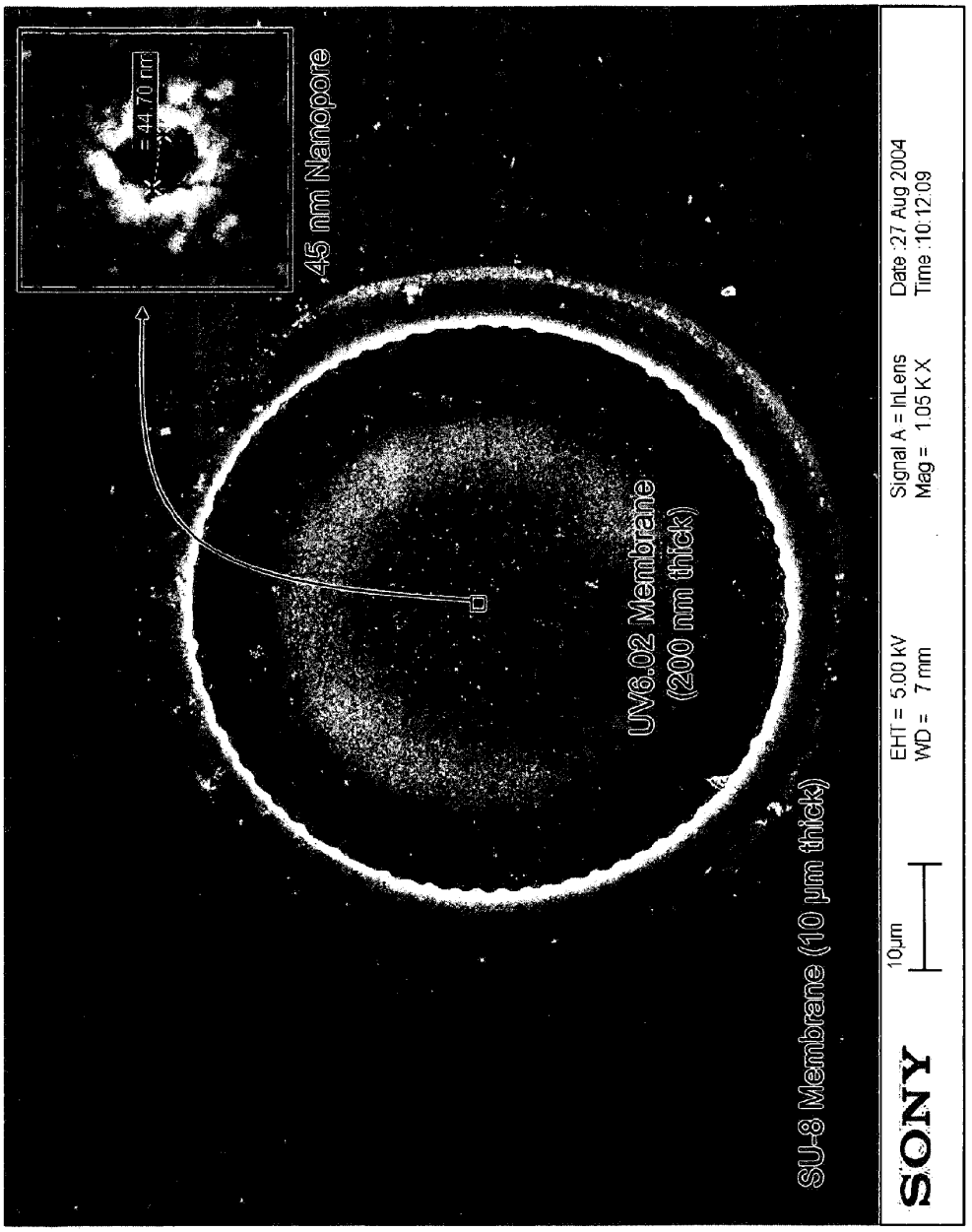
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Figure 3b



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Figure 4



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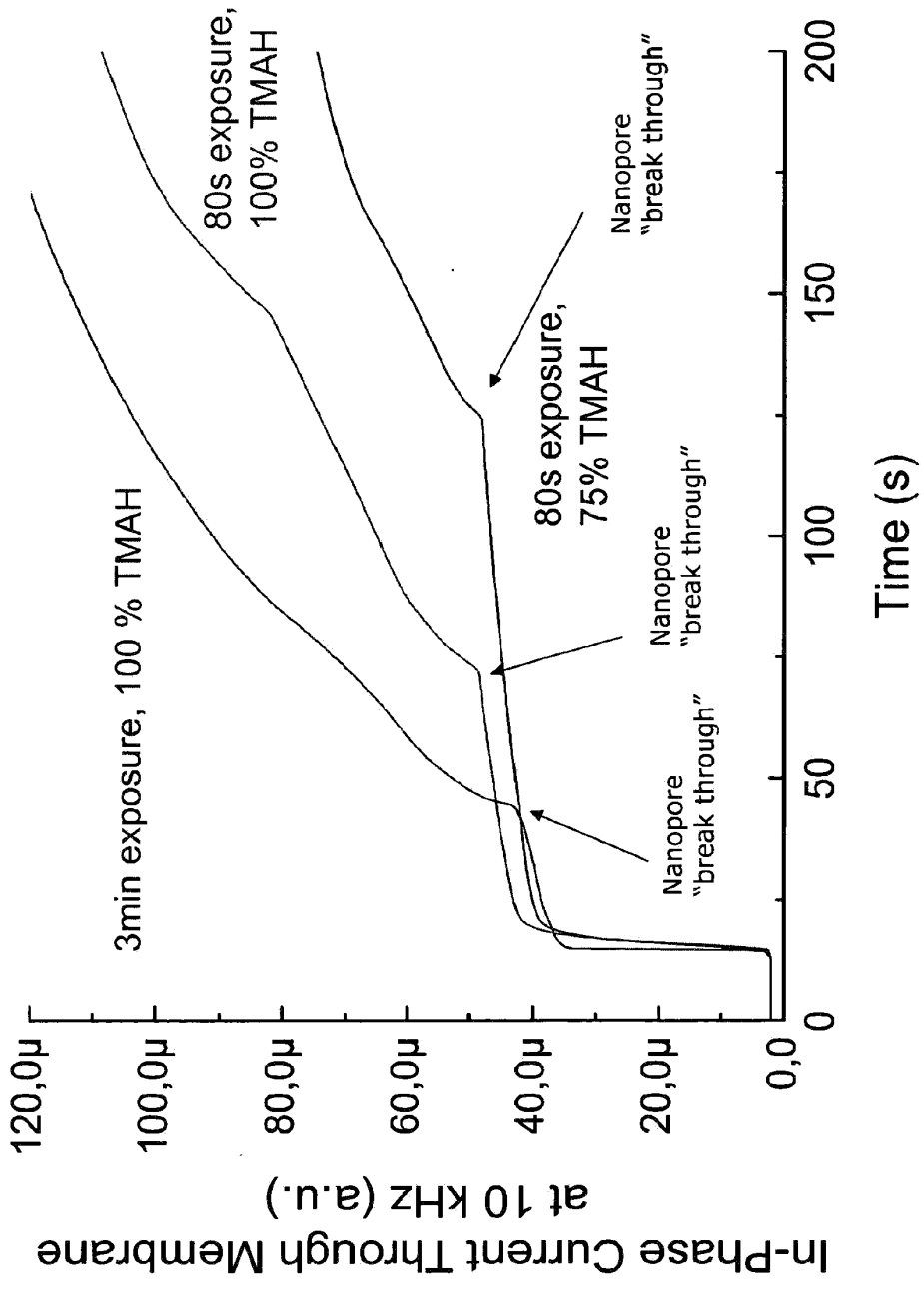
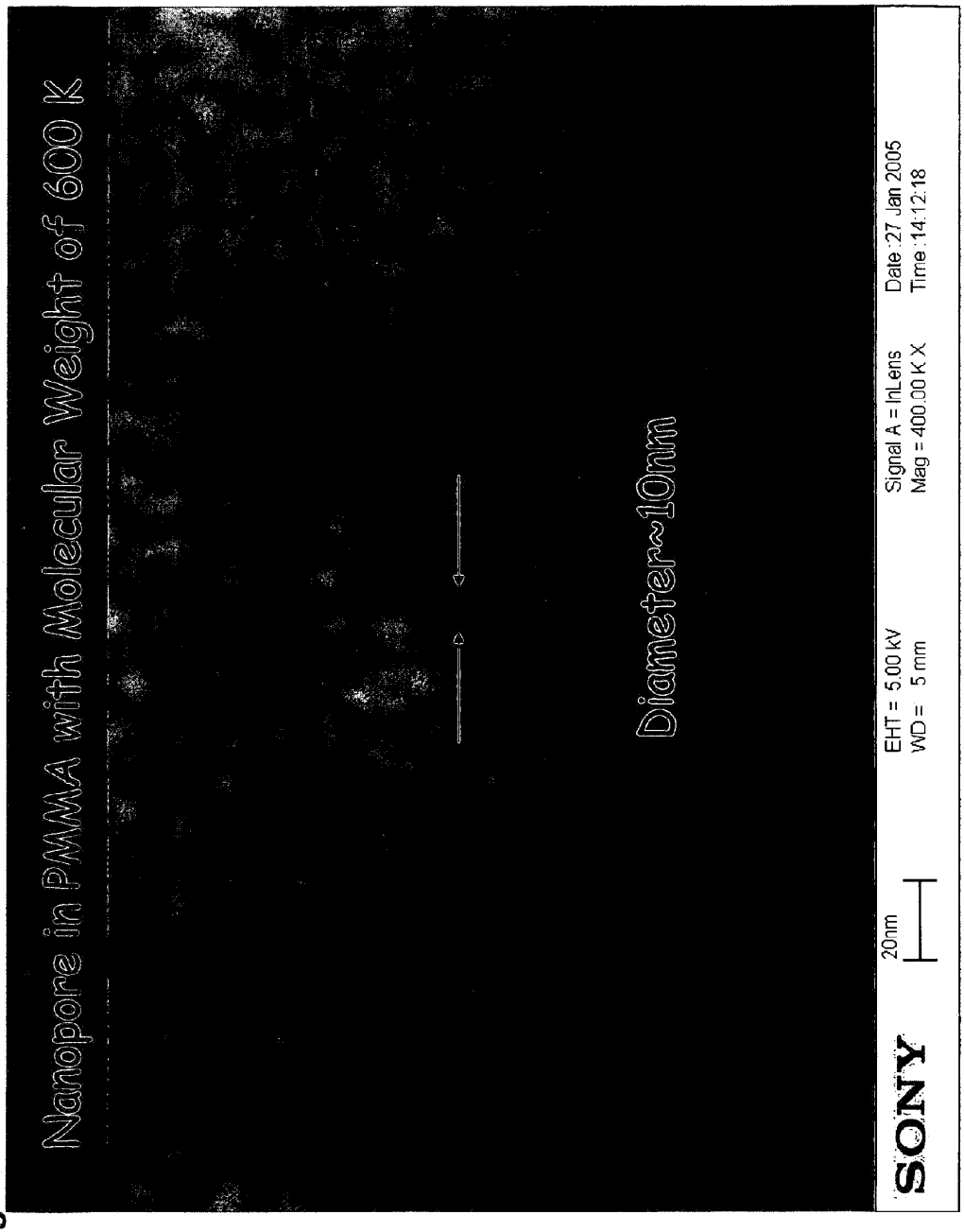


Figure 5

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Figure 6



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European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 05 01 0486

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
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A	EP 0 203 577 A (THE DOW CHEMICAL COMPANY) 3 December 1986 (1986-12-03) * column 24, lines 1-14 *	1	
A	PATENT ABSTRACTS OF JAPAN vol. 010, no. 172 (C-354), 18 June 1986 (1986-06-18) & JP 61 021702 A (TERUMO KK), 30 January 1986 (1986-01-30) * abstract *	1	
A	GB 2 199 786 A (* FUJI PHOTO FILM CO LTD) 20 July 1988 (1988-07-20) * page 11, lines 28-31 *		TECHNICAL FIELDS SEARCHED (Int.Cl.7) B01D B82B B81C C12Q
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1 The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
The Hague	19 October 2005	Lançon, E

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
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European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 05 01 0486

DOCUMENTS CONSIDERED TO BE RELEVANT

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			TECHNICAL FIELDS SEARCHED (Int.Cl.7)

The present search report has been drawn up for all claims

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The Hague	19 October 2005	Lançon, E

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ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 05 01 0486

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19-10-2005

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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REFERENCES CITED IN THE DESCRIPTION

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(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 2 267 451 A2

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G01N 33/53 *(2006.01)*

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01928376.1 / 1 282 734

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Remarks:
This application was filed on 27-09-2010 as a divisional application to the application mentioned under INID code 62.

(54) Two-dimensional spectral imaging system and method

(57) Devices, systems, methods, and compositions of matter can track and/or identify a library of elements, particularly for use with fluids, particulates, cells, and the like. Signals from one or more semiconductor nanocrystals may be combined to define spectral codes. Separation of signal wavelengths within dedicated wavelength ranges or windows facilitates differentiation of spectral codes, while calibration signals within the spectral codes can avoid ambiguity. Modeling based on prior testing can help derive libraries of acceptable codes.

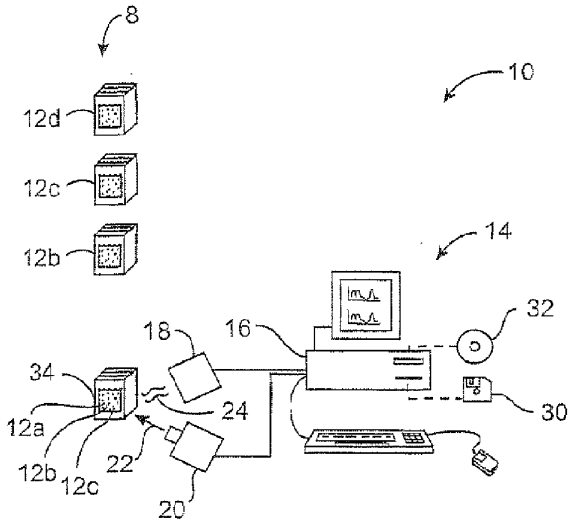


FIG. 1

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Description

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] The application claims the benefit of priority from co-pending U,S. Provisional Patent Application No. 60/195,520, entitled "Method for Encoding Materials with Semiconductor Nanocrystals, Compositions Made Thereby, and Devices for Detection and Decoding Thereof," filed April 6, 2000, the full disclosure of which is incorporated herein by reference.

[0002] The subject matter of the present application is related to the following co-pending patent applications, the disclosures of which are also incorporated herein by reference: U.S. Patent Application No. 09/160,458 filed September 24, 1998 and entitled, "Inventory Control"; U.S. Patent Application No. 09/397,432 filed September 17, 1999, and also entitled "Inventory Control"; PCT Patent Application No. WO 99/50916 as published on April 1. 1999, entitled "Quantum Dot White and Colored Light Emitting Diodes"; and U.S. Patent Application No. 09/259,982 filed March 1, 1999, and entitled "Semiconductor Nanocrystal Probes for Biological Applications and Process for Making and Using Such Probes".

BACKGROUND OF THE INVENTION

[0003] The present invention generally provides devices, systems, methods, and kits for labeling and/or tracking inventories of elements. In a particular embodiment, the invention provides improved identification systems and methods which make use of labels that emit differentiable spectra, the spectra preferably including a number of signals having measurable wavelength maxima, minima, and/or intensities.

[0004] Tracking the locations and/or identities of a large number of items can be challenging in many settings. Barcode technology in general, and the Universal Product Code in particular, has provided huge benefits for tracking a variety of objects. Barcode technologies often use a linear array of elements printed either directly on an object or on labels which may be affixed to the object. These barcode elements often comprise bars and spaces, with the bars having varying widths to represent strings of binary ones, and the spaces between the bars having varying widths to represent strings of binary zeros.

[0005] Barcodes can be detected optically using devices such as scanning laser beams or handheld wands. Similar barcode schemes can be implemented in magnetic media. The scanning systems often electro-optically decode the label to determine multiple alphanumerical characters that are intended to be descriptive of (or otherwise identify) the article or its character. These barcodes are often presented in digital form as an input to a data processing system, for example, for use in point-of-sale processing, inventory control, and the like.

[0006] Barcode techniques such as the Universal Product Code have gained wide acceptance, and a variety of higher density alternatives have been proposed. Unfortunately, these known barcodes are often unsuitable for labeling many "libraries" or groupings of elements. For example, small items such as jewelry or minute electrical components may lack sufficient surface area for convenient attachment of the barcode. Similarly, emerging technologies such as combinatorial chemistry, genomics and proteomics research, microfluidics, micromachines, and other nanoscale technologies do not appear well-suited for supporting known, relatively large-scale barcode labels. In these and other ongoing work, it is often desirable to make use of large numbers of fluids, and identifying and tracking the movements of such fluids using existing barcodes is particularly problematic. While a few chemical encoding systems for chemicals and fluids had been proposed, reliable and accurate labeling of large numbers of small and/or fluid elements remained a challenge.

[0007] Small scale and fluid labeling capabilities have recently advanced radically with the suggested application of semiconductor nanocrystals (also known as Quantum Dot particles), as detailed in U.S. Patent Application No. 09/397,432, the full disclosure of which is incorporated herein by reference. Semiconductor nanocrystals are microscopic particles having size-dependent electromagnetic signal generation properties. As the band gap energy of such semiconductor nanocrystals vary with a size, coating and/or material of the crystal, populations of these crystals can be produced having a variety of spectral emission characteristics. Furthermore, the intensity of the emission of a particular wavelength can be varied, thereby enabling the use of binary or higher order encoding schemes. A label generated by combining semiconductor nanocrystals having differing emission signals can be identified from the characteristics of the spectrum emitted by the label when the semiconductor nanocrystals are energized.

[0008] While semiconductor nanocrystal-based inventory control schemes represent a significant advancement for tracking and identifying many elements of interest, still further improvements would be desirable. In general, it would be desirable to provide improved identification systems, methods for identifying elements, and/or identifiable groups or libraries of elements. It would be particularly beneficial if these improved inventory and identification systems enhanced the accuracy, reliability and robustness of the identifications provided by the system. Ideally, these improvements should allow enhanced differentiation of the labeled elements without significantly increasing the overall costs, complexity and/or size of the labels and associated system components. At least some of these objectives may be provided by the inventions described hereinbelow.

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SUMMARY OF THE INVENTION

[0009] The present invention generally provides improved devices, systems, methods, kits, and compositions of matter for identification of elements of interest. The techniques of the present invention will often be adapted for use in tracking and/or identifying a large number or library of elements. These tracking or identification techniques are particularly well suited for use with fluids (such as liquids, solutions, gases, chemicals, biological fluids, and the like) and small items (such as jewelry, calls, components for assembly, and the like), but may also be used with a wide range of identifiable elements (including consumer products, powders, biological organisms, compositions of matter, and the like.)

[0010] The inventory control techniques of the present invention will often make use of signals generated by one or more semiconductor nanocrystals. Semiconductor nanocrystals can be fabricated to absorb and/or emit signals at discrete wavelengths and intensities. The discrete signals from semiconductor nanocrystals can be combined to form a large number of differentiable spectral codes. More specifically, semiconductor nanocrystals can have absorption and emission characteristics that vary with their size and composition. By fabricating populations of semiconductor nanocrystals to generate signals at discrete wavelengths, the differing populations of semiconductor nanocrystals can be selectively combined to define labels having differentiable spectral codes.

[0011] While semiconductor nanocrystals are particularly advantageous for defining complex spectral codes, the labels may also comprise any of a wide variety of alternative signal-emitting or other markers, including organic or inorganic fluorescent dyes, Raman scattering materials, and the like. Regardless, the invention enhances the robustness of the spectral codes by a variety of techniques, including the addition of calibrating signals within the label spectra as an aid for code interpretation. This calibration signal can compensate for overall signal variability in wavelength and/or in intensity. Surprisingly, the invention also enhances the number of differentiable codes by limiting the label spectra to a series of signals having discrete wavelengths within separated wavelength ranges or windows, thereby facilitating identification of substantially isolated peak wavelengths from among tightly spaced discrete allowable wavelength increments. The invention also provides methods for establishing an inventory of acceptable labels by either physically testing candidate spectral labels, or by modeling the characteristics of the spectral labels and/or label interpreting system to help insure that the system can accurately differentiate between the different elements of the inventory based on the spectra of the labels.

[0012] In a first aspect, the invention provides an identification system. The identification system comprises a plurality of identifiable elements and a plurality of labels. Each label is associated with an identifiable element, and the labels include reference markers and other markers, the labels generating spectra in response to excitation energy. An analyzer identifies the elements from the spectra of the associated labels by calibrating the spectra using reference signals generated by the reference markers.

[0013] In many embodiments, the labels comprise semiconductor nanocrystals, with the reference markers often including at least one reference semiconductor nanocrystal. The reference markers may include a plurality of reference semiconductor nanocrystals, the reference markers of each label generating a reference wavelength with a reference intensity. Similarly, the other markers may comprise other semiconductor nanocrystals generating associated signals at associated wavelengths and with associated intensities. The spectra may define an identifiable spectral code, with the other markers optionally comprising code signal markers, the spectra comprising a combination of the reference signals from the reference markers in combination with code signals from the code signal markers

[0014] Optionally, the analyzer can, for each label, discretely quantify the other signals of the label by comparison of the other signals with the reference signal. For example, where the reference signal has a reference intensity and the other signals of the label have other intensities, the analyzer can discretely quantify the other intensities by comparison to the reference intensity of that label. This is particularly beneficial when interpreting spectral codes which employ variations in both wavelength and intensity. The intensities may define discrete intensity ratios relative to the associated reference intensities. For example, the other intensities may be integer multiples of the reference intensity, or a variety of regular intensity ratio increments may be provided, with each signal from the other markers having an intensity selected from ½, 1, 1.5, ... or 3.5 times the intensity of the reference signal. In some embodiments, the signals from the other markers may have an arbitrary, and typically known, intensity relative to the intensity of the reference signal.

[0015] To facilitate identification of the reference signal, the reference intensity may be a highest or lowest intensity of the label spectra. Alternatively, at least some of the labels may have a common reference signal wavelength. A variety of alternative reference signal identification criteria may be established, such as selecting a shortest or longest wavelength of the spectra of the label as the reference wavelength, and the like. The analyzer may be adapted to identify the reference signal based on a pre-determined criteria.

[0016] Advantageously, the spectral identification system of the present invention is particularly well-suited for use with large numbers of identifiable elements. Typically, the identification system will include at least 10 elements, often having at least 100 elements, often having at least 1,000 elements, and in many embodiments, having at least 10,000 identifiable elements. As the invention enhances the robust interpretation of complex spectral codes, large numbers of such codes may be reliably interpreted with the aid of a calibration signal within the spectra. Nonetheless, the invention

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may find uses with identification systems having fewer numbers of labels.

[0017] In many embodiments, the analyzer will comprise a tangible media embodying a machine-readable code comprising a listing of a plurality of distinguishable labels. Often times, the code will also include a listing of the identifiable elements, and can provide a correlation between each label and an associated identifiable element having the label. The identifiable elements may comprise compositions of matter, fluids, articles of manufacture, consumer products, components for an assembly, and a wide variety of alternative items of interest.

[0018] In a related method aspect, the invention provides a method for sensing a plurality of identifiable elements. The method comprises labeling each identifiable element with a reference marker and at least one associated other marker. The markers of a first label from a first identifiable element are energized so that the markers generate signals. A spectrum defined by the combined signals is measured from the first identifiable element. The first identifiable element is identified from the measured spectrum by calibrating the spectrum with reference to a reference signal from the reference marker of the first label.

[0019] In another related aspect, the invention provides a library of elements. The library comprises a plurality of identifiable elements, each identifiable element having an associated label with a reference marker. The labels generate spectra in response to an excitation energy. Each spectrum includes a wavelength calibration reference signal from the reference marker.

[0020] In another aspect, the invention provides a method comprising labeling an identifiable element with a label, and measuring a spectrum generated by the label. The spectrum comprises a plurality of signals. The element is identified by selecting a first wavelength range encompassing a first signal of the spectra, and by determining a wavelength of the first signal within the first range. Typically, the wavelength of the signal will comprise wavelength having a local and/or overall maximum intensity, often referred to as the peak wavelength of the signal.

[0021] Often times, the element will be labeled by applying at least one semiconductor nanocrystal to the element. The semiconductor nanocrystals generate at least some of the signals of the spectra in response to excitation energy.

[0022] The method will often further comprise selecting a second wavelength range encompassing a second signal of the spectra, and determining a wavelength of the second signal within the second range. Still further additional wavelength ranges may be selected, and wavelengths identified for each additional signal of the spectra within its associated range. Preferably, no more than one signal of the spectra will be disposed within each wavelength range. By limiting the number of signals within a wavelength range, the various signals of the spectra can be sufficiently separated to avoid excessive overlap of adjacent signals. This allows wavelengths of the signals within each associated wavelength range to be readily identified, even when the signals can occupy any of a plurality of relatively tightly spaced discrete wavelength increments. Accurate selection of an appropriate predetermined wavelength for each signal is significantly facilitated by limiting spacing between adjacent signals.

[0023] Typically, the wavelengths of the first and second signals will be determined by selecting the wavelengths of the signals from a plurality of discrete wavelengths within the ranges. The discrete wavelengths within each range can be sufficiently close that if two signals were at adjacent discrete wavelengths within the range they would substantially overlap, optionally overlapping sufficiently to make identification of either or both wavelength peaks of the signals difficult and/or impossible.

[0024] In many embodiments, the discrete wavelengths within the ranges will be predetermined. These discrete wavelengths may be separated by about 1 nanometer or more, generally being separated by about 5 nanometers or more, optionally being separated by about 15 nanometers or more, and in many embodiments, being separated by about 30 nanometers or more.

[0025] Typically, the wavelength ranges will be separated, ideally being sufficiently separated so that a pair of signals at adjacent discrete wavelengths within two different ranges can have their discrete wavelengths identified independently and relatively easily. Typically, the ranges will be separated by at least about 30 nm, often by at least about 50 nm, and each wavelength range will include at least 1, and often at least 5 predetermined discrete wavelengths. To accommodate relatively large numbers of spectral codes, there will often be at least three non-overlapping ranges, with the wavelength ranges optionally being predetermined. In other embodiments, the wavelength ranges may not be separated. In such embodiments, the encoding system may optionally have signals within two adjacent wavelength ranges separated by at least a predetermined wavelength separation, wavelengths within adjacent ranges typically being separated by at least 30 nm, often being separated by at least about 50 nm.

[0026] Optionally, the wavelength may be determined by deciding whether a discrete wavelength is present or absent. Such binary methods will often make use of labels having at least one different signal for each different label. Alternatively, a discrete intensity of at least one wavelength may be measured to provide a higher-order code with more information for a given spectral range.

[0027] Where differing materials and their associated labels are to be intermingled, the signals of a first label may optionally be encompassed within a first wavelength range, while the signals of a second label are encompassed within another wavelength range. Separation of the first and second wavelength ranges can help avoid confusion between the intermingled markers of the two labels, allowing each identifiable element to be independently identified.

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[0028] In a related method aspect, the invention provides a method for sensing a plurality of intermingled labels. The method comprises energizing the labels so that the labels generate signals. A first label is identified by measuring a first discrete wavelength from among a plurality of discrete wavelengths within a first wavelength range. A second label is identified by measuring a second discrete wavelength from among a plurality of discrete wavelengths within a second wavelength range, the first and second wavelength ranges being separated.

[0029] Separation of differing wavelengths of differing labels into separate wavelength ranges or windows is particularly useful when tracking fluids which are to be combined. For example, when any of a first plurality of fluids is to be added to any of a second plurality of fluids, maintaining the spectral codes of each group of fluids within a dedicated window can significantly facilitate identification of each fluid in the combination. Similarly, labels may be attached at each process step of a multi-step method so as to indicate the specific processes performed.

[0030] In yet another aspect, the invention provides an inventory system comprising a plurality of identifiable elements. A plurality of labels have markers, with each label associated with an element. Each marker generates a signal when energized so that each label emits an identifiable spectrum. At least some of the spectra comprise a plurality of signals, with each signal of the spectra having a discrete wavelength selected within a dedicated wavelength range. The ranges are sufficiently separated so that the signals in different ranges are independently identifiable.

[0031] In yet another aspect, the invention provides an inventory label method. The method comprises generating a plurality of candidate labels, and selecting a plurality of acceptably distinguishable labels from among the candidate labels. The acceptable labels are selected by determining spectra emitted by the candidate labels when the candidate labels are energized, and by comparing the spectra of the candidate labels.

[0032] Candidate labels may be generated by physically combining a plurality of markers, where each marker emits a marker signal at an associated signal wavelength in response to excitation energy. This also allows the spectra of the labels to be measured by directing excitation energy towards the markers. Alternatively, the spectra of the candidate labels may be determined by modeling a combination of a plurality of marker signals. The individual marker signals may be separately measured, or the marker signals may be calculated by modeling emissions from a manufacturable marker. Preferably, the calculated signals may be adjusted for spectra analyzer characteristics and/or variations of measured marker signals. Often times, at least some of the candidate codes will be compared with a library of distinguishable codes to determine if the candidate codes are acceptable, and the acceptable candidate codes can be added to the library.

[0033] In yet another aspect, the invention provides a method for identifying a plurality of identifiable elements. The method comprises energizing a plurality of labels so that a first marker of each label generates a first signal with a first wavelength peak. At least some of the labels comprise multiple-signal labels, and each multiple-signal label has a second marker generating a second signal with a second wavelength peak. The first wavelength peaks are measured, and the second wavelength peak of each multiple-signal label can be measured at a predetermined minimum wavelength separation (or more) from the associated first peak. The labels can then be identified in response to the measured peaks. Generally, each predetermined minimum wavelength separation is at least as large as a full width half maximum (FWHM) of one ore both of the peaks separated thereby.

BRIEF DESCRIPTION OF THE DRAWINGS

[0034]

Fig. 1 schematically illustrates an inventory system and identification method according the principles of the present invention.

Fig. 1A schematically illustrates an exemplary processor for the system of claim 1.

Figs. 1B, 1C, and 1D schematically illustrate a simple single-signal spectral code.

Figs. 2A-E schematically illustrate a spectral code having a plurality of signals.

Fig. 3 schematically illustrates a spectral code having a single signal within each of a plurality of separated windows.

Fig. 4 graphically illustrates rules used to establish a series of spectral codes so that the signals are sufficiently separated to allow tighter candidate discrete peak wavelengths.

Fig. 5 illustrates one method for spectral deconvolution which may be used to interpret binary and/or higher order codes according the principles of the present invention.

Fig. 6A schematically illustrates a potentially ambiguous multi-signal spectral code.

Fig. 6B schematically illustrates an unambiguous code having a calibration reference signal.

Fig. 7 illustrates calibration of an intensity and/or wavelength of a spectral code using a reference signal.

Figs. 8A and 8B schematically illustrate two-dimensional imaging using a spectrometer to read a plurality of spectral codes with reference to a calibration reference signal.

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