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(54) WIDE RANGE LUMINESCENT IMMUNOASSAYS

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ESSAIS IMMUNOLOGIQUES LUMINESCENTS DE PLAGE LARGE

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- **HAGAN ET AL.: 'Lanthanide-based time-resolved luminescence immunoassays' ANALYTICAL & BIOANALYTICAL CHEMISTRY vol. 400, no. 9, 11 May 2011, pages 2847 - 2864, XP019909258**

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EP 2 839 283 B1

Description

BACKGROUND OF THE INVENTION

[0001] In many situations in clinical diagnostics, certain samples quantified by immunoassays can generate out of high range values, i.e. the analyte concentration is higher than the level where the assay can produce accurate and reproducible results. There is a need to quantify these samples because patients producing such out of high range analyte samples often have a high incidence of morbidity and mortality demanding medical attention. Immunoassays are also useful tools in these clinical situations not only for diagnosis but as well to monitor therapy.

[0002] Standard laboratory practice with an out of high range sample is for the laboratory personnel to further dilute the sample so that the analyte concentration falls within the quantification range followed by a repeat second assay. This protocol is problematic in that the reassay requires more time to result, which can be detrimental in acute care or emergency rooms, and further incurs additional reagent cost to the laboratory. Some common lateral flow immunoassay devices do not have a means to dilute samples, which compels the user to perform the subsequent assay on another instrument enabling sample dilution protocols.

[0003] Solid phase immunoassays have limitations with high range samples because analyte concentrations can exceed the binding capacity of the immobilized antibody. Some clinical assays further require the combination of ultra sensitive detection of low analyte levels and quantification of high amounts of analyte, consequentially a wide quantification range is desirable. B-type naturetic peptide, NTproBNP, and procalcitonin are such examples.

[0004] Development of solid phase immunoassays with a wide quantification range with low and high level detection are opposed technical goals. For example, in order not to exceed the immobilized antibody binding capacity with high analyte levels, samples are highly diluted (1/10 - 1/100) or have short incubation times with the solid phase. Sensitive assays often require minimal sample dilutions (undiluted, 1/2 - 1/3) and relatively long incubation times with solid phase to effect binding of detectable amounts of trace analyte. The net result is often a compromise with less than adequate quantification range with either the low or high end of the analyte range having suboptimal clinical performance.

[0005] Arylsulfonate cyanine fluorescent dyes are described in Mujumdar et al. (1993) Bioconjugate Chemistry, 4:105-111; Southwick et al. (1990) Cytometry, 11:418-430; and U.S. Pat. No. 5,268,486. Cy5 is described in each of the references and is commercially available from Biological Detection Systems, Inc., Pittsburgh, PA, under the tradename FLUOROLINK™ Cy5™. The arylsulfonate cyanine fluorescent dyes have high extinction coefficients (typically from 130,000 L/mole to 250,000 L/mole), good quantum yields, fluorescent emission spectra in a range (500 nm to 750 nm) outside of the autofluorescence wavelengths of most biological materials and plastics, good solubilities, and low non-specific binding characteristics.

[0006] Despite these excellent properties, arylsulfonate cyanine fluorescent dyes suffer from certain limitations. In particular, these dyes have a relatively narrow Stokes shift which results in significant overlap between the excitation and emission spectra of the dye. The overlap of excitation and emission spectra, in turn, can cause self-quenching of the fluorescence when the dye molecules are located close to each other when excited. Such self-quenching limits the number of arylsulfonate dye molecules which can be conjugated to a single antibody molecule for use in immunoassays. In the case of Cy5, an exemplary arylsulfonate cyanine fluorescent dye, the Stokes shift is 17 nm (which is the difference between an excitation wavelength of 650 nm and an emission wavelength of 667 nm). Optimal fluorescent yield is obtained when from two to four Cy5 molecules are conjugated to a single antibody molecule. The fluorescent signal output drops rapidly when more than four dye molecules are conjugated to a single antibody molecule. The inability to conjugate more than four dye molecules to individual antibody molecules significantly limits the sensitivity of immunoassays using Cy5-labelled antibodies and other binding substances.

[0007] U.S. Publication 2011/0312105 discloses a detection system and fluorescent immunoassays. WO 2017/145270 A2, published after the effective date of this application, discloses a luminescent immunoassay method for detecting an analyte in a liquid sample with high sensitivity, which includes cycling a probe having an immunocomplex formed thereon back to the reagent vessel and amplification vessel 1-10 times and repeating the reaction with the reagent and the amplification polymer, to improve the sensitivity of detection level. WO 97/08552 A1 discloses fluorescent and chemiluminescent labelling compositions comprise a linear polysaccharide backbone molecule having a plurality of target-binding molecules, such as antibodies or nucleic acids, attached at spaced-apart intervals thereon.

[0008] There is a need for a method for quantitating an analyte having a wide range concentration in a single assay without having to dilute the sample and repeat the assay with fresh reagents.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009]

FIG. 1 illustrates an optical detecting system for detecting fluorescent signal from the sensing surface of the probe.

FIG. 2 illustrates an electrochemiluminescent detecting system for detecting chemiluminescent signal on the probe tip.

FIG. 3 illustrates an immunoassay format of the first embodiment of the invention for detecting an antigen analyte. Ab: antibody, Ag: antigen, Sa: streptavidin, B: biotin, F: fluorescent label.

FIG. 4 illustrates a wide range protocol of the first embodiment of the invention.

FIG. 5 illustrates an immunoassay format of the second embodiment of the invention for detecting an antigen analyte. Ab: antibody, Ag: antigen, F: fluorescent label.

FIG. 6 illustrates a wide range protocol of the second embodiment of the invention.

FIG. 7 shows a flow chart of the preparation crosslinked FICOLL® 400-SPDP.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[0010] Terms used in the claims and specification are to be construed in accordance with their usual meaning as understood by one skilled in the art except and as defined as set forth below.

[0011] "About," as used herein, refers to within ± 10% of the recited value.

[0012] An "analyte-binding molecule", as used herein, refers to any molecule capable of participating in a specific binding reaction with an analyte molecule.

[0013] An "aspect ratio" of a shape refers to the ratio of its longer dimension to its shorter dimension.

[0014] A "binding molecule," refers to a molecule that is capable to bind another molecule of interest.

[0015] A "binding pair," as used herein, refers to two molecules that are attracted to each other and specifically bind to each other. Examples of binding pairs include, but not limited to, an antigen and an antibody against the antigen, a ligand and its receptor, complementary strands of nucleic acids, biotin and avidin, biotin and streptavidin, biotin and neutravidin (a deglycosylated version of avidin), lectin and carbohydrates. Preferred binding pairs are biotin and streptavidin, biotin and avidin, biotin and neutravidin, fluorescein and anti-fluorescein, digioxigenin/anti-digioxigenin, DNP (dinitrophenol)/anti-DNP.

[0016] A "branched polymer," as used herein, refers to a non-linear polymer having a 2- or 3-dimensional structure, which can be either a naturally occurring branched polymer, or a synthetically crosslinked polymer.

[0017] "Chemiluminescence," as used herein, refers to the emission of energy with limited emission of luminescence, as the result of a chemical reaction. For example, when luminol reacts with hydrogen peroxide in the presence of a suitable catalyst, it produces 3-aminophthalate in an excited state, which emits light when it decays to a lower energy level.

[0018] A "dendrimer," as used herein, refers to repetitively organic, branched molecules. A dendrimer is typically symmetric around the core, and often adopts a spherical three-dimensional morphology.

[0019] "Electrochemiluminescence," (ECL), as used herein, refers to luminescence produced during electrochemical reactions in solutions. In ECL, electrochemically generated intermediates undergo a highly exergonic reaction to produce an electronically excited state and then emits light. ECL excitation is caused by energetic electron transfer (redox) reactions of electrogenerated species. ECL is usually observed during application of potential (several volts) to electrodes of electrochemical cell that contains solution of luminescent species

[0020] "Immobilized," as used herein, refers to reagents being fixed to a solid surface. When a reagent is immobilized to a solid surface, it is either be non-covalently bound or covalently bound to the surface.

[0021] A "monolithic substrate," as used herein, refers to a single piece of a solid material. A "probe," as used herein, refers to a substrate coated with a thin-film layer of analyte-binding molecules at the sensing side. A probe has a distal end and a proximal end. The proximal end (also refers to probe tip in the application) has a sensing surface coated with a thin layer of analyte-binding molecules.

[0022] A "wide range concentration", as used herein, refers to a concentration range over at least 500-fold, 1000-fold, 2000-fold or 5000-fold.

[0023] The present invention is directed to a method for quantitating an analyte that has a wide range concentration in a single assay without having to dilute the sample and repeating the assay. The feature of the invention has two cycles of events each including sample binding to probe, binding reactions, and detection. In general, the assay conditions of the first cycle are optimized for samples at the high concentration end of the relevant clinical range, and the assay conditions of the second cycle are optimized for low concentration end of the relevant clinical range. After the first cycle of binding and detecting, the probe is re-dipped into the same sample vessel to bind additional analyte in the sample vessel to the probe in a more favorable binding condition (e.g., longer reaction time and/or agitation) than the binding condition in the first cycle. The analyte concentration is detected in both cycles, and the combined results provide the ability of quantitating an analyte that has a wide range concentration in a single assay without having to dilute the sample and re-do the assay. Another advantage of the present invention is that the wide range protocol uses the same sample and reagents in both cycles and does not require additional sample or reagents for the second cycle.

First Embodiment

[0024] In the first embodiment, the present method comprises the steps in the order of: (a) obtaining a probe having a first antibody immobilized on the tip of the probe, wherein the diameter of the tip surface area is ≤ 5 mm; (b) dipping the probe tip into a sample vessel containing

a sample solution having an analyte for 10 seconds to 2 minutes and flowing the sample solution laterally in the sample vessel at 0-500 rpm, preferably 0-200 rpm, to bind the analyte to the first antibody on the probe tip; (c) dipping the probe tip into a reagent vessel containing a reagent solution comprising a reagent of a second antibody conjugated with a first member of a binding pair to bind the reagent to the analyte; (d) dipping the probe tip into a first washing vessel containing a wash solution to wash the probe tip; (e) dipping the probe tip into an amplification vessel containing an amplification solution comprising a second member of the binding pair conjugated with one or more luminescent labels, to form an immunocomplex of the analyte, the first antibody, the second antibody, and the first and the second members of the binding pair on the probe tip; (f) dipping the probe tip into a second washing vessel containing a wash solution to wash the probe tip; (g) obtaining a first result by measuring the luminescent signal of the immunocomplex formed on the probe tip; (h) dipping the probe tip into the same sample vessel for a time period longer than in (b) and between 1-30 minutes and flowing the sample solution laterally in the sample vessel at an increased flow rate from that of (b) and between 200-1200 rpm, preferably 200-1000 rpm, to bind additional analyte in the sample to the first antibody on the probe tip; (i) repeating steps (c) to (f) 1-10 times; (j) obtaining a second result by measuring luminescent signal of the final immunocomplex formed on the probe tip; and (k) combining the two results and analyzing the analyte concentration in a wide range; wherein the first antibody and the second antibody are antibodies against the analyte.

[0025] In step (a), the probe can be any shape such as rod, cylindrical, round, square, triangle, etc., with an aspect ratio of length to width of at least 5 to 1, preferably 10 to 1. A rod-shape is preferred. Because the probe is dipped in a sample solution and one or more assay solutions during an immunoassay, it is desirable to have a long probe with an aspect ratio of at least 5 to 1 to enable the probe tip's immersion into the solutions. For fluorescent assay, the probe can be a monolithic substrate.

[0026] The probe has a small tip for binding analytes. The tip has a smaller surface area with a diameter $\leq$ 5 mm, preferably $\leq$ 2 mm or $\leq$ 1 mm, e.g., 0.5-2 mm. The small surface of the probe tip provides several advantages. First, a small surface has less non-specific binding and thus produces a lower background signal. Second, the reagent or sample carry over on the probe tip is extremely small due to the small surface area of the tip. This feature makes the probe tip easy to wash, and causes negligible contamination in the wash solution since the wash solution has a larger volume. Further, small surface area of the probe tip has a small binding capacity. Consequently, when the probe tip is immersed in a reagent solution, the binding of the reagent does not consume a significant amount of the reagent. The reagent concentration is effectively unchanged. Negligible contamination of the wash solution and small consumption of the reagents enable the reagent solution, the amplification solution, and the wash solution to be re-used many times, for example, 1-10 times or 3-5 times.

[0027] The sensing surface of the probe is coated with first antibody which binds to the analyte in a sample. Methods to immobilize reagents to the solid phase (the sensing surface of the probe tip) are common in immunochemistry and involve formation of covalent, hydrophobic or electrostatic bonds between the solid phase and reagent. The first antibody can be directly immobilized on the sensing surface. Alternatively, the first antibody can be indirectly immobilized on the sensing surface through a binding pair. For example, anti-fluorescein can be first immobilized either by adsorption to the solid surface or by covalently binding to aminopropylsilane coated on the solid surface. Then the first antibody that is labeled with fluorescein can be bound to the solid surface through the binding of fluorescein and anti-fluorescein (binding pair).

[0028] In step (b), the probe tip is dipped into a sample vessel for 10 seconds to 2 minutes, preferably 30 seconds to 1 minute, to bind the analyte to the first antibody on the probe tip.

[0029] After step (b), the probe is optionally washed 1-5 times, preferably 1-3 times in a wash vessel containing a wash solution. This extra washing step may not be required because the amount of the carried-over solution is minimal due to a small binding surface area. The wash solution typically contains buffer and a surfactant such as Tween® 20.

[0030] In step (c), the probe tip is dipped into a reagent vessel for 20 seconds to 10 minutes, preferably 20 seconds to 2 minutes to bind the reagent to the analyte on the probe tip. The reagent solution comprises a reagent of a second antibody conjugated with a first member of a binding pair.

[0031] The binding pair is typically a hapten and its antibody, a ligand and its receptor, complementary strands of nucleic acids, or lectin and carbohydrates. For example, the binding pair is biotin and streptavidin, biotin and avidin, biotin and neutravidin, fluorescein and anti-fluorescein, digioxigenin and anti-digioxigenin, and DNP (dinitrophenol) and anti-DNP. Preferably, the first member of the binding pair is biotin and the second member of the binding pair is streptavidin.

[0032] In Step (d), the probe is washed 1-5 times, preferably 1-3 times in a first wash vessel containing a wash solution. The wash solution typically contains buffer and a surfactant such as Tween® 20.

[0033] In step (e), the probe is dipped into an amplification vessel containing an amplification solution for 20 seconds to 5 minutes, preferably 20 seconds to 2 minutes, to form an immunocomplex of the analyte, the first antibody, the second antibody, and the first and the second members of the binding pair on the probe tip. The amplification solution comprises a second member of the binding pair conjugated with one or more luminescent labels.

[0034] To improve the sensitivity of the assay, the amplification solution may comprise a polymer conjugated with at least 5 molecules of second member of the binding pair and at least 25 luminescent labels. The polymer is preferably branched and/or crosslinked. The polymer has a molecular weight of at least 500,000, preferably 1 million Daltons. The polymer can be a polysaccharide (e.g. FICOLL® (copolymers of sucrose and epichlorohydrin) or dextran), a polynucleotide, a dendrimer, a polyols, or polyethylene glycol. The polymer is preferably branched or crosslinked to have a 2- or 3- dimensional structure. The polymer preferably comprises 5-50 or 5-100 binding molecules and 25-100 or 25-500 luminescent molecules.

[0035] The luminescent label useful for this invention has a molecular weight of < 5,000, preferably < 2,000, such as 500-2000 or 100-2000 Daltons. In one embodiment, the luminescent label is a fluorescent dye selected from the group consisting of: cyanine, coumarin, xanthene and a derivative thereof. For example, the fluorescent dye is Cy5 (molecule weight MW 792), Alexa FIuor® 647, DyLight® 350 (MW 874), DyLight® 405 (MW793), DyLight® 488 (MW 71011), DyLight® 550 (MW 982), DyLight® 594 (MW 1078), DyLight® 633 (MW 1066), DyLight® 650 (MW 1008), DyLight® 680 (MW 950), DyLight® 755 (MW 1092), DyLight® 800 (MW 1050), an Oyster fluorescent dye, IRDye, or organic compounds comprising multiple rings chelated with a rare earth metal such as a lanthanide (Eu, Th, Sm, or Dy).

[0036] In another embodiment, the luminescent label is a chemiluminescent marker selected from the group consisting of: Ruthenium(II)tris-bipyridine (MW 1057), luminol (MW 177), acridinium ester (9[[4-[3-[(2,5-dioxo-1-pyrrolidinyl)oxy]-3-oxopropyl]phenoxy]carbonyl]-10-methyl-acridinium trifluoromethane sulfonate, MW 632), hemin (MW 652).

[0037] When the binding molecule is a polypeptide or protein, the luminescent label can covalently bind to it through a variety of moieties, including disulfide, hydroxyphenyl, amino, carboxyl, indole, or other functional groups, using conventional conjugation chemistry as described in the scientific and patent literature.

[0038] Covalent binding of a binding molecule to a polynucleotide can be effected through a variety of moieties, including aldehyde, ketone, isothiocyanate, imidate, inosine, acyl, and alkyl, using conventional conjugation chemistry, while derivatization with biotin is taught in many references. (Leary et al. (1983) Proc. Natl. Acad. Sci. USA 80:4045-4049; WO86/02929; EP063 879; Langer et al. (1981) Proc. Natl. Acad. Sci. USA 78:6633-6637).

[0039] In each step (b), (c), and (e), the reaction can be accelerated by agitating or mixing the solution in the vessel. For example, a lateral flow (orbital flow) of the solution across the probe tip can be induced at 1-500 rpm, preferably 1-200 rpm, which accelerates the capture of target molecules by its binding partner immobilized to solid phase. For example, the reaction vessel can be mounted on an orbital shaker and the orbital shaker is rotated at a speed at least 50 rpm, preferably at least 200 rpm. Optionally, the probe tip can be moved up and down and perpendicular to the plane of the orbital flow, at a speed of 0.01 to 10 mm/second, in order to induce additional mixing of the solution above and below the probe tip.

[0040] In Step (f), the probe is washed 1-5 times, preferably 1-3 times in a second wash vessel containing a second wash solution. The wash solution typically contains buffer and a surfactant such as Tween® 20. The first and the second wash vessels can be the same vessel or different vessels. The first and the second wash solutions can be the same or different solutions.

[0041] In Step (g), the immunocomplex is detected by reading the luminescent signal on the probe. For a fluorescent label, the probe is placed in a clear-bottom well and read by a detector, such as those described in US 2011/0312105 (FIG. 1).

[0042] For a chemiluminescent label, the probe is placed in a clear-bottom well containing a measurement solution having a co-reactant. For example, if the chemiluminescent label is Ruthenium(II)tris-bipyridine, the co-reactant is tripropylamine. If the chemiluminescent label is luminol, the co-reactants are hydrogen peroxide and a hydroxide salt in water. The light emitted is measured by a photomultiplier tube (PMT).

[0043] For electrochemiluminescence (ECL), the mechanism and the principal components of the ECL analyzer is described by Blackburn et al (Clin. Chem. 37: 1534-1539 (1991)). After the probe is placed in a clear-bottom well containing a measurement solution having a co-reactant, a voltage is applied to the working electrode and counter electrode, and the emitted light is measured by PMT.

[0044] In a preferred embodiment, the antibody-coated probe serves as the working electrode of ECL analyzer (FIG. 2). This offers the advantage of efficient luminescence generation since the Ru(II)/tripropylamine red-ox reaction needs to occur at the electrode surface or very close proximity.

[0045] Step (h) starts the second cycle of events. Step (h) dips the probe tip back into the same sample vessels for a longer time of 1-30 minutes, preferably 2-30 minutes, or 3-30 minutes, and optionally agitates the same to increase the binding of additional analyte to the first antibody on the probe tip.

[0046] Step (i) is cycling amplification by repeating steps (c)-(f) 1-10 times, preferably 1-5 times, 1-3 times, or 2-3 times. When the amplification solution comprises a polymer conjugated with at least 5 molecules of second member of the binding pair and at least 25 luminescent labels, steps (c)-(f) can be repeated 2-10 times to increase assay signal and sensitivity. Each cycle consists of placing the probe back to the same reagent vessel, the same first wash vessel, the same amplification vessel, and the same second wash vessel. When the amplification solution does not comprise a high molecular

weight polymer, steps (c)-(f) are typically repeated only one time.

[0047] Step (j) detects the final immunocomplex formed by measuring the luminescent signal on the probe tip and combining the two detection results to analyze the analyte concentration in a wide range and then combines the two detection results to analyze the analyte concentration in a wide range.

[0048] FIG. 3 illustrates an immunoassay format of the first embodiment of the invention for detecting an antigen analyte.

[0049] FIG. 4 illustrates the probe transfer in wide range protocols of the first embodiment of the invention. In FIG. 4, the wide range protocol consists of two assay sequences with the same sample and reagents. The first sequence entails immersion of the antibody (Ab) coated probe in an antigen (Ag) sample vessel followed by immersion in a biotinylated-antibody (B-AB) reagent vessel and then immersion in a vessel comprising streptavidin conjugated with a fluorescent label (CyS-SA). Signal is read on the distal tip of the probe after the labeled streptavidin binding. For the second sequence, the probe is then transferred back to the same sample vessel where binding conditions are altered to effect greater binding and higher sensitivity. Typically, increasing the incubation time and/or increasing the orbital flow rate of the probe improves sensitivity of the sample binding. The probe is then transferred to the same biotinylated-antibody reagent vessel and then labeled streptavidin reagent vessel, followed by a second measurement.

Second Embodiment

[0050] In the second embodiment, the present method comprises the steps in the order of: (i) obtaining a probe having a first antibody immobilized on the tip of the probe, wherein the diameter of the tip surface area is $\leq$ 5 mm; (ii) dipping the probe tip into a sample vessel containing a sample solution having an analyte for 10 seconds to 2 minutes and flowing the sample solution laterally in the sample vessel at 0-500 rpm, to bind the analyte to the first antibody on the probe tip; (iii) dipping the probe tip into a reagent vessel containing a reagent solution comprising a second antibody conjugated with fluorescent labels, to form an immunocomplex of the analyte, the first antibody, and the second antibody; (iv) dipping the probe tip into a washing vessel containing a wash solution to wash the probe tip; (v) obtaining a first result by measuring the luminescent signal of the first immunocomplex formed on the probe tip; (vi) dipping the probe tip into the same sample vessel for a time period longer than that in (ii) and between 1-30 minutes and flowing the sample solution laterally in the sample vessel at an increased flow rate that in (ii) and between 200-1200 rpm, preferably 200-1000 rpm, to bind additional analyte in the sample to the first antibody on the probe tip; (vii) repeating steps (iii) and (iv); (viii) obtaining a second result by measuring the luminescent signal of the final immunocomplex formed on the probe tip; and (ix) combining the two results and analyzing the analyte concentration in a wide range; wherein the first antibody and the second antibody are antibodies against the analyte.

[0051] Steps (i) and (ii) of the second embodiment are similar to Steps (a) and (b) of the first embodiment.

[0052] In step (iii), the probe tip is dipped into a reagent vessel for 20 seconds to 10 minutes, preferably 20 seconds to 2 minutes to bind the reagent to the analyte on the probe tip. The reagent solution comprises a reagent of a second antibody conjugated with fluorescent labels. In one embodiment, the reagent solution comprises a polymer conjugated with at least 5 molecules of the second antibody and at least 25 luminescent labels, wherein the polymer has a molecular weight of at least 1 million Daltons, and the luminescent labels has a molecular weight of less than 2,000 Daltons. Suitable polymers are similar to those described in the first embodiment.

[0053] Steps (iv), (v), (vi), (viii) and (ix) are similar to steps (d), (g), (h), (j), and (k) of the first embodiment, respectively.

[0054] FIG. 5 illustrates of an immunoassay format of the second embodiment of the invention for detecting an antigen analyte.

[0055] FIG. 6 illustrates the probe transfer in wide range protocols of the second embodiment of the invention. In FIG. 6, the wide range protocol consists of two assay sequences with the same sample and reagents. The first sequence entails immersion of the antibody (Ab) coated probe in an antigen (Ag) sample vessel followed by immersion in a reagent vessel comprising an antibody conjugated with a fluorescent label (Ab-Cy5). Signal is read on the distal tip of the probe after the labeled antibody binding. For the second sequence, the probe is then transferred back to the same sample vessel where binding conditions are altered to effect greater binding and higher sensitivity. Typically, increasing the incubation time and/or increasing the orbital flow rate of the probe improves sensitivity of the sample binding. The probe is then transferred to the same reagent vessel, followed by a second measurement.

[0056] In general, the assay conditions of the first sequence are optimized for samples at the high concentration end of the relevant clinical range with low concentration samples being undetectable. The assay conditions of the second sequence are optimized for low concentration clinical samples with high concentration samples saturating the binding capacity of the probe. Cyclic amplification can be employed in either sequence, but rarely in the first sequence since high sensitivity is not required at that step.

[0057] The wide range protocol using a small surface area probe features two assay sequences using the same sample and reagents to extend an immunoassay's analytical range. The present invention has unexpected advantages over other heterogeneous immunoassay formats such as microwells, magnetic particles, or beads, which are commonly employed as the solid phase since

they have relatively high surface areas to effect rapid capture of antigen. Their protocols entail adding, and after an incubation period, withdrawing sample and reagents from the solid phase. In between each reagent addition to the solid phase, a wash sequence is performed. The wash sequence also consists of adding then withdrawing the wash reagent from the solid phase. It adds to the complexity of performing the assay to have extra pipetting systems to enable re-use of sample and reagents. Secondly, the high surface area of the solid phase in other protocols may deplete the reagents or cause carry over in the wash cycles which could reduce assay performance.

[0058] The invention is illustrated further by the following examples that are not to be construed as limiting the invention in scope to the specific procedures described in them.

EXAMPLES

Example 1: Preparation of Probe Having Immobilized First Antibody

[0059] Basic natriuretic peptide (BNP) is a 32 amino acid polypeptide secreted by the ventricles of the heart in response to excessive stretching of heart muscle cells. The N-terminal prohormone of brain natriuretic peptide (NT-proBNP) is a 76 amino acid N-terminal fragment. Both BNP and NT-proBNP levels in the blood are used for screening, diagnosis of acute congestive heart failure and may be useful to establish prognosis in heart failure,

[0060] Procalcitonin (PCT) is a peptide precursor of the hormone calcitonin, the latter being involved with calcium homeostasis. It is composed of 116 amino acids and is produced by parafollicular cells of the thyroid and by the neuroendocrine cells of the lung and the intestine.

[0061] Quartz probes, 1 mm diameter and 2 cm in length, were coated with aminopropylsilane using a chemical vapor deposition process (Yield Engineering Systems, 1224P) following manufacturer's protocol. The probe tip was then immersed in a solution of murine monoclonal anti-fluorescein (BiosPacific Inc.), 10 μg/ml in PBS (phosphate-buffered saline) at pH 7.4. After allowing the antibody to adsorb to the probe for 20 minutes, the probe tip was washed in PBS.

[0062] Capture antibodies for BNP, NT-proBNP and PCT (HyTest, Finland) were labeled with fluorescein by standard methods. Typically, there were about 4 fluorescein substitutions per antibody. Anti-fluorescein coated probes were immersed in fluorescein labeled capture antibody solution, 5 μg/ml, for 5 minutes followed by washing in PBS.

Example 2: Preparation of biotinylated Antibodies

[0063] Anti-BNP, anti-NT-proBNP and anti-PCT antibodies were labeled with biotins by standard methods. For example, biotinylated -NHS was reacted with the antibody at a molar ratio about 15 to 1 at room temperature in PBS (pH 7) for 1 hour. The biotinylated antibody was purified by Sephadex® G-25 column. Typically, there were about 3-6 biotins per antibody.

Example 3. Preparation of Crosslinked FICOLL® 400-SPDP

[0064] Crosslinked FICOLL® 400-SPDP (succinimydyl 6-[3-[2-pyridyldithio]-proprionamido]hexanoate, Invitrogen) was prepared according to Example 1 of US 2011/0312105. FIG. 7 shows a flow chart of its preparation.

Example 4. Preparation of Cy5-Streptavidin

[0065] 32 μL of Cy 5-NHS (GE Healthcare) at 5 mg/ml in DMF reacted with 1 ml of streptavidin (Scripps Labs) at 2.4 mg/ml in 0.1 M sodium carbonate buffer pH 9.5 for 40 minutes at 30°C. Applying the mixture to a PD 10 column (Pharmacia) removed unconjugated Cy 5. Spectral analysis indicated 2.8 Cy 5 linked per streptavidin molecule.

Example 4a. Preparation of Cy5-Antibody

[0066] Cy5-antibody is prepared according to Example 4 by replacing streptavidin with an antibody.

Example 5. Preparation of Cy5-Streptavidin-Crosslinked FICOLL®

[0067] 5.8 μL of SMCC (succinimidyl 4-[N-maleimidomethyl]cyclohexan-1-carboxylate, Pierce Chemical) at 10 mg/ml in DMF reacted with 2 mg Cy5-streptavidin (Example 4) in 1 ml PBS pH 7.4 for 1 hour at room temperature. Applying the mixture to a PD 10 column removed unbound SMCC.

[0068] The thiols on crosslinked FICOLL® 400-SPDP were deprotected by adding 30 μL DTT at 38 mg/ml to 1 mg crosslinked FICOLL® 400-SPDP in 1 ml PBS and reacting for 1 hour at room temperature followed by a PD 10 column to purify the crosslinked FICOLL®

[0069] The Cy5-streptavidin-SMCC was mixed with crosslinked FICOLL® 400-SH and reacted overnight at room temperature. 10 μL NEM (Aldrich) at 12.5 mg/ml was then added and reacted for ½ hour at room temperature. The conjugate was then purified on a Sepharose® 4B CL column. It was estimated that the conjugate carried about 20 to 30 streptavidins per FICOLL® (2 million Daltons), and 2-3 Cy5s per streptavidin.

Example 5a. Preparation of Cy5-Streptavidin-Crosslinked FICOLL®

[0070] Cy5-antibody-crosslinked FICOLL® is prepared according to Example 5 by replacing streptavidin with an antibody.

Example 6: Wide Range Protocol for BNP

[0071] For the first sequence of the wide range assay, BNP calibrators (Hytest) were spiked into normal, pooled human plasma then diluted 1 to 3 in PBS with 5 mg/ml BSA and 0.05% Tween® 20 (assay buffer). The probe tip was immersed in the BNP sample wells and incubated for 1 minute at room temperature with the sample wells subjected to orbital movement (1 mm diameter stroke) at 50 rpm. The probe was held stationary. The probe was washed 3 times for 10 seconds in PBS, 0.05% Tween® 20. After the wash sequence, the probe was immersed in a reagent solution containing biotinylated anti-BNP at 10 μg/ml in assay buffer, followed with 0.5 min incubation at 500 rpm, then a 3x wash sequence. Probes were then transferred to an amplification solution Cy5-streptavidin-Cx FICOLL® After 0.5 minute incubation at 500 rpm, the probes were taken through a wash sequence. Fluorescence at the distal tip of the probe was then measured and results shown in Table 1 under 1st Read.

[0072] The second sequence consisted of transferring the probe back to the same sample wells and performed the assay with the cyclic amplification procedure. The probe was incubated for 5 min. at 750 rpm in the sample, followed by a wash sequence. Three cycles were then performed, where for each cycle the probe was immersed in the same biotinylated anti-BNP solution for 2 min at 500 rpm, followed by a wash sequence, immersed in the same Cy5-streptavidin-Cx FICOLL® solution for 1 minute at 500 rpm, followed by a wash sequence. After the first cycle (Amp1) and the third cycle (Amp3), the fluorescence on the probe tip was measured. The data are shown on Table 1. Each data point is a mean of duplicates. "Sat" refers to saturated signal.

Table 1.

[BNP]	1st Read	2nd Read	
(ng/ml)		Amp 1	Amp 3
50	5.2	12.6	Sat
25	3.5	10.2	Sat
12	2.1	7.3	Sat
6	1.4	6.6	Sat
3	0.7	3.7	14.3
1	0.27	1.85	8.43
0.3	0.1	0.83	3.93
0.1	0.004	0.3	1.13
0.05	0.01		0.43
0.012	0.01		0.28
0	0.01	0	0.09

[0073] The results of Table 1 show that the quantification range for the first sequence (1st Read) is from about 1- 50 ng/ml, for the second sequence with one amplification cycle (Amp 1) is from 0.3-25 ng/ml, and for the second sequence with three amplification cycles (Amp 3) is from 0.012-3 ng/ml. The combination of the results of the first sequence and the second sequence yield a much greater overall range (0.01-50 ng/mL, 5000 fold) than relying on a single sequence.

Example 7: Wide Range Protocol for NT-proBNP

[0074] NT-proBNP calibrators were obtained from Hytest, Finland. The Assays were performed similarly to that of Example 5, except two amplification cycles (Amp 2) were carried out in the second sequence.

[0075] The data are shown on Table 2.

Table 2.

NTProBNP	1st Read	2nd Read
ng/ml		Amp 2
135	7.25	20
45.3	4.66	20
15.1	2.01	20
5.04	0.81	16.4
1.67	0.23	9.29
0.56	0.12	5.25
0.18	0.07	2.25
0.06	0.05	0.94
0	0.06	0.13

[0076] The results of Table 2 show that the quantification range for the first sequence (1st Read) is from about 0.56-135 ng/ml, for the second sequence with two amplification cycles (Amp 2) is from 0.06-5.04 ng/ml. The combination of the results of the first sequence and the second sequence yield a much greater overall range (0.06-135 ng/mL, 2250 fold) than relying on a single sequence.

[0077] As a comparison with an industry standard, Roche Cobas NTproBNP assay range is 0.06-35 ng/mL (583 fold). Roche's quantification range is about 4-fold lower than that of the present invention.

Example 8: Wide Range Protocol for PCT

[0078] PCT calibrators were obtained from Hytest, Finland. The Assays were performed similarly to that of Example 5, except two amplification cycles (Amp 2) were carried out in the second sequence.

[0079] The data are shown on Table 3.

Table 3.

[PCT], ng/ml	1st Read	2nd Read
ng/ml		Amp 2
400	5.84	20
133	2.11	20
44	0.81	20
14.8	0.28	20
4.9	0.11	11.82
1.64	0.07	4.54
0.54	0.04	1.68
0.18	0.04	0.83
0.06	0.04	0.54
0	0.04	0.38

[0080] The results of Table 3 show that the quantification range for the first sequence (1st Read) is from about 4.9-400 ng/ml, for the second sequence with two amplification cycles (Amp 2) is from 0.06-14.8 ng/ml. The combination of the results of the first sequence and the second sequence yield a much greater overall range (0.06-400 ng/mL, 6667 fold) than relying on a single sequence.

[0081] As a comparison with an industry standard, Roche Cobas PCT assay range is 0.06-100 ng/mL (1667 fold). Roche's quantification range is about 4-fold lower than that of the present invention.

Claims

1. A method of detecting an analyte in a wide concentration range in a liquid sample, comprising the steps in the order of:

(a) obtaining a probe having a first antibody immobilized on the tip of the probe, wherein the diameter of the tip surface area is < 5 mm;
(b) dipping the probe tip into a sample vessel containing a sample solution having an analyte for 10 seconds to 2 minutes and flowing the sample solution laterally in the sample vessel at 0-500 rpm, to bind the analyte to the first antibody on the probe tip;
(c) dipping the probe tip into a reagent vessel containing a reagent solution comprising a reagent of a second antibody conjugated with a first member of a binding pair to bind the reagent to the analyte;
(d) dipping the probe tip into a first washing vessel containing a first wash solution to wash the probe tip;
(e) dipping the probe tip into an amplification vessel containing an amplification solution comprising a second member of the binding pair conjugated with one or more luminescent labels, to form an immunocomplex of the analyte, the first antibody, the second antibody, and the first and the second members of the binding pair on the probe tip;
(f) dipping the probe tip into a second washing vessel containing a second wash solution to wash the probe tip;
(g) obtaining a first result by measuring the luminescent signal of the immunocomplex formed on the probe tip;
(h) dipping the probe tip into the same sample vessel for a time period longer than in (b) and between 1 -30 minutes and flowing the sample solution laterally in the sample vessel at an increased flow rate from that of (b) and between 200-1200 rpm, to bind additional analyte in the sample to the first antibody on the probe tip;
(i) repeating steps (e) to (f) 1-10 times; and
(j) obtaining a second result by measuring the luminescent signal of the final immunocomplex formed on the probe tip; and
(k) combining the two results and analyzing the analyte concentration in a wide range; wherein the first antibody and the second antibody are antibodies against the analyte.

2. The method according to Claim 1, wherein said amplification solution comprises a polymer conjugated with at least 5 molecules of second member of the binding pair and at least 25 luminescent labels, wherein the polymer has a molecular weight of at least 1 million Daltons, and the luminescent labels has a molecular weight of less than 2,000 Daltons.

3. The method according to Claim 1, wherein the binding pair is a hapten and its antibody, a ligand and its receptor, complementary strands of nucleic acids, or lectin and carbohydrates.

4. The method according to Claim 3, wherein the binding pair is biotin and streptavidin, biotin and avidin, fluorescein and antifluorescein, digioxigenin/anti-digioxigenin, or DNP-anti-DNP,

5. The method according to Claim 4, wherein the first, member of the binding pair is biotin and the second member of the binding pair is streptavidin.

6. The method according to Claim 1, wherein the step (i) repeats steps (c) to (f) 1-3 times.

7. A method of detecting an analyte in a wide concen-

tration range in a liquid sample, comprising the steps in the order of:

(i) obtaining a probe having a first antibody immobilized on the tip of the probe, wherein the diameter of the tip surface area is < 5 mm;
(ii) dipping the probe tip into a sample vessel containing a sample solution having an analyte for 10 seconds to 2 minutes and flowing the sample solution laterally in the sample vessel at 0-500 rpm, to bind the analyte to the first antibody on the probe tip;
(iii) dipping the probe tip into a reagent vessel containing a reagent solution comprising a second antibody conjugated with fluorescent labels, to form an immunocomplex of the analyte, the first antibody, and the second antibody;
(iv) dipping the probe tip into a washing vessel containing a wash solution to wash the probe tip;
(v) obtaining a first result by measuring the luminescent signal of the first immunocomplex formed on the probe tip;
(vi) dipping the probe tip into the same sample vessel for a time period longer than that in (ii) and between 1-30 minutes and flowing the sample solution laterally in the sample vessel at an increased flow rate from that in (ii) and between 200-1200 rpm, to bind additional analyte in the sample to the first antibody on the probe tip;
(vii) repeating steps (iii) and (iv);
(viii) obtaining a second result by measuring the luminescent signal of the final immunocomplex formed on the probe tip; and
(ix) combining the two results and analyzing the analyte concentration in a wide range; wherein the first antibody and the second antibody are antibodies against the analyte.

8. The method according to Claim 7, wherein said reagent solution comprises a polymer conjugated with at least 5 molecules of the second antibody and at least 25 luminescent labels, wherein the polymer has a molecular weight of at least 1 million Daltons, and the luminescent labels has a molecular weight of less than 2,000 Daltons.

9. The method according to Claim 1 or 7, wherein the diameter of the tip surface area is < about 2 mm.

10. The method according to Claim 1, wherein the polymer is a polysaccharide, a polynucleotide, a dendrimer, a polyols, or polyethylene glycol.

11. The method according to Claim 10, wherein the polymer is a branched polysaccharide.

12. The method according to Claim 1 or 7, wherein the luminescent label is a fluorescent dye selected from the group consisting of: cyanine, coumarin, xanthene and a derivative thereof.

13. The method according to Claim 1 or 7, wherein the luminescent label is a chemiluminescent label of Ruthenium(II)tris-bipyridine or luminol.

Patentansprüche

1. Ein Verfahren zum Nachweis eines Analyten in einem breiten Konzentrationsbereich in einer flüssigen Probe, das die folgenden Schritte in der folgenden Reihenfolge beinhaltet:

(a) Erhalten einer Sonde mit einem ersten auf der Spitze der Sonde immobilisierten Antikörper, wobei der Durchmesser des Spitzenoberflächeninhalts < 5 mm beträgt;
(b) Eintauchen der Sondenspitze in ein Probengefäß, das eine Probenlösung enthält, die einen Analyten aufweist, für 10 Sekunden bis 2 Minuten und laterales Strömenlassen der Probenlösung in dem Probengefäß bei 0-500 U/min, um den Analyten an den ersten Antikörper auf der Sondenspitze zu binden;
(c) Eintauchen der Sondenspitze in ein Reagenzgefäß, das eine Reagenzlösung enthält, die ein Reagens eines zweiten Antikörpers, der mit einem ersten Mitglied eines Bindungspaars konjugiert ist, beinhaltet, um das Reagens an den Analyten zu binden;
(d) Eintauchen der Sondenspitze in ein erstes Waschgefäß, das eine erste Waschlösung enthält, um die Sondenspitze zu waschen;
(e) Eintauchen der Sondenspitze in ein Amplifikationsgefäß, das eine Amplifikationslösung enthält, die ein zweites Mitglied des Bindungspaars, das mit einem oder mehreren Lumineszenzmarkierungen konjugiert ist, beinhaltet, um einen Immunkomplex des Analyten, des ersten Antikörpers, des zweiten Antikörpers und des ersten und des zweiten Mitglieds des Bindungspaars auf der Sondenspitze zu bilden;
(f) Eintauchen der Sondenspitze in ein zweites Waschgefäß, das eine zweite Waschlösung enthält, um die Sondenspitze zu waschen;
(g) Erhalten eines ersten Ergebnisses durch Messen des Lumineszenzsignals des auf der Sondenspitze gebildeten Immunkomplexes;
(h) Eintauchen der Sondenspitze in das gleiche Probengefäß für einen Zeitraum, der länger ist als in (b) und zwischen 1-30 Minuten beträgt, und laterales Strömenlassen der Probenlösung in dem Probengefäß bei einer Durchflussgeschwindigkeit, die gegenüber der von (b) erhöht ist und zwischen 200-1200 U/min beträgt, um zusätzlichen Analyten in der Probe an den ers-

ten Antikörper auf der Sondenspitze zu binden;
(i) 1- bis 10-maliges Wiederholen der Schritte (e) bis (f); und
(j) Erhalten eines zweiten Ergebnisses durch Messen des Lumineszenzsignals des endgültigen, auf der Sondenspitze gebildeten Immunkomplexes; und
(k) Kombinieren der zwei Ergebniosse und Analysieren der Analytenkonzentration in einem breiten Bereich; wobei der erste Antikörper und der zweite Antikörper gegen den Analyten gerichtete Antikörper sind.

2. Verfahren gemäß Anspruch 1, wobei die Amplifikationslösung ein Polymer, das mit mindestens 5 Molekülen eines zweiten Mitglieds des Bindungspaars konjugiert ist, und mindestens 25 Lumineszenzmarkierungen beinhaltet, wobei das Polymer ein Molekulargewicht von mindestens 1 Million Dalton aufweist und die Lumineszenzmarkierungen ein Molekulargewicht von weniger als 2000 Dalton aufweisen.

3. Verfahren gemäß Anspruch 1, wobei das Bindungspaar ein Hapten und sein Antikörper, ein Ligand und sein Rezeptor, komplementäre Stränge von Nukleinsäuren oder Lectin und Kohlenhydrate ist.

4. Verfahren gemäß Anspruch 3, wobei das Bindungspaar Biotin und Streptavidin, Biotin und Avidin, Fluorescein und Antifluorescein, Digitoxigenin/Antidigitoxigenin oder DNP/Anti-DNP ist.

5. Verfahren gemäß Anspruch 4, wobei das erste Mitglied des Bindungspaars Biotin ist und das zweite Element des Bindungspaars Streptavidin ist.

6. Verfahren gemäß Anspruch 1, wobei der Schritt (i) Schritte (c) bis (f) 1- bis 3-mal wiederholt.

7. Ein Verfahren zum Nachweis eines Analyten in einem breiten Konzentrationsbereich in einer flüssigen Probe, das die folgenden Schritte in der folgenden Reihenfolge beinhaltet:

(i) Erhalten einer Sonde mit einem ersten auf der Spitze der Sonde immobilisierten Antikörper, wobei der Durchmesser des Spitzenoberflächeninhalts < 5 mm beträgt;
(ii) Eintauchen der Sondenspitze in ein Probengefäß, das eine Probenlösung enthält, die einen Analyten aufweist, für 10 Sekunden bis 2 Minuten und laterales Strömenlassen der Probenlösung in dem Probengefäß bei 0-500 U/min, um den Analyten an den ersten Antikörper auf der Sondenspitze zu binden;
(iii) Eintauchen der Sondenspitze in ein Reagenzgefäß, das eine Reagenzlösung enthält, die einen zweiten Antikörper, der mit Fluoreszenzmarkierungen konjugiert ist, beinhaltet, um einen Immunkomplex des Analyten, des ersten Antikörpers und des zweiten Antikörpers zu bilden;
(iv) Eintauchen der Sondenspitze in ein Waschgefäß, das eine Waschlösung enthält, um die Sondenspitze zu waschen;
(v) Erhalten eines ersten Ergebnisses durch Messen des Lumineszenzsignals des ersten, auf der Sondenspitze gebildeten Immunkomplexes;
(vi) Eintauchen der Sondenspitze in das gleiche Probengefäß für einen Zeitraum, der länger ist als der in (ii) und zwischen 1-30 Minuten beträgt, und laterales Strömenlassen der Probenlösung in dem Probengefäß bei einer Durchflussgeschwindigkeit, die gegenüber der von (ii) erhöht ist und zwischen 200-1200 U/min beträgt, um zusätzlichen Analyten in der Probe an den ersten Antikörper auf der Sondenspitze zu binden;
(vii) Wiederholen der Schritte (iii) und (iv);
(viii) Erhalten eines zweiten Ergebnisses durch Messen des Lumineszenzsignals des endgültigen, auf der Sondenspitze gebildeten Immunkomplexes; und
(ix) Kombinieren der zwei Ergebnisse und Analysieren der Analytenkonzentration in einem breiten Bereich; wobei der erste Antikörper und der zweite Antikörper gegen den Analyten gerichtete Antikörper sind.

8. Verfahren gemäß Anspruch 7, wobei die Reagenzlösung ein Polymer, das mit mindestens 5 Molekülen des zweiten Antikörpers konjugiert ist, und mindestens 25 Lumineszenzmarkierungen beinhaltet, wobei das Polymer ein Molekulargewicht von mindestens 1 Million Dalton aufweist und die Lumineszenzmarkierungen ein Molekulargewicht von weniger als 2000 Dalton aufweisen.

9. Verfahren gemäß Anspruch 1 oder 7, wobei der Durchmesser des Spitzenoberflächeninhalts < etwa 2 mm beträgt.

10. Verfahren gemäß Anspruch 1, wobei das Polymer ein Polysaccharid, ein Polynucleotid, ein Dendrimer, ein Polyol oder Polyethylenglycol ist.

11. Verfahren gemäß Anspruch 10, wobei das Polymer ein verzweigtes Polysaccharid ist.

12. Verfahren gemäß Anspruch 1 oder 7, wobei die Lumineszenzmarkierung ein Fluoreszenzfarbstoff ist, ausgewählt aus der Gruppe, bestehend aus: Cyanin, Cumarin, Xanthen und einem Derivat davon.

13. Verfahren gemäß Anspruch 1 oder 7, wobei die Lu-

mineszenzmarkierung eine Chemilumineszenzmarkierung von Ruthenium(II)trisbipyridin oder Luminol ist.

Revendications

1. Procédé de détection d'une substance à analyser dans une large plage de concentrations dans un échantillon liquide, comportant les étapes consistant à :

(a) obtenir une sonde ayant un premier anticorps immobilisé sur la pointe de sonde, dans lequel le diamètre de la surface de la pointe est inférieur à 5 mm;
(b) tremper la pointe de sonde dans une cuve à échantillon contenant une solution d'échantillon ayant une substance à analyser pour dix secondes à deux minutes et faire couler la solution d'échantillon latéralement dans la cuve à échantillon à 0 à 500 tr/min, pour lier la substance à analyser au premier anticorps sur la pointe de sonde ;
(c) tremper la pointe de sonde dans une cuve à réactifs contenant une solution de réactif comprenant un réactif d'un second anticorps conjugué à un premier élément d'une paire de liaison pour lier le réactif à la substance à analyser ;
(d) tremper la pointe de sonde dans une première cuve de lavage contenant une première solution de lavage pour laver la pointe de sonde ;
(e) tremper la pointe de sonde dans une cuve d'amplification contenant une solution d'amplification comprenant un second élément de la paire de liaison conjuguée avec un ou plusieurs marqueurs luminescents, pour former un immunocomplexe de la substance à analyser, le premier anticorps, le second anticorps et les premier et second éléments de la paire de liaison sur la pointe de sonde ;
(f) tremper la pointe de sonde dans une seconde cuve de lavage contenant une seconde solution de lavage pour laver la pointe de sonde ;
(g) obtenir un premier résultat en mesurant le signal luminescent de l'immunocomplexe formé sur la pointe de sonde ;
(h) tremper la pointe de sonde dans la même cuve à échantillon pour une période supérieure à celle en (b) et entre 1 à 30 minutes et faire couler la solution d'échantillon latéralement dans la cuve à échantillon à un débit accru de celui de (b) et entre 200 à 1200 tr/min, pour lier une substance à analyser supplémentaire dans l'échantillon au premier anticorps sur la pointe de sonde ;
(i) répéter les étapes (e) à (f) une à dix fois ; et
(j) obtenir un second résultat en mesurant le signal luminescent de l'immunocomplexe final formé sur la pointe de sonde ; et
(k) combiner les deux résultats et analyser la concentration de la substance à analyser dans une large plage ; dans lequel le premier anticorps et le second anticorps sont des anticorps contre la substance à analyser.

2. Procédé selon la revendication 1, dans lequel ladite solution d'amplification comprend un polymère conjugué avec au moins 5 molécules du second élément de la paire de liaison et au moins 25 marqueurs luminescents, dans lequel le polymère a un poids moléculaire d'au moins 1 million de Daltons et les marqueurs luminescents ont un poids moléculaire de moins de 2 000 Daltons.

3. Procédé selon la revendication 1, dans lequel la paire de liaison est un haptène et son anticorps, un ligand et son récepteur, des brins complémentaires d'acides nucléiques, ou une lectine et des hydrates de carbone.

4. Procédé selon la revendication 3, dans lequel la paire de liaison est une biotine et une streptavidine, une biotine et une avidine, une fluorescéine et une anti-fluorescéine, une digoxygénine/anti-digoxygénine ou un DNP-anti-DNP.

5. Procédé selon la revendication 4, dans lequel le premier élément de la paire de liaison est une biotine et le second élément de la paire de liaison est une streptavidine.

6. Procédé selon la revendication 1, dans lequel l'étape (i) répète les étapes (c) à (f) une à trois fois.

7. Procédé de détection d'une substance à analyser dans une large plage de concentrations dans un échantillon liquide, comportant les étapes consistant à :

(i) obtenir une sonde ayant un premier anticorps immobilisé sur la pointe de sonde, dans lequel le diamètre de la surface de la pointe est inférieur à 5 mm;
(ii) tremper la pointe de sonde dans une cuve à échantillon contenant une solution d'échantillon ayant une substance à analyser pour dix secondes à deux minutes et faire couler la solution d'échantillon latéralement dans la cuve à échantillon à 0 à 500 tr/min, pour lier la substance à analyser au premier anticorps sur la pointe de sonde ;
(iii) tremper la pointe de sonde dans une cuve à réactifs contenant une solution de réactif comprenant un second anticorps conjugué avec des marqueurs fluorescents, pour former un immu-

nocomplexe de la substance à analyser, le premier anticorps et le second anticorps ;
(iv) tremper la pointe de sonde dans une cuve de lavage contenant une solution de lavage pour laver la pointe de sonde ;
(v) obtenir un premier résultat en mesurant le signal luminescent du premier immunocomplexe formé sur la pointe de sonde ;
(vi) tremper la pointe de sonde dans la même cuve à échantillon pour une période supérieure à celle en (ii) et entre 1 à 30 minutes et faire couler la solution d'échantillon latéralement dans la cuve à échantillon à un débit accru de celui en (ii) et entre 200 à 1200 tr/min, pour lier une substance à analyser supplémentaire dans l'échantillon au premier anticorps sur la pointe de sonde ;
(vii) répéter les étapes (iii) et (iv) ;
(viii) obtenir un second résultat en mesurant le signal luminescent de l'immunocomplexe final formé sur la pointe de sonde ; et
(ix) combiner les deux résultats et analyser la concentration de la substance à analyser dans une large plage ; dans lequel le premier anticorps et le second anticorps sont des anticorps contre la substance à analyser.

8. Procédé selon la revendication 7, dans lequel ladite solution de réactif comprend un polymère conjugué avec au moins 5 molécules du second anticorps et au moins 25 marqueurs luminescents, dans lequel le polymère a un poids moléculaire d'au moins 1 million de Daltons, et les marqueurs luminescents ont un poids moléculaire de moins de 2 000 Daltons.

9. Procédé selon la revendication 1 ou 7, dans lequel le diamètre de la surface de la pointe est inférieur à environ 2 mm.

10. Procédé selon la revendication 1, dans lequel le polymère est un polysaccharide, un polynucléotide, un dendrimère, un polyol ou un polyéthylèneglycol.

11. Procédé selon la revendication 10, dans lequel le polymère est un polysaccharide ramifié.

12. Procédé selon la revendication 1 ou 7, dans lequel le marqueur luminescent est un colorant fluorescent choisi parmi le groupe constitué de la cyanine, de la coumarine, du xanthène et d'un dérivé de ceux-ci.

13. Procédé selon la revendication 1 ou 7, dans lequel le marqueur luminescent est un marqueur chimioluminescent de ruthénium(ii)tris-bipyridine ou de luminol.

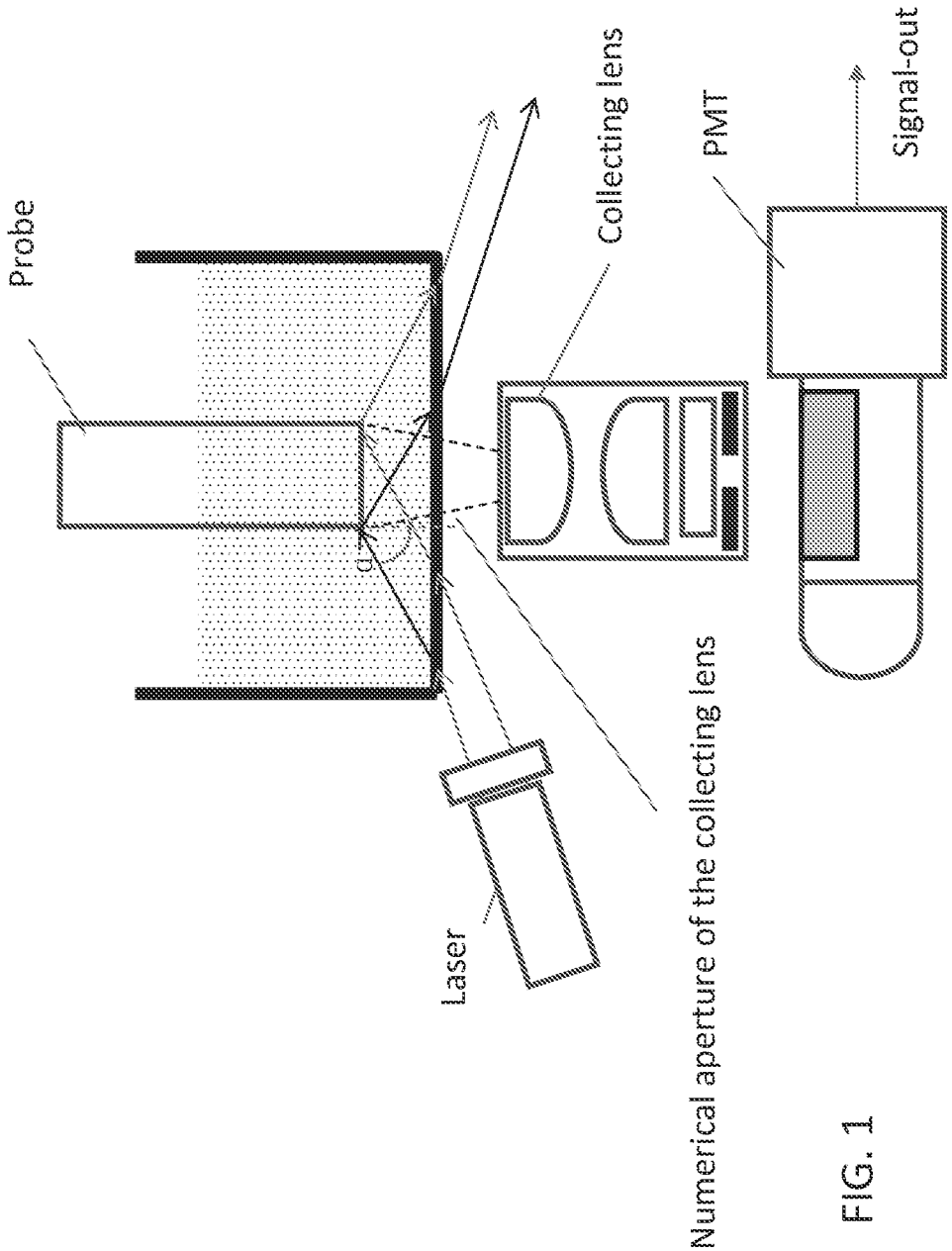

Probe
Collecting lens
PMT
Signal-out
Laser
Numerical aperture of the collecting lens

FIG. 1

FIG. 2

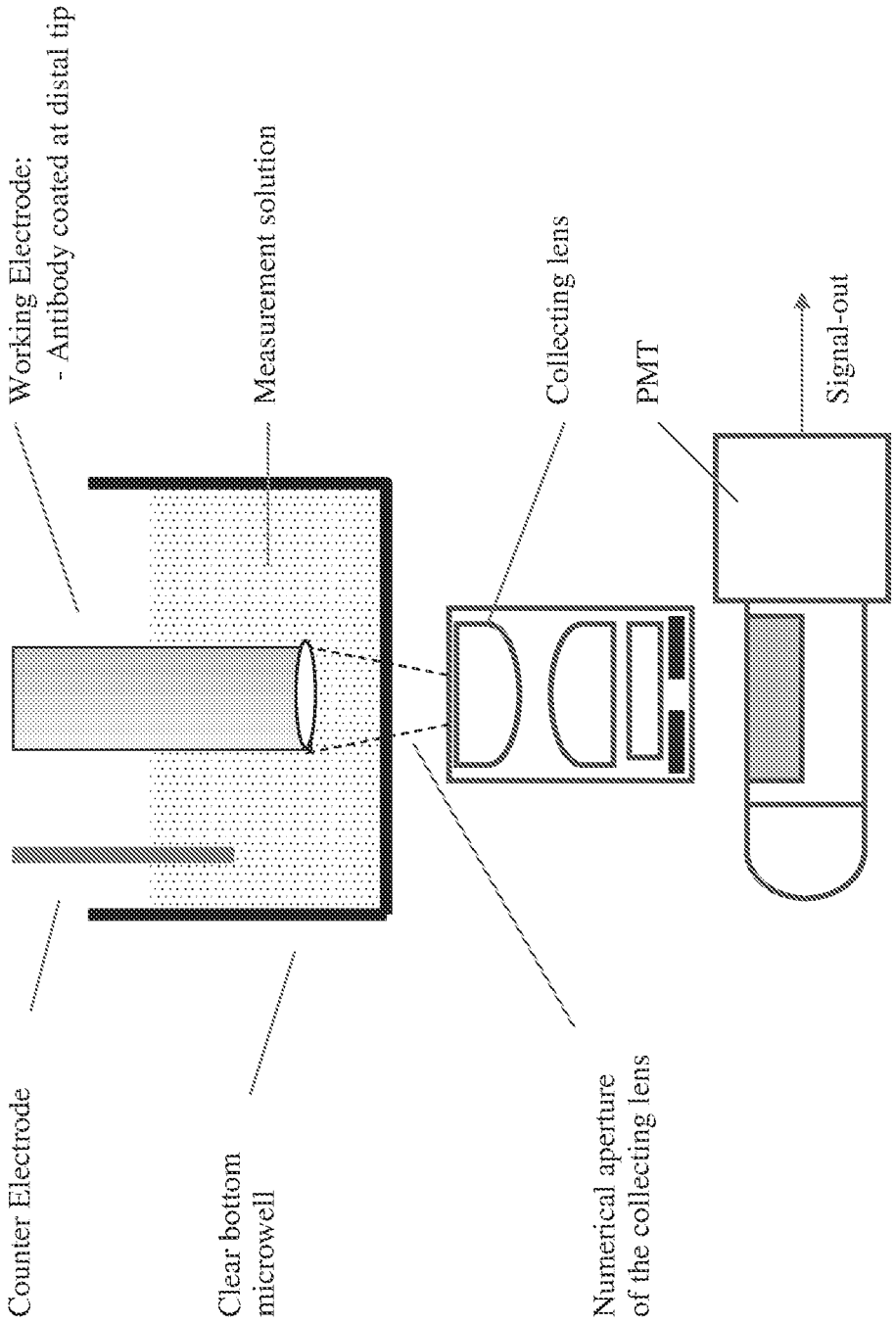
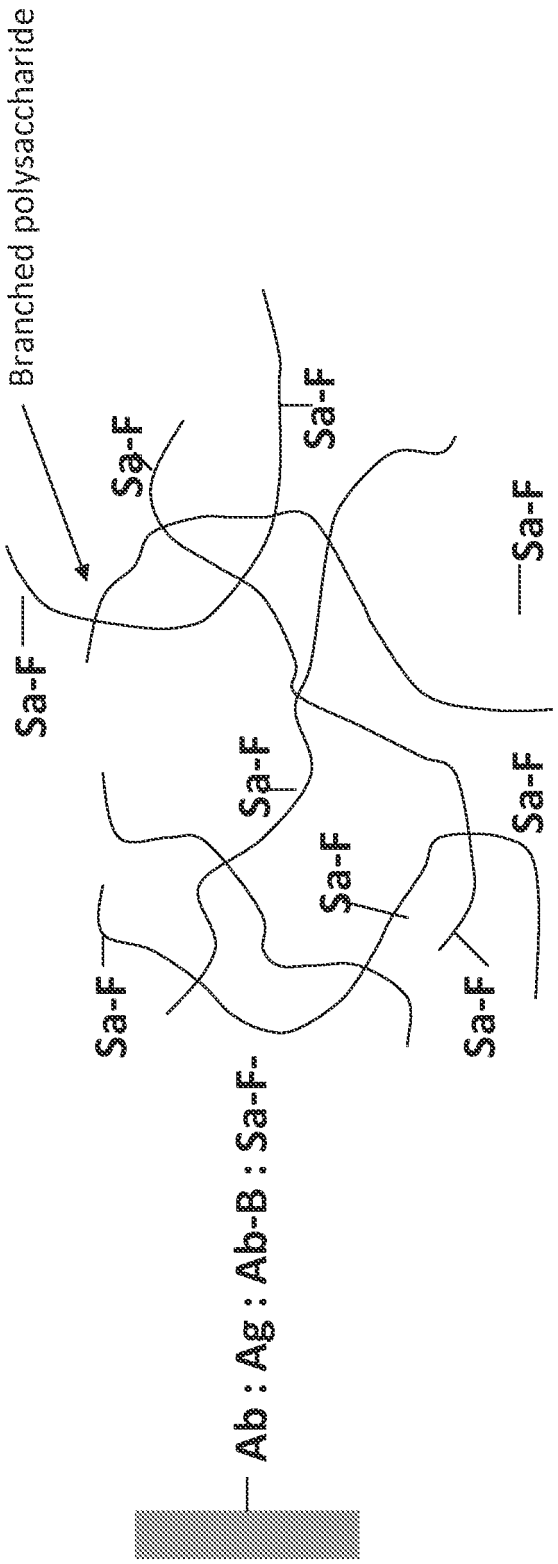

Working Electrode:
- Antibody coated at distal tip
Measurement solution
Collecting lens
PMT
Signal-out
Counter Electrode
Clear bottom microwell
Numerical aperture of the collecting lens

FIG. 3



F: fluorescent label

B: biotin

Sa: streptavidin

Ab: antibody

Ag: antigen analyte

Wide Range Protocol: Two Probe Transfer Sequences

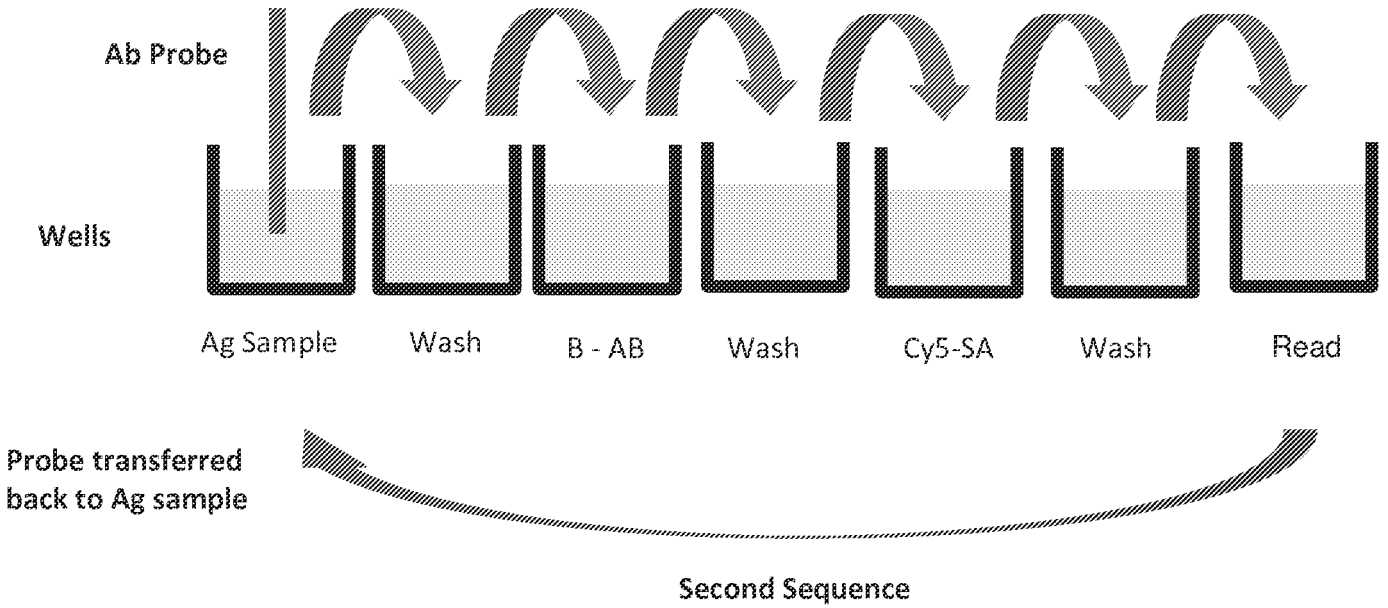
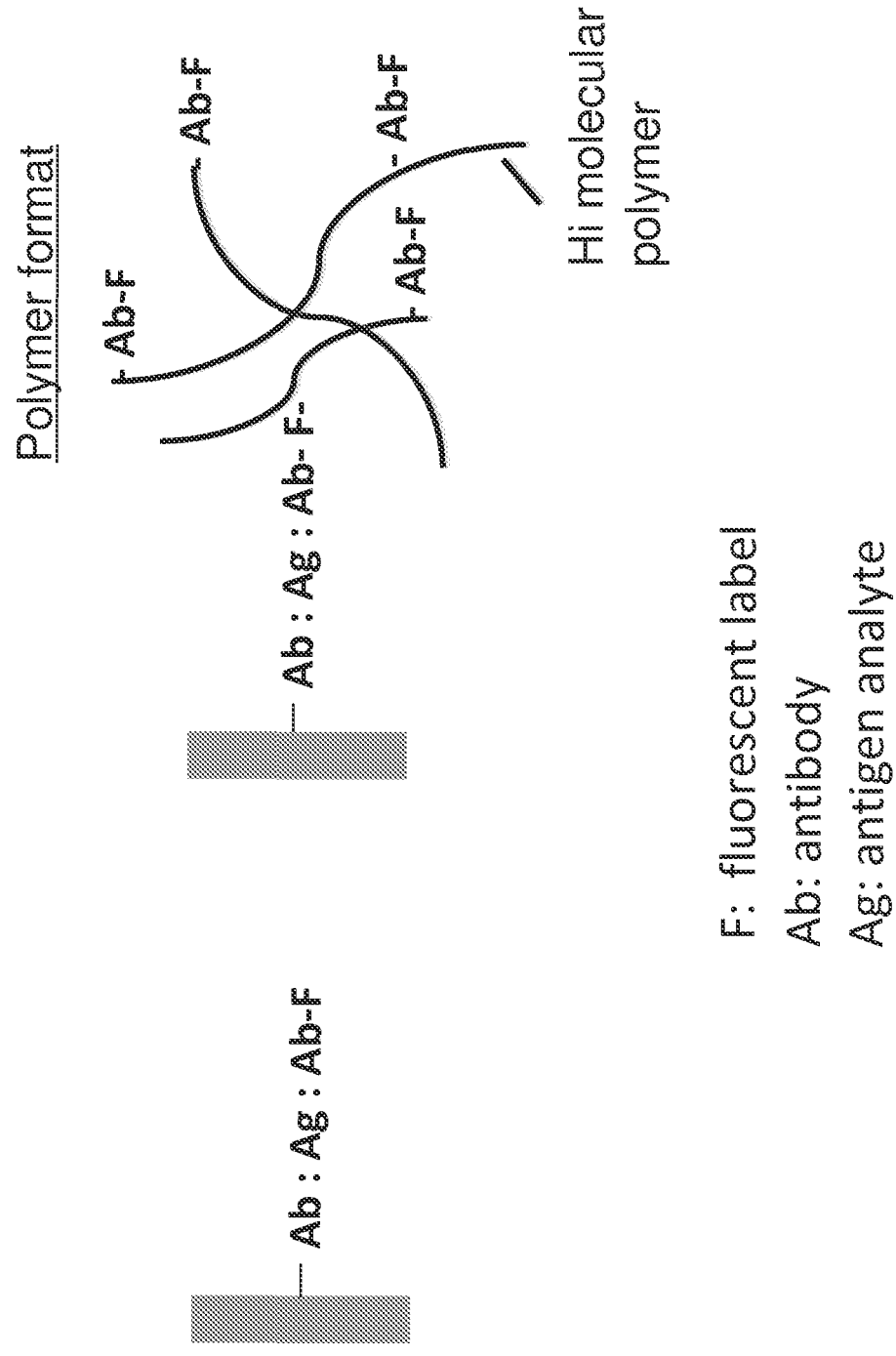


FIG. 4

FIG. 5


Polymer format
Ab-F
Ab-F
Ab-F
Ab-F
Ab : Ag : Ab- F-
Hi molecular polymer
Ab : Ag : Ab-F
F: fluorescent label
Ab: antibody
Ag: antigen analyte

Wide Range Protocol: Two Probe Transfer Sequences

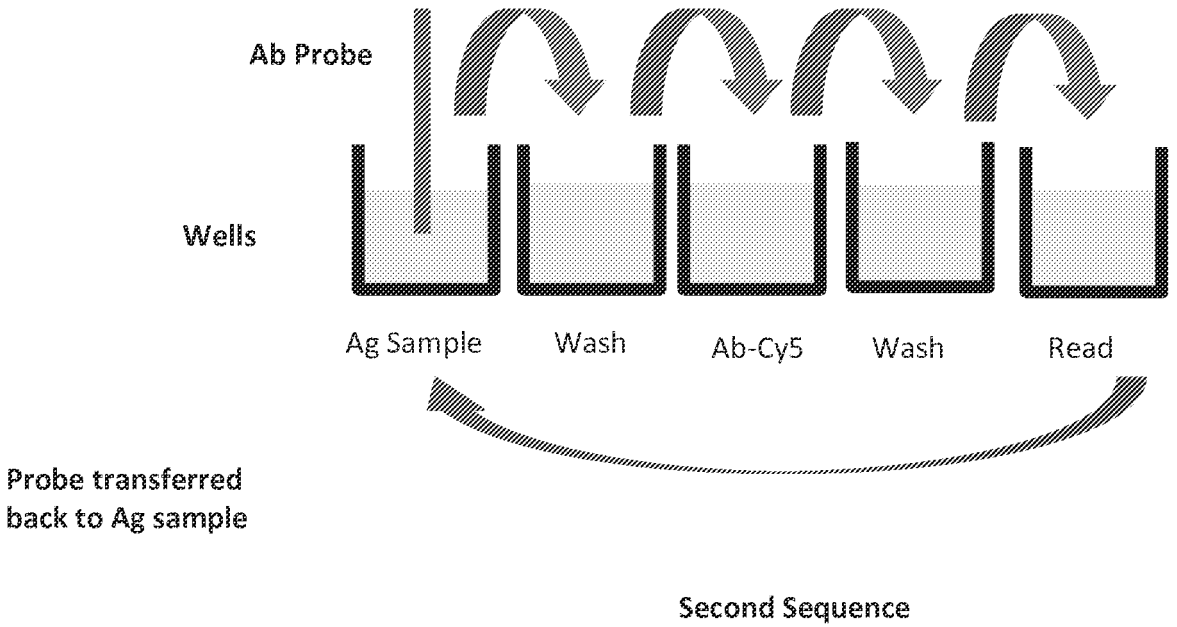


FIG. 6

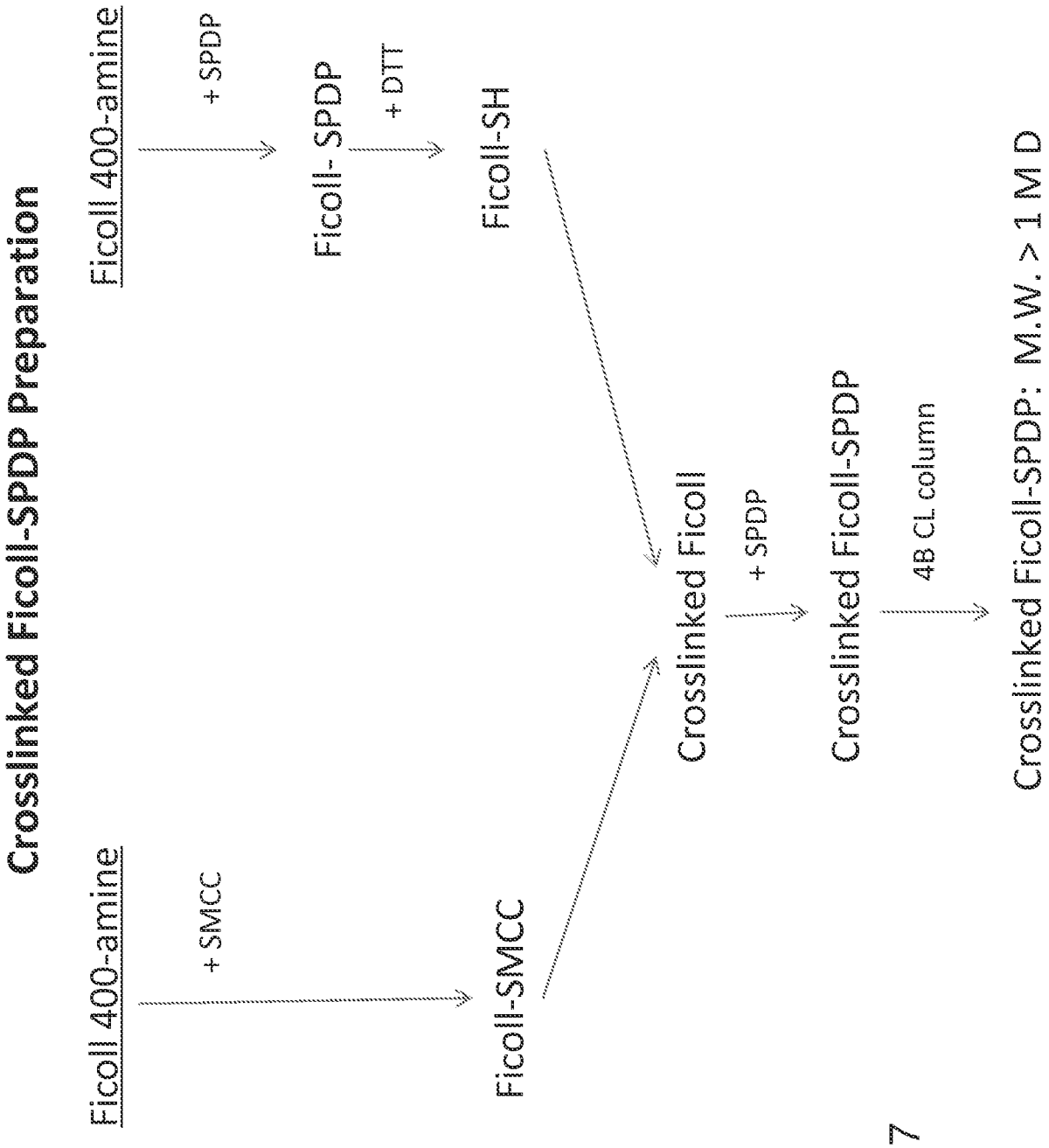

Crosslinked Ficoll-SPDP Preparation
Ficoll 400-amine
+ SMCC
Ficoll-SMCC
Ficoll 400-amine
+ SPDP
Ficoll- SPDP
+ DTT
Ficoll-SH
Crosslinked Ficoll
+ SPDP
Crosslinked Ficoll-SPDP
4B CL column
Crosslinked Ficoll-SPDP: M.W. > 1 M D

FIG. 7

REFERENCES CITED IN THE DESCRIPTION

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- **BLACKBURN et al.** *Clin. Chem.,* 1991, vol. 37, 1534-1539 **[0043]**

专利名称(译)	广泛的发光免疫分析		
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发明人	ZUK, ROBERT F.		
IPC分类号	G01N33/53 G01N33/532 G01N33/543 G01N33/58		
优先权	61/624924 2012-04-16 US 13/794080 2013-03-11 US		
其他公开文献	EP2839283B1 EP2839283A4		
外部链接	Espacenet		

摘要(译)

本发明涉及一种在单次测定中定量具有宽范围浓度的分析物的方法，而不必稀释样品并重复测定。本发明的关键特征是具有两个循环的事件，包括样品与探针的结合，结合反应和检测。在结合和检测的第一个循环之后，将探针浸入相同的样品容器中以在比第一个循环中的条件更有利于结合的条件下结合样品容器中的另外的分析物。