UNITED STATES DISTRICT COURT FOR THE DISTRICT OF MASSACHUSETTS

SAPIDYNE INSTRUMENTS INC. Plaintiff, v. GYROS US, INC. Defendant.	Civil Action No. _____ JURY TRIAL DEMANDED

COMPLAINT

Sapidyne Instruments Inc. ("Sapidyne"), by and through undersigned counsel, assert this action for patent infringement against Defendant Gyros US, Inc ("Gyros").

PARTIES

1. Sapidyne is a Delaware Corporation with a principle place of business at 967 E. ParkCenter Blvd., Boise, Idaho.

2. Upon information and belief, Gyros is a Delaware Corporation with a principle place of business in Monmouth Junction, New Jersey.

JURISDICTION AND VENUE

3. This Court has subject matter jurisdiction over this action pursuant to 28 U.S.C. §§ 1331, and 1338(a) because it arises under the patent laws of the United States, 35 U.S.C.

4. The Court has personal jurisdiction over the Defendant because the Defendant knowingly transact business in the state.

5. Venue is proper in this Court, pursuant to 28 U.S.C. §§ 1391(b), (c) and §1400(b), in that the Defendant transacts business in this district.

THE PATENTS

6. On September 9, 2000, U.S. Patent No. 6,120,734, entitled "Assay System," was lawfully issued ("the '734 Patent.") The '734 Patent relates to a system for assaying a fluid sample. A true and accurate copy of the '734 Patent is attached hereto as **Exhibit A**.

7. Sapidyne is the owner of the entire right, title, and interest in, and is the assignee of the '734 Patent.

8. On December 16, 2003, U.S. Patent No. 6,664,114, entitled "Solid Phase Assay for Detection of Ligands," was lawfully issued ("the '114 Patent.") The '114 Patent relates to an improved system for detecting the presence or level of an analyte in a sample. A true and accurate copy of the '114 Patent is attached hereto as **Exhibit B.**

9. Sapidyne is the owner of the entire right, title, and interest in, and is the assignee of the '114 Patent.

CLAIMS FOR RELIEF

COUNT ONE
Infringement of the '734 Patent

10. Plaintiff realleges, and incorporates herein by reference, the allegations of paragraphs 1 through 9 of this Complaint as if fully set forth herein.

11. Upon information and belief, Gyros' business is focused on developing miniaturized and non-mechanical systems for conducting assays used in scientific research and the drug development process. Gryos has developed and currently markets the Gyros Bioaffy CD micro laboratory.

12. On information and belief, Gyros has sold its Gyros Bioaffy product to customers in Massachusetts.

13. Gyros has infringed, and continues to infringe, the '734 Patent, either directly or under the doctrine of equivalents, by making, using, offering to sell, or selling products that infringe the '734 Patent, including Gyros' Bioaffy product.

14. On information and belief, Gyros actively has induced, and continues to induce, others to infringe the '734 Patent.

15. Upon information and belief, Gyros' infringement of the '734 Patent has been, and continues to be, willful and deliberate. Gyros' willful conduct provides a basis for this Court to award enhanced damages pursuant to 35 U.S.C. § 284 and makes this an exceptional case within the meaning of 35 U.S.C. § 285.

16. Gyros' infringing conduct has caused, is causing, and unless enjoined, will continue to cause, substantial and irreparable injury and damage to the plaintiff.

COUNT TWO
Infringement of the '114 Patent

17. Plaintiff realleges, and incorporates herein by reference, the allegations of paragraphs 1 through 16 of this Complaint as if fully set forth herein.

18. Upon information and belief, Gyros' business is focused on developing miniaturized and non-mechanical systems for conducting assays used in scientific research and the drug development process. Gryos has developed and currently markets the Gyros Bioaffy CD micro laboratory.

19. On information and belief, Gyros has sold its Gyros Bioaffy product to customers in Massachusetts.

20. Gyros has infringed, and continues to infringe, the '114 Patent, either directly or under the doctrine of equivalents, by making, using, offering to sell, or selling products that infringe the '114 Patent, including Gyros' Bioaffy product.

21. On information and belief, Gyros actively has induced, and continues to induce, others to infringe the '114 Patent.

22. Upon information and belief, Gyros' infringement of the '114 Patent has been, and continues to be, willful and deliberate. Gyros' willful conduct provides a basis for this Court to award enhanced damages pursuant to 35 U.S.C. § 284 and makes this an exceptional case within the meaning of 35 U.S.C. § 285.

23. Gyros' infringing conduct has caused, is causing, and unless enjoined, will continue to cause, substantial and irreparable injury and damage to the plaintiff.

PRAYER FOR RELIEF

WHEREFORE, the plaintiff requests:

A. That the Court determine that Gyros has infringed, and is infringing, one or more claims of the '734 and '114 Patents;

B. That, after trial, the Court enter a permanent injunction, ordering Gryos, its officers, directors, agents, servants and employees, and all persons in active concert or participation with them, to refrain from conduct that infringes the '734 and '114 Patents.

C. That the Court determine the amount of damages to the plaintiff caused by Gyros' infringement and enter judgment for the plaintiff in that amount, plus interests and costs;

D. That the Court determine that Gyros' infringement has been willful and deliberate, and award up to treble damages to the plaintiff pursuant to 35 U.S.C. § 284;

E. That the Court determine that this case is exceptional, within the meaning of 35 U.S.C. § 285, and order Gyros to play plaintiff's reasonable attorneys' fees pursuant to 35 U.S.C. § 285; and

F. That the Court grant such other and further relief as it deems appropriate in the circumstances.

DEMAND FOR JURY TRIAL

Pursuant to Fed. R. Civ. P. 38, the plaintiff hereby demands a jury trial on all issues triable of right by a jury.

Dated: July 2, 2008

Respectfully submitted,

SAPIDYNE INSTRUMENTS INC.

By its counsel,

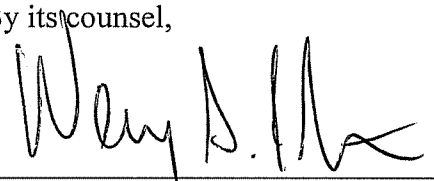

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Exhibit A

US006120734A

United States Patent [19]

Lackie

[11] Patent Number: 6,120,734

[45] Date of Patent: *Sep. 19, 2000

[54] ASSAY SYSTEM

[75] Inventor: Steve J. Lackie, Lexington, Mass.

[73] Assignee: Sapidyne, Inc., Boise, Id.

[*] Notice: This patent is subject to a terminal disclaimer.

[21] Appl. No.: 08/265,648

[22] Filed: Jun. 24, 1994

Related U.S. Application Data

[63] Continuation of application No. 07/924,720, Aug. 3, 1992, Pat. No. 5,372,783.

[51] Int. Cl.[7] G01N 21/00

[52] U.S. Cl. 422/68.1; 422/82.05; 422/82.07; 422/82.08; 356/246; 435/808; 436/805

[58] Field of Search 422/82.07, 82.08, 422/82.05, 68.1, 99, 104, 102; 55/387, 485; 359/665; 436/164–165, 172, 527, 538, 531, 546–547, 805; 435/808; 356/246, 417, 410, 440

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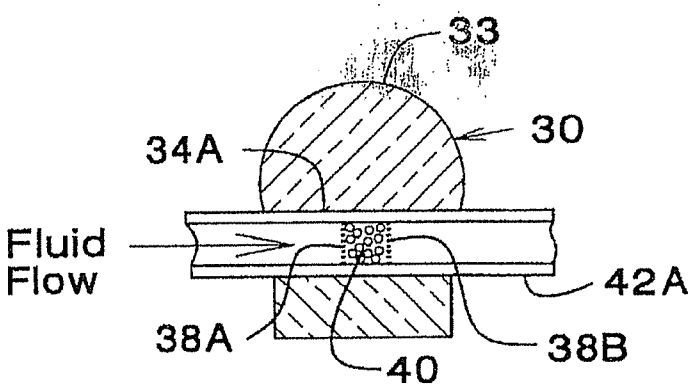
Primary Examiner—Hien Tran
Attorney, Agent, or Firm—Brenda H. Jarrell

[57] ABSTRACT

A system for assaying a fluid sample, typically employing a fluorescent tag, the system comprising a lens capable of focussing both excitation and fluorescent radiation, a fluid-flow conducting conduit being provided in the lens extending transversely of the optical axis of and through the focal region of the latter. One or more mechanical screens are disposed adjacent to the focal region in the conduit to arrest passage of beads as a function of bead diameter. The beads, precoated with at least a moiety of a ligand/conjugate complex, e.g. a specific-binding ligand, are preferably substantially transparent to both the excitation and fluorescent radiation.

16 Claims, 2 Drawing Sheets



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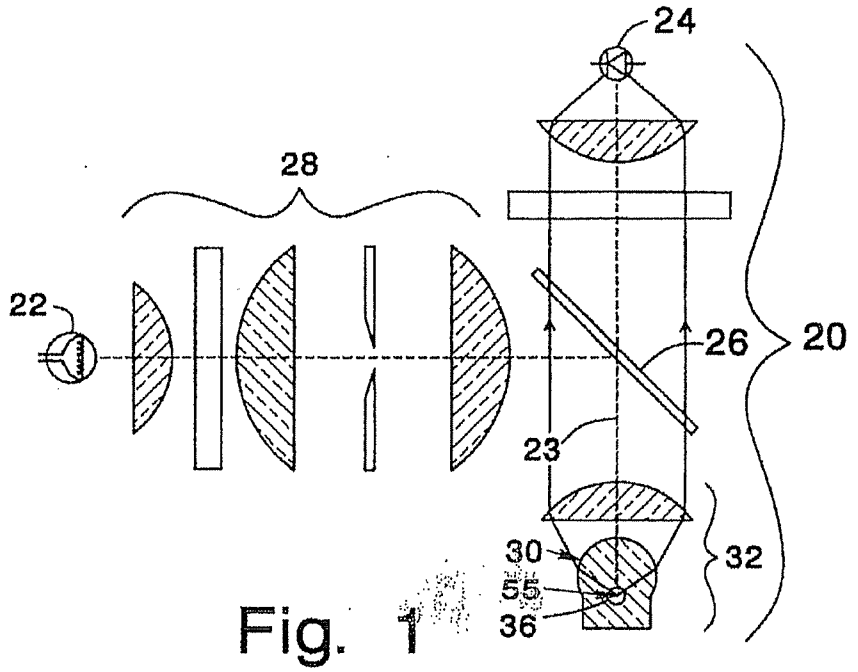

Fig. 1

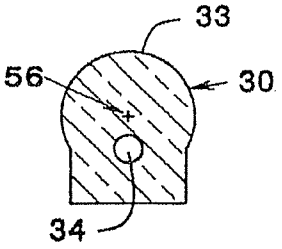

Fig. 2

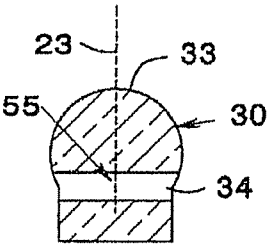

Fig. 3

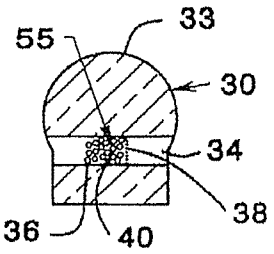

Fig. 4

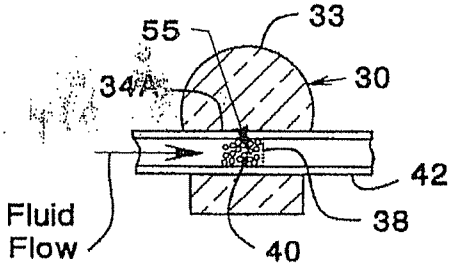

Fig. 5

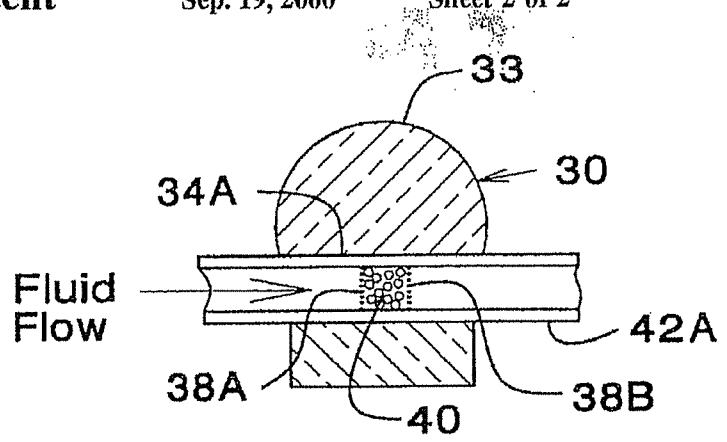

Fig. 6

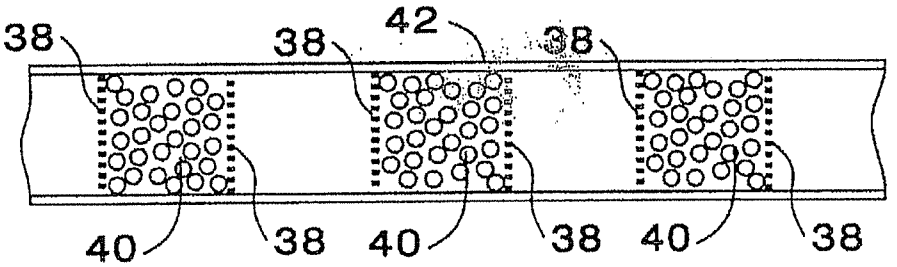

Fig. 7

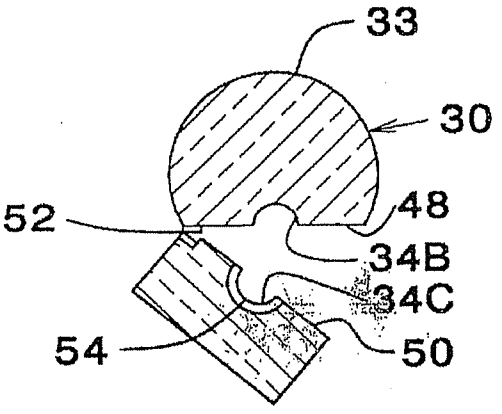

Fig. 8

ASSAY SYSTEM

This is a continuation of application(s) Ser. No. 07/924,720 filed on Aug. 3, 1992, now U.S. Pat. No. 5,372,783.

This invention relates to chemical and biochemical assays, and more particularly to an improved optical apparatus and methods for fluorescent assays.

Assays in which aliquots of sample-under-test and one or more reagents are variously reacted in highly specific reactions to form ligand/conjugate complexes such as antigen/antibody or similar complexes which may then be observed in order to assay the sample for a titer of a predetermined moiety from the sample, are well known. Typically, an antibody is used to assay for the presence of an antigen for which the antibody is specific, but such assays have been extended to quantitate haptens such as hormones, alkaloids, steroids, antigens, antibodies, nucleic acids, and fragments thereof, and it is in this broad sense that the term "ligand/conjugate" as used herein should be understood.

Sensitive immunoassays typically use tracer techniques in which a tagged constituent of the complex is incorporated, for example in the reagent, the non-complexed tagged reagent then being separated from the complexed reagent. The complexed can be thereafter quantitated by observing a signal from the tag. Radioisotopes, fluorescent and chemiluminescent molecules, colorimetric tags, and other markers have been used to label constituents or moieties of the complex, appropriate apparatus being employed to detect and measure the radiation from the label.

In such assays where at least one component of the conjugate complex is initially bound to a solid substrate preparatory to formation of the complex, a basic problem arises because of the typically lengthy time required to bind that component to the substrate. For example, fluorescent assays such as those performed in the usual 96 well microtiter plate, require time in the order of hours for binding of a component to the solid phase to occur notwithstanding such expedients as heating, shaking and the like. It will be appreciated that by increasing the surface area of the solid phase made available to binding or coating with a ligand, the binding delay may be considerably reduced. Consequently, the prior art relating to such solid phase assays (such as microtiter well assays, dipstick assays and the like) also teaches using small particles or beads as the solid phase.

Flowing the sample through a packed particulate bed speeds reactions between the sample ligand being assayed and a conjugate immobilized on the surface of the particles. Several factors probably contribute to this enhanced reactivity: the reduced diffusion distance, the constant stirring of sample due to turbulent flow, and the high density of binding sites in the reaction volume due to the high surface area exposed.

Known particle assays include the well-known bead agglutination test including quantitative or semiquantitative slide agglutination and techniques in which the agglutinated beads are separated from non-agglutinated beads by passage through a mechanical filter. Another known particle assay is that described in U.S. Pat. No. 4,780,423 in which particles with controlled porosity having ligand immobilized thereon are incubated in suspension and washed. Washing can involve sedimentation and resuspension of the particles. The resulting fluorescence can be read either from the concentrated or the suspended particles. In yet another known assay, the particles are bound to a membrane or filter through which the sample is then poured. This technique, is believed to have been limited to enzyme-colorimetric detection. Where the particles are incubated in a water suspension, the

average diffusion distances which the free ligand in the sample must traverse and the time required to bring complex formation to completion tend to be quite large.

A principal object of the present invention is therefore to provide an improved optical assay system in which the kinetics and sensitivity are improved by increasing the surface area of the solid phase, decreasing diffusion distances, and enhancing the optical coupling among the solid phase to the excitation light source and the coupling of the solid phase to the detector. Another object of the present invention is to provide a novel flow cell that provides the desired enhancement between the sample and a detector. Yet other objects of the present invention are to provide such an assay system that requires small sample volume and is particularly suitable for assay of whole blood; to provide such an assay system in which the ligand/conjugate reaction is confined within a disposable item that is readily insertable and removable from the optical system of the flow cell; and to provide such an assay system in which all of the components of the desired complex other than the sample moiety to be assayed, are preprovided.

Other objects of the present invention will in part be obvious and will in part appear hereinafter. Generally, the foregoing and other objects of the present invention are achieved by a system for assaying a fluid sample, typically employing a tag or label intended to emit electromagnetic radiation when excited, the system comprising a flow cell comprising hollow, light-transparent conduit means adapted for fluid flow therethrough, and one or more separate porous masses of light-transparent material disposed in the conduit means, the porosity of the mass of transparent material being selected to permit fluid flow of the sample therethrough, at least a moiety of a respective ligand/conjugate complex e.g. a specific-binding ligand, being immobilized, as by precoating, on the surfaces of each mass.

In one embodiment, the mass comprises a plurality of particles preferably substantially transparent to light, particularly, where the complex formed includes a fluorescent label, transparent to both radiation required to excite fluorescence and the excited fluorescence. The particles are typically beads dimensioned within a specified range of diameters and can be preformed, as by sintering or the like. Alternatively, the mass can be formed by accretion against a fluid-porous barrier means disposed in the conduit means. In the latter case, the barrier means is disposed within the conduit means so as to define at least one wall of a chamber, the porosity of the barrier means being sufficiently smaller than said range so that particles entrained in a fluid flow through the conduit means are trapped by the barrier means and accrete to form the porous mass in the chamber.

A preferred embodiment of the present invention includes focussing optical lens means through which the conduit means forms a hollow, tubular passage extending transversely to the optical axis of and through the focal region of the lens means. Typically, the lens means comprises a plurality of lenses and the conduit means extends through one of those lenses. Where the system is to be used with a tag or label intended to emit electromagnetic radiation when excited, the lens means must be capable of focussing both the excitation and the emission radiation.

The invention accordingly comprises the apparatus possessing the construction, combination of elements and arrangement of parts, and the method comprising the several steps and the relation of one or more of such steps with respect to each of the others, all as exemplified in the following detailed disclosure, and the scope of the application of which will be indicated in the claims.

For a fuller understanding of the nature and objects of the present invention, reference should be had to the following detailed description taken in connection with the accompanying drawings in which like numerals in the several drawings are employed to denote like parts, and wherein:

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a diagrammatic representation, in cross-section, of assay apparatus embodying the principles of the present invention;

FIG. 2 is a schematic cross-section of one embodiment of the flow cell of the present invention;

FIG. 3 is a transverse cross-section of the flow cell of FIG. 2;

FIG. 4 is a schematic cross-section of a variation of the flow cell of FIG. 2;

FIG. 5 is a schematic cross-section of another variation of the flow cell of the present invention;

FIG. 6 is a schematic cross-section of a variation of the flow cell of FIG. 5;

FIG. 7 is a schematic cross-section of another variation of the flow cell of FIG. 5; and

FIG. 8 is a schematic cross-section of yet another embodiment of the flow cell of the present invention.

In FIG. 1 there is shown exemplary apparatus **20** for assaying a fluid sample and which may typically employ an optical system including light source **22** for providing excitation radiation, light detector **24** for detecting light stimulated by the excitation radiation, beam splitter means such as dichroic or semitransparent mirror **26** and collimator means **28**. The embodiment of FIGS. 1, 2 and 3 will be described, for ease of exposition, for use particularly in the context of fluorescence immunoassay, but it should be understood is not so limited. The term "light" as used herein will be understood to include wavelengths in the visible spectrum as well as those in the near infra-red and ultraviolet as well. Similarly, the term "excitation" will be understood to include excitation of fluorescence, polarized or not, as by radiation, excitation of chemiluminescence by chemical agents, emission by reflection of light from chromogens, and the like. In FIG. 2, reference numeral **56** points out the center of curvature of the solid focusing lens means **33**.

The foregoing elements of the optical system are typically disposed in a frame (not shown) in fixed optical relationship to one another, as described more fully hereinafter. The invention further includes a flow cell **30**, shown particularly in enlarged form in FIGS. 2 and 3, and in this embodiment, formed from a focussing optical lens means **32** shown as a compound lens system including solid focussing lens **33**, typically made of glass, high molecular weight polymer or the like. Lens **33** is characterized by having an elongated hollow channel or fluid-flow conducting conduit **34** therein directed transversely to the optical axis of lens means **32**, and comprising a tubular passage, typically of circular cross-section, through lens **33**. At least a portion of such cylindrical conduit, reaction chamber **36**, is disposed at the focal region **55** of lens means **32**.

Thus, for example assume that fluid containing a ligand that can be excited, per se or through an appropriate tag, into emission such as fluorescence, traverses chamber **36** and is appropriately excited into emission there by excitation radiation focussed onto chamber **36** by lens means **32**. That fluorescent emission is then directed by lens means **32** to detector **24** where, assuming that the detector for example is electrical, appropriate electrical signals are produced and can be assessed to evaluate the fluorescence.

In order to provide a better signal-to-ligand ratio, the embodiment shown in FIG. 4 includes mechanical, fluid-porous barrier or screen **38** dimensioned and disposed in conduit **34** adjacent to the focal region **55** of lens means **32** so as to arrest transport of particles or beads **40** of predetermined size in a flow stream through the conduit. Such beads are substantially transparent to both the excitation radiation and the excited fluorescence, and to that end are typically formed of polymethylmethacrylate, styrene-divinylbenzene copolymer or the like. Beads **40** are coated with at least a moiety of the antibody/antigen complex, e.g. a specific-binding ligand, for example an antigen and an antibody thereto, disposed at least on a portion of the surface of the bead.

The mesh or porosity of screen **38** is selected to allow free flow of sample fluid and its constituents therethrough while arresting flow of the coated beads, and thereby accreting a mass of beads **40** against the screen and in the focal region of the lens means **32**. The particle size of the beads is selected to be minimized, provided however that when a mass of beads is accreted against screen **38**, the sample constituents may still pass freely through the accretion mass. Typically, a bead size that works well with whole blood as a sample is in the range of 50 μm to 250 μm, preferably around 98 μm. Bead size, of course, depends to some extent on the nature of the sample (e.g. blood, food, urine, process stream and the like). Mesh size, of course, depends upon the range of diameters of the beads to be employed in the system, but typically, for beads of about 98 μm diameter, a mesh size of about 50 μm is appropriate. Thus, as sample fluid is flowed through conduit **34**, it must pass through the interstices of the accreted mass of coated beads **40**, resulting in a very small diffusion distance over which the assayed moiety must pass to complex with the coating on the beads. This small diffusion distance, coupled with the long, tortuous path of the sample through the accreted mass and the high surface to volume ratio of the beads, enables very efficient scavenging of the assayed moiety from the sample. This characteristic of the present invention is significant inasmuch as the diffusion time is reduced by the square of the diffusion distance. It should also be noted that the entire solid phase is contained in the accreted mass, a very small volume (e.g. about 0.02 cm^3 for a typical conduit of 0.18 cm diameter), and is "immersed" in lens **32** thus providing a high numerical aperture, optical coupling between the excitation and detection systems. Because the fluorescent signal is increased by the fourth power of the numerical aperture, high numerical aperture optical coupling is very important.

In operation of the invention shown in FIG. 4, a quantity of beads **40** are preferably preloaded with an appropriate ligand immobilized onto the bead surfaces by adsorption or other known immobilizing techniques and suspended in a suspending fluid. Where the beads will ordinarily not readily form a stable suspension in the suspending fluid, they may be placed into a vortexer (not shown) or similar mixer which maintains the beads in a suspension, typically aqueous, by agitation. A desired portion of the bead suspension is sucked out of the vortexer as by a pump (not shown) and injected into conduit **34** where the flow of the beads is arrested by screen **38**, creating an accretion or mass of beads **40** within reaction chamber **36**. An aliquot of sample solution being assayed is then flowed through conduit **34** and the mass of beads **40** in reaction chamber **36**, effecting the formation of a ligand/conjugate complex on the surface of the beads. As is well known, for competitive assays, prior to flowing the sample solution through the flow cell, typically the sample solution is first treated with a tagging reagent and allowed to

incubate. Where the assay is a sandwich assay, the sample solution is passed through the flow cell, then tagged antibody is passed through the cell, and the bead mass is subjected to a wash step. As is well known in the art, a tagged, typically fluorescent, component may be either the complement or conjugate to or an analog of the immobilized ligand, depending upon whether a competitive or sandwich assay is to be performed. The tag or label is typically a fluorescent dye such as a fluorescein dye, acridine dye or the like, all as well known in the art. In either case, the resulting ligand/conjugate complex should include desired dye moieties bound to the complex. Flowing a wash buffer through the bead mass then washes out any unreacted materials and particularly any free dye components, leaving only those dyed moieties as are immobilized on the beads. Light source 22 is then activated to generate excitation light beam 23 (shown in broken lines) which, in turn, directed to mirror 26 by collimating lens 28 so that the collimated beam is reflected onto lens means 32. The latter focusses the excitation beam to a focal region at which the mass of beads 40 in reaction chamber 36 is located, and the excitation radiation excites the fluorophores on beads 40 into fluorescence. That fluorescence is transmitted through lens 32 and directed through beam splitter mirror 26 to detector 24. After measurements are made, the mass of beads 40 can be readily removed from reaction chamber 36 simply by back-flushing through conduit 34.

As thus described, the technique of filling the reaction chamber from a suspension or pool of preloaded beads is clearly amenable to automation, where the components for specific assays, such as the type of preloaded beads, sample solution, tagging reagent and the like, are selectable by appropriate valves controlling the flow of materials from respective storage containers. However, the present invention also is readily adaptable for more portable systems in which the bead mass and reagents are disposables.

For example, while conduit 34 is shown in FIG. 3 to be simply a passageway through the focal region 55 of lens means 32 transverse to the optical axis 23 of the lens means 32, in the embodiment shown in FIG. 5, conduit 34 is formed of elongated bore 34A of uniform diameter provided similarly through lens 33 and elongated light-transparent tube 42 having a uniform diameter slightly less than that of bore 34A so that tube 42 may be inserted and removed from the bore. Screen 38 is so disposed within tube 42 that the latter can be positioned within bore 34A adjacent to the focal region of the lens.

In yet another embodiment of the flow cell of the present invention, as shown in FIG. 6, conduit 34 is similarly formed of elongated bore 34A of uniform diameter through lens 33 and elongated light-transparent tube 42A having a uniform diameter slightly less than that of bore 34A so that tube 42A may be inserted and removed from the bore. Screens 38A and 38B are so disposed within tube 42A in spaced-apart relation to one another so as to define reaction chamber 44 within the tube. As in the embodiment of FIG. 5, reaction chamber 44 can be positioned within bore 34A adjacent to the focal region of the lens. Included within chamber 44 is a plurality of beads 40 dimensioned within a specified range of diameters, the mesh of screens being sufficiently smaller than the range of bead diameters so that the latter are trapped by the screens in chamber 44 to form a porous mass positionable substantially at the lens focal region 55. The beads in the embodiment of FIG. 6 are preferably precoated with the desired specific binding ligand before installation in chamber 44.

In both the embodiments of FIGS. 5 and 6, it will be appreciated that tubes 42 and 42A are preferably readily insertable and removable in and from bore 34A as the case may be, hence may be considered to be "disposables". Particularly, the "disposable" shown in FIG. 6 lends itself to laboratory preloading and packaging in hermetically sealed containers from convenient distribution and use. In both the embodiments of FIGS. 5 and 6, the materials forming both lens 32 and tube 42 or 42A are selected so that the respective indices of refraction thereof are substantially matched. In order to provide the optimum optical coupling between tube 42, 42A and lens 32, a refractive index-matching fluid is preferably disposed around the tube in the interspace between the tube and the interior wall of bore 34A.

It should be understood that bead mass 40 of the embodiment of FIG. 6 can be formed by, for example, the same technique used to create the bead mass of FIG. 5, i.e. by flowing a suspension of beads through tube 42A to accrete against a screen such as 38B, the other screen then being emplaced to capture the bead mass. Alternatively, the porous bead mass may also be formed of a plurality of beads adhered lightly to one another as by sintering or adhesives. For example, the bead mass can be formed by providing a thick layer of beads which may be free-standing, or by coating a porous substrate or forming a sandwich between a pair of porous substrates, with the thick layer of beads, which bead layers include a minor amount of adhesive that will not materially reduce the porosity of the resulting mass. After curing, the coating can be precoated with an appropriate specifically reactive ligand and minute cylinder of the coating punched out and inserted into appropriately dimensioned tubes 42A. Alternatively, sheets of high-molecular weight polymeric material of the desired porosity are commercially available, and after treatment to immobilize the requisite ligand within the porous structure, can be punched to produce the desired cylinders for insertion into tubes 42A. Thus, one may provide a plurality of bead masses, each coated with a different ligand. The resulting plurality of bead masses can be emplaced in a single tube 42A, as shown in FIG. 7, so that one may assay a sample flowing through the tube for several different ligands separately but substantially simultaneously.

As shown in FIG. 8, conduit 34 can be formed in part as a shallow elongated channel 34B or hemi-tubular portion of, for example, semicircular cross-section cut or molded into planar surface 48 of lens 33 which extends perpendicularly to the optical axis of the lens and through the focal region of lens means 32. The remainder of conduit 34 is formed by another hemi-tubular elongated channel 34C, similarly of semicircular cross-section, provided in plate 50. The latter is attached to lens 32 adjacent to surface 48, typically by hinging 52 such that plate 50 can be rotated to match channels 34C and 34B into coaxial relation to form a combined conduit of substantially circular cross-section. In the preferred embodiment, the inner surface of channel 34C is provided with highly reflective coating 54.

Since certain changes may be made in the above process and apparatus without departing from the scope of the invention herein involved, it is intended that all matter contained in the above description or shown in the accompanying drawing shall be interpreted in an illustrative and not in a limiting sense.

What is claimed is:

1. An apparatus comprising, in combination:

light-transparent conduit means for allowing fluid flow of a fluid sample therethrough; and

a porous mass of light-transparent material disposed in said conduit means, the porosity of said mass being

selected to permit fluid flow of said fluid sample therethrough, said mass having immobilized thereon at least a moiety of a ligand/conjugate complex, said mass being arranged and constructed such that said at least a moiety is localized within only a portion of said conduit means; and

measuring means positioned relative to said portion of said conduit means so that said measuring means quantitatively measures an amount of radiation emanating from within said portion of said conduit means.

2. Apparatus as defined in claim 1 wherein said porous mass comprises a plurality of particles dimensioned within a specified range of diameters, said apparatus further including:

fluid-porous barrier means disposed within said conduit means, the porosity and location of said barrier means being selected so that said particles are trapped by said barrier means to form said porous mass.

3. Apparatus as defined in claim 2 wherein said barrier means comprises at least a pair of screens spaced apart from one another so as to define a reaction chamber within said conduit means, the mesh of said screens being sufficiently smaller than said range of diameters that said particles are trapped between said screens in said chamber to form said porous mass, said particles having immobilized thereon said at least a moiety of a ligand/conjugate complex.

4. Apparatus as defined in claim 1, wherein said at least a moiety of a ligand/conjugate complex comprises a plurality of distinct moieties.

5. Apparatus as defined in claim 4, wherein each moiety of said plurality of distinct moieties is localized within a different portion of said conduit means.

6. An apparatus comprising, in combination;

light-transparent conduit means for allowing fluid flow of a fluid sample therethrough;

a plurality of pairs of screens, the screens of each said pair being spaced apart from one another, so that each said pair defines a reaction chamber within said conduit means, and said plurality of pairs defines a plurality of reaction chambers within said conduit means;

a plurality of porous masses of light-transparent material, the porosity of said masses being selected to permit fluid flow of said fluid sample therethrough, each said porous mass being disposed within one said reaction chamber defined by one said pair of screens, at least one of said masses having immobilized thereon at least a moiety of a ligand/conjugate complex; and

measuring means positioned relative to at least one said reaction chamber so that said measuring means quantitatively measures radiation emanating therefrom.

7. Apparatus as defined in claim 6 wherein each said porous mass of said plurality of porous masses comprises a plurality of particles dimensioned within a specified range of diameters, and wherein the mesh of said screens is sufficiently smaller than said range of diameters that each said plurality of particles is trapped by one said pair of screens to form one said porous mass in one said reaction chamber.

8. An apparatus comprising, in combination:

focussing optical lens means; and

a conduit means of substantially uniform cross-sectional dimension disposed within said lens means for fluid flow of a fluid sample therethrough, and extending transversely to an optical axis of said lens means through a focal region of said lens means, said apparatus being arranged and constructed such that said lens means focuses light rays that emanate from within said conduit means said lens means focussing said light rays by refraction.

9. An apparatus comprising:

lens means having conduit means therein, said conduit means allowing fluid flow of a fluid sample therethrough and extending transversely of an optical axis of said lens means through a focal region of said lens means so that, when a fluid sample including a tag that emits electromagnetic radiation is flowed through said conduit means, said lens means focusses said electromagnetic radiation; and

fluid-porous barrier means disposed adjacent to said focal region in said conduit means for limiting passage of particles through said fluid-porous barrier means as a function of particle size.

10. Apparatus as defined in claim 9 including exciting means for exciting emission of said electromagnetic radiation, said exciting means being positioned relative to said focal region so that, when a fluid sample including a tag that emits electromagnetic radiation is flowed through said conduit means and passes through said focal region, said exciting means excites said tag to emit said electromagnetic radiation.

11. Apparatus as defined in claim 10 wherein said means for exciting said emission comprises a source of excitation radiation and directing means for directing said excitation radiation, said directing means being oriented relative to said conduit means so that said excitation radiation is directed at said conduit means at said focal region.

12. Apparatus as defined in claim 9 including a plurality of particles dimensioned within a specified range of diameters, said fluid-porous barrier means having pores of lesser diameter than said range of diameters so that said particles are accreted in said conduit means against said fluid-porous barrier means to form a porous mass disposed substantially at said focal region.

13. Apparatus as defined in claim 12 wherein said particles are substantially transparent to both said excitation radiation and fluorescent radiation, and are at least partly coated with immobilized specific binding ligand.

14. Apparatus as defined in claim 13 wherein said ligand has formed a complex in a ligand/conjugate reaction, said complex being tagged with molecules that fluoresce when excited by appropriate excitation radiation.

15. Method of assaying a fluid sample by measuring radiation emitted from a ligand/conjugate complex, said method comprising the steps of:

providing a hollow, light-transparent conduit means containing a porous mass of light-transparent material disposed in said conduit means, the porosity of said mass of transparent material being selected to permit fluid flow of a fluid sample therethrough, said porous mass having immobilized thereon at least a moiety of a ligand/conjugate complex, said mass being arranged and constructed such that said moiety is localized within only a portion of said conduit means;

treating said porous mass, including flowing at least said fluid sample therethrough, so as to allow formation of said ligand/conjugate complex on said porous mass within said portion of said conduit means;

stimulating said complex so that characteristic radiation arises therefrom; and

quantitatively measuring an amount of said characteristic radiation that emanates from within said portion of said conduit means.

16. An apparatus comprising, in combination:

focussing optical lens means;

a conduit means of substantially uniform cross-sectional dimension disposed within said lens means for fluid

flow of a fluid sample therethrough, and extending transversely to an optical axis of said lens means through a focal region of said lens means, said apparatus being arranged and constructed such that said lens means focuses light rays that emanate from within said conduit means as said light rays exit said lens means.

* * * * *

Exhibit B

US006664114B1

(12) **United States Patent**
Lackie et al.

(10) **Patent No.: US 6,664,114 B1**
(45) **Date of Patent: *Dec. 16, 2003**

(54) **SOLID PHASE ASSAY FOR DETECTION OF LIGANDS**

(75) Inventors: **Steve J. Lackie**, Boise, ID (US); **Thomas R. Glass**, Idaho City, ID (US)

(73) Assignee: **Sapidyne Instruments, Inc.**, Boise, ID (US)

(*) Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **08/277,225**

(22) Filed: **Jul. 18, 1994**

Related U.S. Application Data

(63) Continuation-in-part of application No. 08/262,741, filed on Jun. 20, 1994, now abandoned, which is a continuation of application No. 08/197,431, filed on Feb. 16, 1994, now abandoned, which is a continuation-in-part of application No. 07/924,720, filed on Aug. 3, 1992, now Pat. No. 5,372,783.

(51) **Int. Cl.**[7] **G01N 33/543**

(52) **U.S. Cl.** **436/518**; 435/7.1; 435/7.92; 435/7.95; 435/962; 435/967; 436/514; 436/523; 436/538; 422/55; 422/56; 422/57

(58) **Field of Search** 435/7.1, 7.92–7.95, 435/962, 967; 436/523, 538, 518; 422/55–57

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Primary Examiner—Bao-Thuy L. Nguyen
(74) *Attorney, Agent, or Firm*—Choate, Hall & Stewart; Brenda Herschbach Jarrell

(57) **ABSTRACT**

The present invention provides an improved system for detecting the presence or level of an analyte in a sample. In "competition-like" assays of the present invention, a sample including an analyte is mixed with a second ligand to which the analyte binds, and the mixture is exposed to a solid phase containing a first ligand that can compete with the analyte for binding to the second ligand. According to the present invention, the time of exposure of the mixture to the solid phase is limited so that substantially no dissociation of analyte/second ligand complex occurs. The competition-like assays of the present invention are preferably performed with a solid phase containing a substantial excess of first ligand. In "sandwich-type" assays of the present invention, a sample including an analyte is contacted with a solid phase including a first ligand that binds the analyte and, simultaneously or subsequently, is contacted with a second ligand that binds the analyte (or the analyte/first ligand complex). The time of contact between the second ligand and the solid phase is limited so that substantially no non-specific binding between the second ligand and the solid phase occurs.

24 Claims, 12 Drawing Sheets

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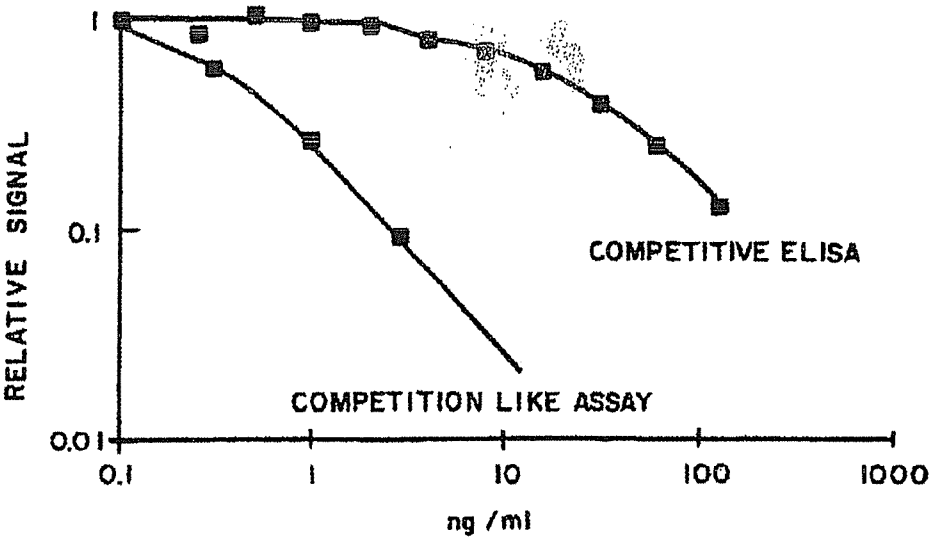
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0.01
COMPETITIVE ELISA
COMPETITION LIKE ASSAY
0.1
1
10
100
1000
ng /ml

FIG. 1

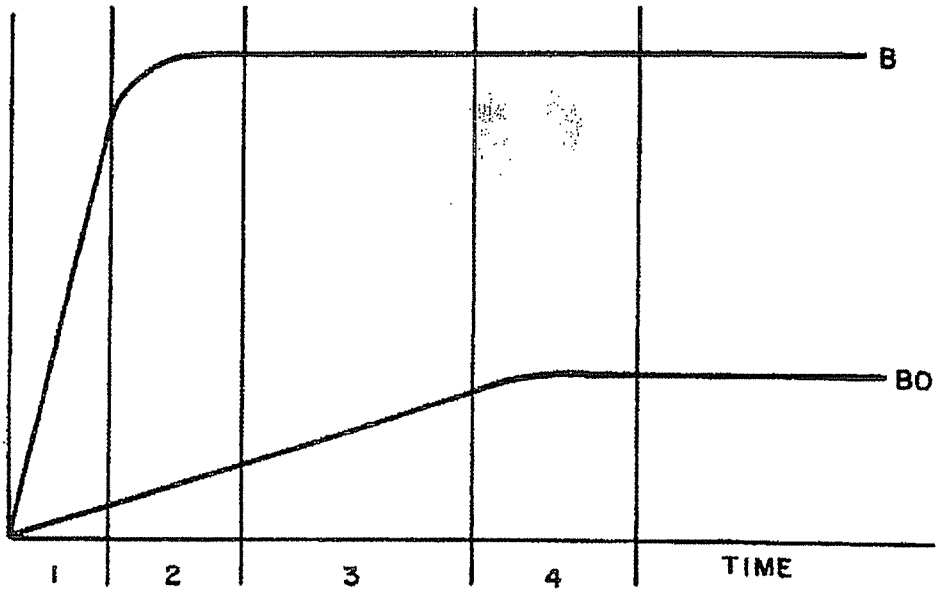


FIG. 2

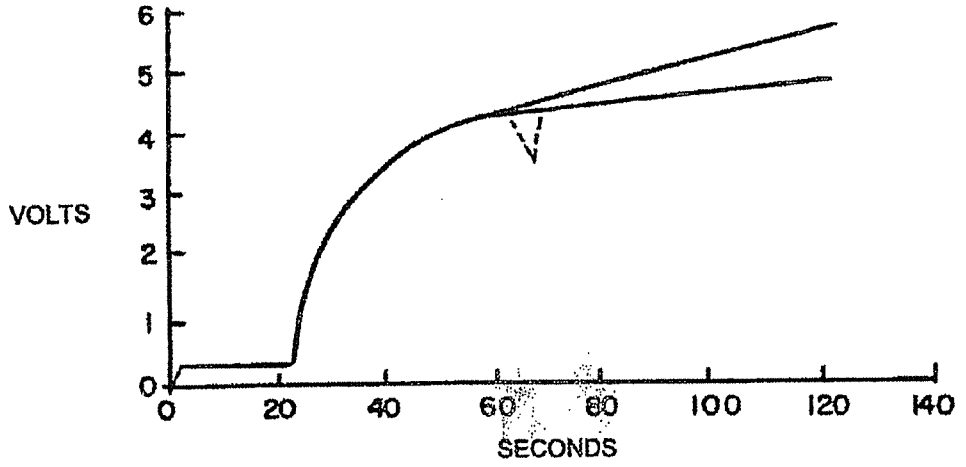
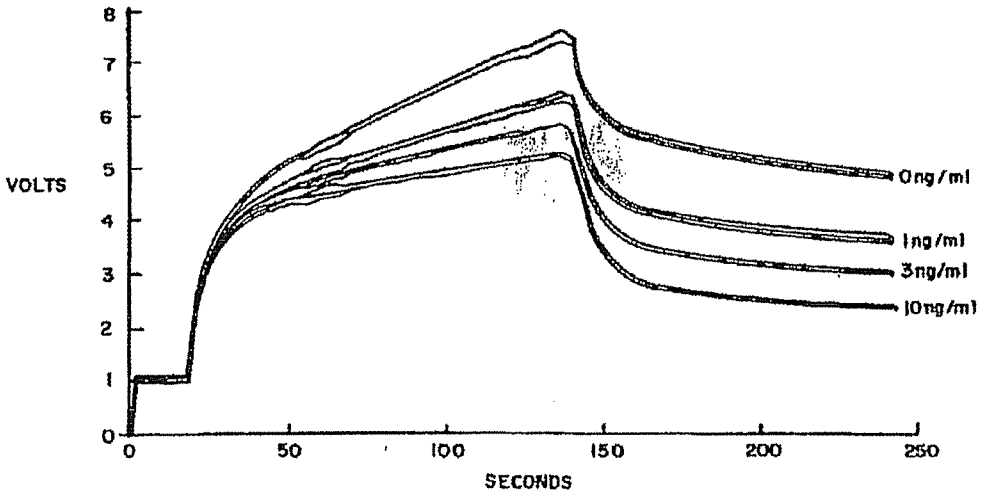


FIG.3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1
2
3
4
5
6
7
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0
50
100
150
200
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SECONDS
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3ng/ml
10ng/ml

FIG. 4

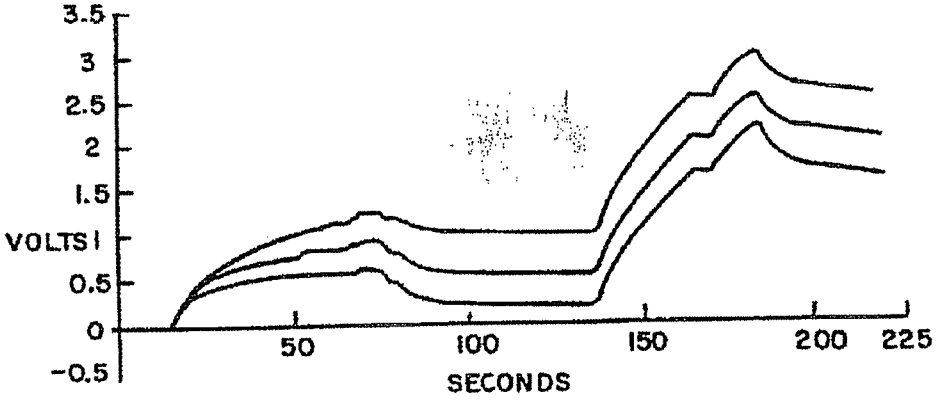
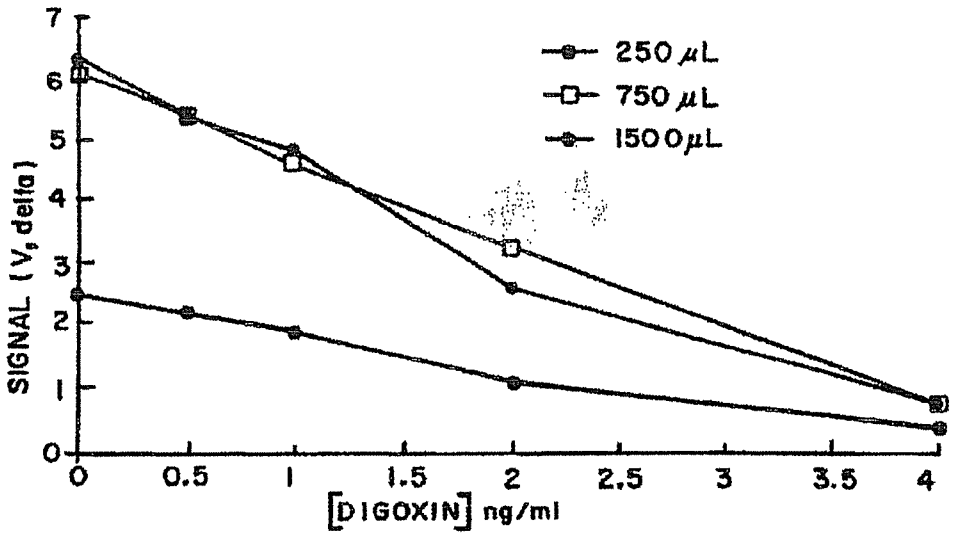


FIG. 5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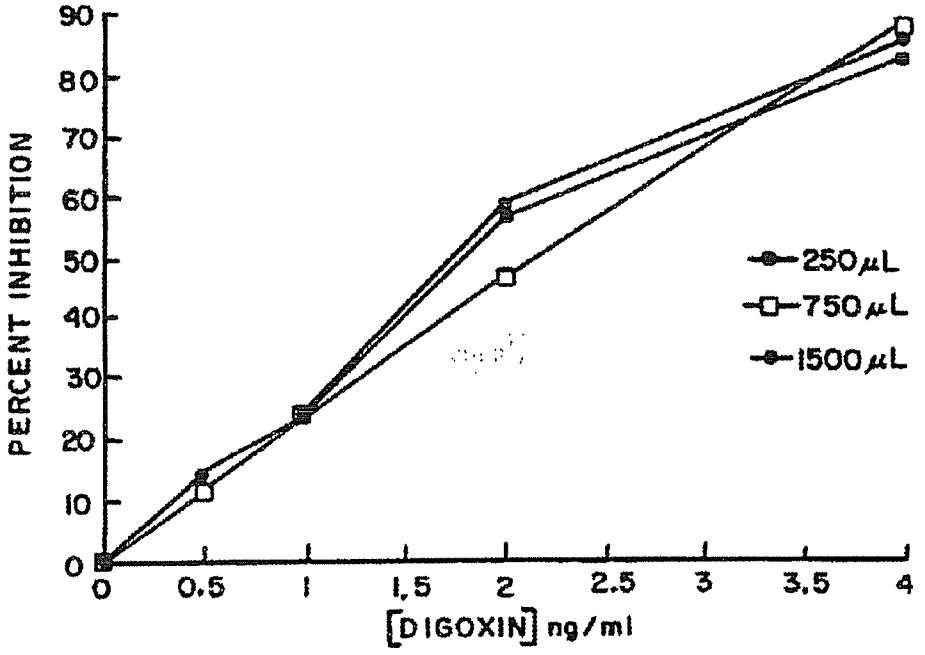
750 µL
1500µL
SIGNAL (V, delta)
[DIGOXIN] ng/ml
0
1
2
3
4
5
6
7
0
0.5
1
1.5
2
2.5
3
3.5
4

FIG.6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80
70
60
50
40
30
20
10
0
0
0.5
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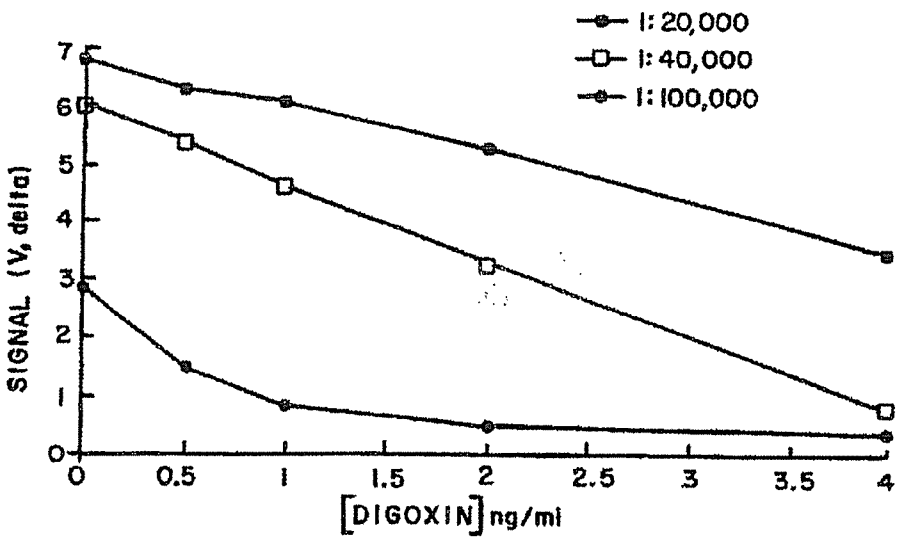
2
2.5
3
3.5
4
[DIGOXIN] ng/ml
250μL
750μL
1500μL

FIG. 7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1: 100,000
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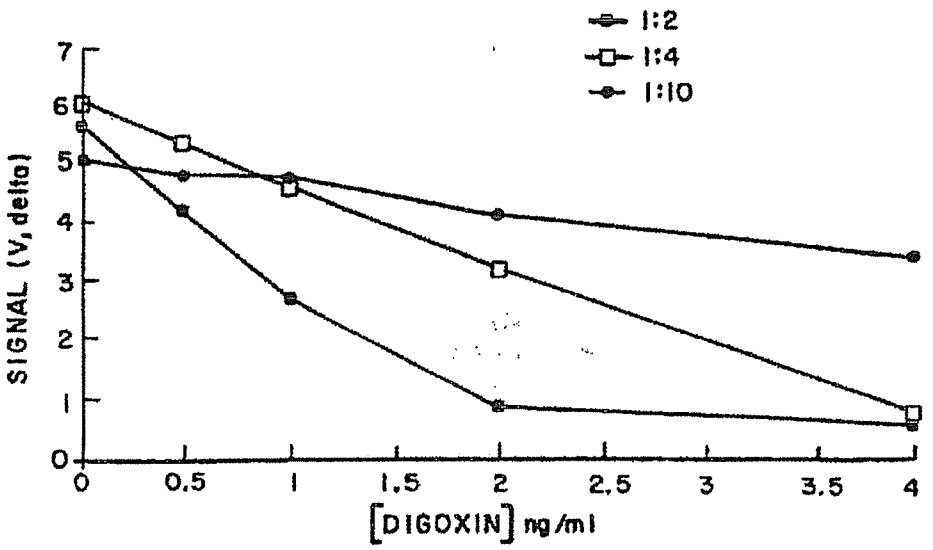
1
1.5
2
2.5
3
3.5
4
[DIGOXIN] ng/ml

FIG. 8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1:10
SIGNAL (V, delta)
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1
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3
4
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0.5
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1.5
2
2.5
3
3.5
4
[DIGOXIN] ng/ml

FIG. 9

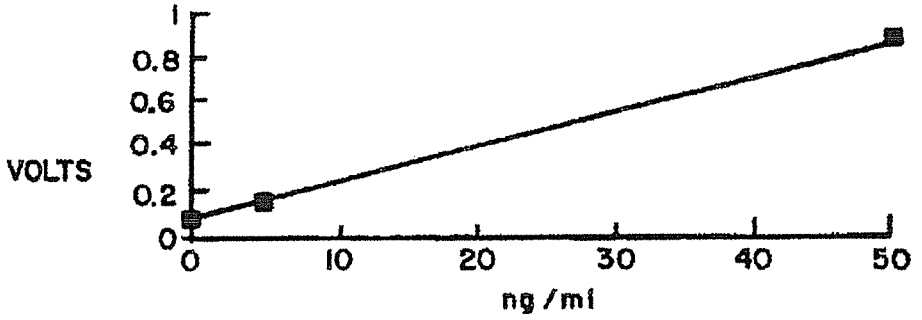

FIG. 10A

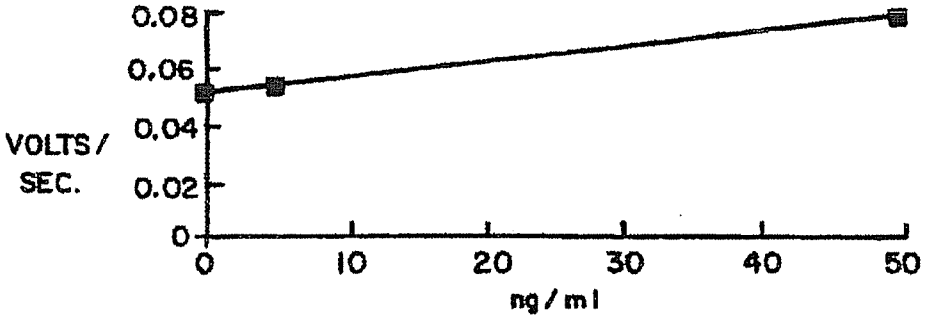

FIG. 10B

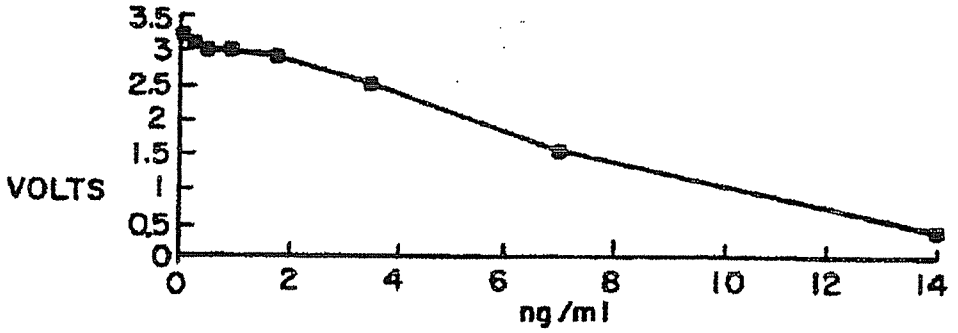

FIG. 11A

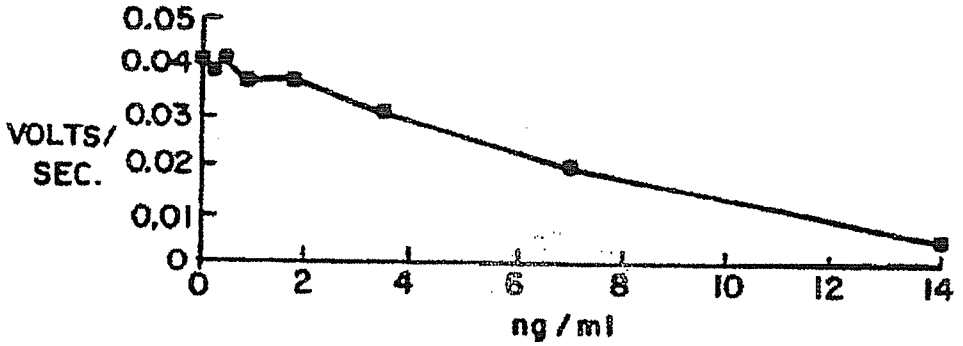

FIG. 11B

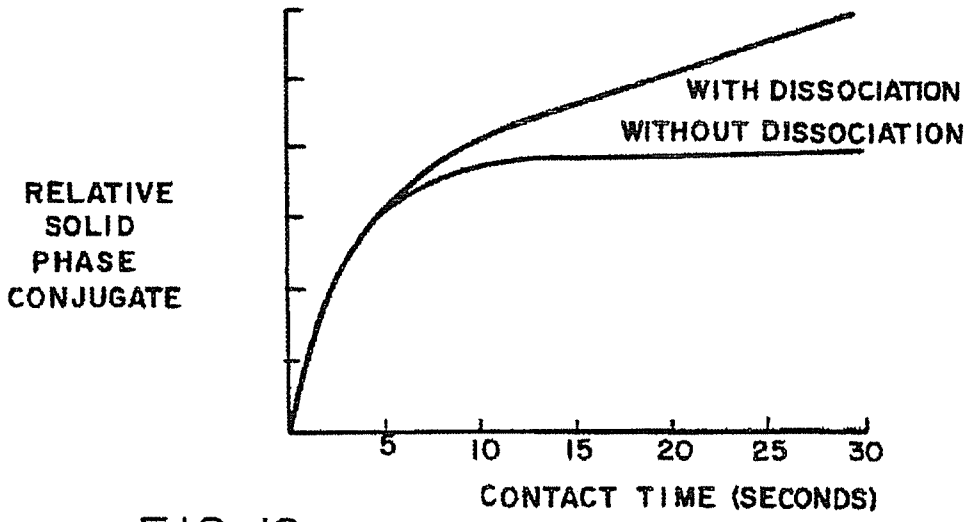
WITH DISSOCIATION
WITHOUT DISSOCIATION
RELATIVE
SOLID
PHASE
CONJUGATE
5
10
15
20
25
30
CONTACT TIME (SECONDS)

FIG. 12

SOLID PHASE ASSAY FOR DETECTION OF LIGANDS

This application is a continuation-in-part of commonly owned U.S. patent application Ser. No. 08/262,741, filed on Jun. 20, 1994 now abandoned, which is a continuation of U.S. patent application Ser. No. 08/197,431, now abandoned, filed on Feb. 16, 1994, which is a continuation-in-part of 07/924,720, filed on Aug. 3, 1992, now U.S. Pat. No. 5,372,783. The entire contents of each of these parent applications are hereby incorporated by reference.

FIELD OF THE INVENTION

The invention relates to chemical, biochemical, and biological assays, and more particularly to solid phase assays for the detection of ligands.

BACKGROUND OF THE INVENTION

Assays in which a sample and one or more reagents are variously reacted to form a ligand/conjugate complex such as an antibody/antigen or similar complex, which may then be observed in order to measure the presence or level of a predetermined moiety in the sample, are well known. Typically, an antibody is used to assay for the presence of an antigen for which the antibody is specific. These assays have been extended to quantitate haptens such as hormones, alkaloids, steroids, antigens, antibodies, nucleic acids, and fragments thereof, enzymes, and cell surface receptors. It is in this broad sense that the term "ligand/conjugate" as used herein should be understood.

Sensitive immunoassays typically use tracer techniques in which a tagged constituent of the complex is incorporated, for example in the reagent, the non-complexed tagged reagent then being separated from the complexed reagent. Thereafter, the complex can be quantitated by observing a signal from the tag. Radioisotopes, fluorescent and chemiluminescent molecules, calorimetric tags, and other markers have been used to label constituents or moieties of the complex, appropriate apparatus being employed to detect and measure the radiation from the label.

In such assays where at least one component of the complex is initially bound to a solid substrate preparatory to formation of the complex, a basic problem arises because of the typically lengthy time required to bind that component to the solid substrate such as a well microtiter plate or bead, sometimes requiring incubation times on the order of hours for binding of a component to the solid phase to occur notwithstanding such expedients as heating, agitation and the like. Consequently, there is a significant amount of prior art regarding attempts to reduce this incubation time, including using microbeads, dipsticks, macrobeads, etc., but nonetheless incubation times on the order of 10 to 20 minutes are typical.

There are numerous formats for solid phase assays, but they can nonetheless be sorted into two types: sandwich and competition, both of which are well known to those skilled in the art. Sandwich assays typically require the antigen to be able to simultaneously bind to more than one conjugate. One of the conjugates is attached to the solid phase while the other conjugate is labeled with a tag. The amount of tagged conjugate attached to the solid phase is then related to the antigen concentration in a sample. A universal problem in sandwich assays is nonspecific binding, i.e., the amount of labeled conjugate that is on the solid phase, but not attached to the antigen. In designing sandwich assays, there is usually a trade-off between signal level and nonspecific binding.

Increasing the concentration of labeled conjugate or incubation time of the labeled conjugate will increase the signal levels, but will also increase the amount of nonspecific binding.

Various approaches have been used to try to reduce the effect of nonspecific binding including wash buffers, detergents, blocking steps, referencing, etc., which are also well known in the art.

In competition assays a labeled moiety, either a conjugate to or an analog of the antigen, can be bound to the solid phase. The presence of antigen reduces or inhibits the binding of the labeled moiety to the solid phase. The amount of inhibition in the signal is a measure of the antigen concentration. For a competition assay to work well the amount of antigen, labeled moiety and solid phase binding sites must be roughly equal. Therefore, competition assays are usually much less sensitive than sandwich assays and also suffer from a small linear range. They are nonetheless useful for small antigens which can only bind with one conjugate at a time.

The results of chemical, biochemical, and biological assays are used to make important decisions, and therefore, the accuracy and reliability of the result is of utmost importance. Heretofore, control samples of known concentration are assayed periodically, or even simultaneously with the sample to be measured, to calibrate and verify the operation of the assay on the unknown sample. This process reduces, but does not eliminate, the possibility of error in the assay of interest.

An object of the present invention is to provide a solid phase assay method which solves many of the problems described above. Another object of the invention is to provide a "competition-like" assay with an increased linear range of determination, improved sensitivity, reduced susceptibility to errors caused by deteriorating reagents or variations in environmental conditions, reduced susceptibility to errors caused by bubbles or other mechanical problems, ease of automation, and reduced time to obtain a final result of the assay, among other things, relative to standard competition assays known in the art. A "competition-like" assay of the invention is similar to a standard competition assay except that at least one step of the competition-like assay of the invention is time limited in a manner described herein so that competitive equilibria typical of standard competition assays are not established.

Another object of the invention is to provide an improved sandwich assay in which at least one step is time limited so that problems that are associated with non-specific binding reactions and are typical of standard sandwich assays are avoided.

Yet another object of the invention is to eliminate time-consuming incubation steps in a solid phase assay method or to significantly reduce the required time to run the assay.

SUMMARY OF THE INVENTION

The above objects are accomplished by the present invention which comprises a method of performing binding assays, utilizing a solid phase, in which at least one or more of the analyte and/or ligand components is or are allowed only a limited contact time with the solid phase component. In the present methods, the solid phase material is preferably coated with a substantial excess of binding ligand. In the preferred embodiments, as described in our co-pending, commonly assigned U.S. patent application Ser. No. 07/924,720, the contact time of the solid phase component with the analyte-containing sample of the reagent-containing sample

is limited by means of flowing the sample relatively rapidly past the solid phase material. The present invention comprises several variations and improvements, described in more detail below, each of which includes the limited solid phase contact time.

The present invention comprises methods of detecting the presence or level of an analyte in a sample by detecting the formation of a binding complex on a solid phase. Preferred "competition-like" methods of the invention comprise the steps of:

(a) mixing the sample with a second ligand capable of binding with said analyte so that an analyte/second ligand complex is formed;

(b) contacting the mixture produced in step (a) with a solid phase having bound thereto a first ligand capable of binding with the second ligand so that a first ligand/second ligand complex is formed, the contacting being performed under conditions and for a time sufficiently limited that dissociation of the analyte/second ligand complex formed in step (a) is substantially inhibited;

(c) binding a detectable tag to the second ligand-either prior to or after step (a) or step (b) so that a portion of the tag is retained on the solid phase upon formation of the first ligand/second ligand complex;

(d) detecting the portion of the tag to detect formation of the first ligand/second ligand complex on the solid phase, so that the presence or level of the analyte in the sample can be determined.

As will be readily appreciated by one of ordinary skill in the art, such competition-like assays could also be used to quantify the binding constant for a particular binding ligand such as, for example, an antibody.

Preferred "sandwich-type" methods of the invention comprise the steps of:

(a) contacting the sample with:

(i) a solid phase having bound thereto a first ligand capable of binding the analyte; and

(ii) a second ligand capable of binding to the first ligand or to a first ligand/analyte complex so that a first ligand/analyte/second ligand complex is formed on the solid phase, the contacting being performed under conditions and for a time sufficiently limited that any non-specific binding between the second ligand and the solid phase is substantially inhibited;

(b) binding a detectable tag to the second ligand either prior to or after formation of the first ligand/analyte/second ligand complex so that a portion of the tag is retained on the solid phase upon formation of the first ligand/analyte/second ligand complex;

(c) detecting the tag to determine the presence or level of the analyte in the sample.

The invention also provides methods for single-point calibration and quality assurance that can be used in conjunction with the above-mentioned competition-like and/or sandwich-type assays.

The present invention involves substantially increasing the sensitivity of standard competition-type and sandwich-type immunoassays by a process wherein at least one or more of the analyte and/or ligand components is flowed over the surface of and contacts a solid phase device (e.g. beads, a capillary tube interior surface, a microtiter plate or other device), the solid surface having been suitably coated or impregnated in advance with a binding partner of the analyte and/or ligand.

As described hereinafter, in some cases the analyte-containing fluid is pre-reacted with a label or a labelled binding partner for the analyte. In other instances, after the analyte-containing sample is flowed over and contacts the solid surface, at least one additional solution containing a tagged second binding partner, a tagging agent or a tag component is also flowed over and contacts the solid surface. The solid surface may then be washed with a suitable washing solution and the signal intensity of the tag immediately measured.

Alternately, as in the preferred embodiment described in the following paragraph, the accumulation of label on the solid phase material may be monitored continuously in real time. In this case, in at least some assays, the wash step is unnecessary as the rate of accumulation of signal may be successfully used to accurately quantify analyte.

In the preferred embodiment, the assays of this invention may be conducted in a system comprising the flow cell system described in co-pending U.S. patent application Ser. No. 07/924,720. In preferred embodiments of the system, at least one separate mass of discrete beads coated with a binding partner, is disposed within a cylindrical capillary conduit, which is preferably transparent and is arranged within and passes through the focal region of a focusing lens means. The cylindrical conduit is positioned transversely to the optical axis of the lens means and behind the center of curvature of the lens means. As already noted, the assays of this invention can also be conducted in various other systems, typically with lower sensitivity as hereinafter described, including immunologically coated capillaries, microtiter plates, etc.

In this system, when the coated mass of beads or the like is translucent and the capillary is translucent or transparent, the development of fluorescence can be monitored and measured as the assay proceeds, using optical measuring equipment and an electrical detector means as proposed in U.S. patent application Ser. No. 07/924,720. Measurement of tagged ligand/conjugate complexes bound to other solid surfaces can be made, depending upon the nature of the tag, using measuring means heretofore utilized in such measurements.

The assays of this invention, in general, contemplate coating the solid surface with a substantial excess of a binding partner (for sandwich assays) or analog (for competition-like assays) of the analyte, flowing the analyte-containing fluid and/or any other fluids over and in contact with the solid surface at a rapid rate and under conditions which expose the surface area of the solid to the analyte- or other reactant-containing fluid to the maximum possible extent.

It is believed that a key element of the invention is that the contact time of the analyte and reagent containing solutions and the solid phase material be relatively short. For example, in the preferred embodiment, only a relatively small volume of the analyte (about 2 microliters) is in contact with the solid phase material at any given time.

For competition-like assays of the present invention, "contact time" refers to the average amount of time that an individual analyte/second ligand complex is in contact with the solid phase. At typical flow rates, each "increment" of pre-mixed sample (where "increment" refers to a volume containing, on average, a single analyte/second ligand complex) is in contact with the solid phase for less than about one minute, preferably less than about ten seconds, and most preferably less than about one second. For example, at a flow rate of 1000 ul/min, each increment of pre-mixed sample is typically in contact with the solid phase for only approximately one five-hundredth of a minute.

For improved sandwich assays of the present invention, "contact time" refers to the total amount of time that second ligand is in contact with the solid phase.

While the optimum contact time may vary with other assay parameters including the binding kinetics of the particular analyte being studied, it is anticipated that the optimum contact time in all cases will be relatively short.

The assays of this invention are applicable to a wide range of analytes including monovalent and polyvalent entities. These assays can be directly conducted successfully on suitably diluted samples of fluids heretofore considered difficult to assay directly, such as, e.g., whole blood, milk, etc.

The assay methodology of this invention has numerous advantages over the methodology conventionally used in the art. These advantages include, but are by no means limited to, faster running time, greater sensitivity especially at low analyte concentrations, elimination in many cases of incubation time and diminution thereof in all cases, ease of automation, and high reproducibility, and in the particular case of competition assays, extended linear range.

As described below, using the present assay methodology, one may establish a specific performance curve for each analyte whereby false negatives and false positives due to abnormally high levels of binding and erratic results caused by performance errors can be readily eliminated.

Using the methodology of the present invention, single point calibration with a fluid of known antigen concentration can be utilized for competition-like assays as well as those of the sandwich type. This is particularly useful in eliminating false negatives and false positives due to abnormally low binding levels (e.g., such as may be caused by the presence of inactive antibodies). Other and further advantages and benefits of the present invention are discussed hereinafter or will be apparent from the detailed discussion below.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a comparison of the present invention to a standard ELISA assay;

FIG. 2 shows representative binding curves for specific (B) and non-specific binding (B_0);

FIG. 3 shows representative output data with and without flaw;

FIG. 4 shows representative family of curves for gentamicin in whole milk;

FIG. 5 shows a graph of sample and calibration signals;

FIG. 6 shows standard curves for variable flow rate;

FIG. 7 shows a graph showing percent inhibition versus concentration for several flow rates;

FIG. 8 shows standard curves for variable antibody dilutions;

FIG. 9 shows standard curves for variable analyte dilutions;

FIG. 10A and FIG. 10B show signal versus ferritin concentration; and

FIG. 11A and FIG. 11B show signal versus gentamicin concentration.

FIG. 12 shows a plot of complex-formation versus time for a typical competition-like assay ("with dissociation"), as compared to an idealized competition-like assay in which there is no dissociation of pre-formed analyte-second binding ligand complexes ("without dissociation").

DETAILED DESCRIPTION OF THE INVENTION

The present invention rests on the finding that competition and sandwich immunoassays can be run, with reproducible and otherwise satisfactory results, within very short time spans, by a process that comprises the step of rapidly flowing a sample containing analyte over and in contact with a solid surface that has been impregnated or coated with a substantial excess of, for example, an immunological binding partner for the analyte.

The presence of a label, while essential, can be effected in a variety of ways. Thus, the diluted analyte sample may be pre-reacted with labeled antibody or the labeled antibody solution may be flowed over and in contact with the solid surface immediately after flowing the analyte thereover. The label may be directly bound to a primary antibody for the analyte or it may be bound to a secondary antibody which is then either (a) pre-reacted with primary antibody and analyte, (b) pre-reacted with primary antibody whereupon the product is then rapidly flowed over and in contact with the solid substrate, to at least some of the active sites of which analyte has already been bound, (c) flowed rapidly over and in contact with the solid substrate, to at least some active sites of which an analyte-primary antibody conjugate has already been bound.

It has been found that in working with samples of analytes of known concentration, different binding curves will be obtained depending upon whether some or all of the immunoreactants are pre-reacted prior to flowing the solution containing the analyte over the solid surface to which its immunological binding partner is coupled. If, however, the binding curve is carefully established based on runs made with analyte samples of known concentration using identical reagents and method steps in each run, it can be successfully utilized with analyte samples of unknown concentration to give results that are highly reproducible and precise.

Stated otherwise, the immunoreactants, including the labeled primary or secondary antibody, must be mixed with the diluted analyte sample in the same order for the same time periods and contacted with the immunologically treated solid surface in the same order at the same flow rates and under the same conditions of contact as well as other conditions that may have an effect such as, e.g., agitation (or lack thereof) and temperature. The maintenance of identical methodology becomes increasingly critical as the time of exposure of the analyte to its solid-bound immunological binding partner is decreased.

In conducting competition-like immunoassays in accordance with this invention, the contact time between any given analyte molecule, or any given analyte/second ligand complex, and the solid surface to which its immunological binding partner is coupled is very small. To date, in the experimental evaluation of the assay method of this invention, solutions containing analyte have been flowed over and in contact with a solid substrate, to which an immunological binding partner of the analyte is coupled, in a cylindrical column (approximately 1.7 mm in diameter), at flow rates that have varied between 250 and 2000 microliters per minute. While slower and faster flow rates may well work under certain circumstances, it is presently believed that this range is optimal for insuring the requisite contact between the analyte and its binding partner immobilized on the solid surface while assuring a relatively short contact time, as described above.

Using the stated range of flow rates for analyte-containing solution, competition-like immunoassays according to this invention have so far been conducted under circumstances where the solid substrate is continuously contacted with sequential incremental volumes of analyte for a period of from about one to about two minutes. This time is believed

to be of less importance to competition-like assays of the invention than the contact time between any single volume increment of analyte and the solid phase material (though, as discussed herein, the situation is different for sandwich assays of the invention). In all competition-like assays investigated so far, the contact time between any single volume increment of analyte and the solid phase material has been less than about one minute, preferably less than about 10 seconds, and most preferably less than about one second.

While it is contemplated that, depending upon the nature of the analyte and its immobilized binding partner, the configuration and arrangement of the solid substrate, the reaction temperature and perhaps other variables as well, contact times of the analyte solution with its immobilized binding partner ranging between about 25 seconds and 5 minutes (i.e., 300 seconds) may in some instances be desirable, it is believed that the time range so far utilized of 60–120 seconds (i.e., 1–2 minutes) is particularly effective when the solid substrate exhibits a high surface area—as, e.g., when it comprises a mass of beads, each of about 100 microns in mean diameter.

It is contemplated that the methodology of the invention is usable with a wide variety of solid substrates and is by no means limited to use with beads. It is noted that preferred solid substrates as alternatives to beads, e.g., synthetic polymer masses suitable as substrates for immunogens are available in the form of foamed and filamentous structures, as loosely woven mat-like structures and in a variety of other forms in addition to beads. Moreover, as is well-known in the art, glass or plastic tubes and other devices coated with immunological reactants are suitable as solid substrates. In the method of the present invention, it is contemplated that capillary tubes with appropriate interior immunological coating and coated microtiter plates, for example, could readily afford the requisite contact with analyte-containing sample during rapid flow.

It is further believed that other devices such as cassettes, microfilters and the like can readily be adapted to serve as chambers within which the rapid flow, contact reaction between analyte in solution and solid-coupled immobilized binding partner can advantageously be carried out.

A reaction chamber which has been found to function efficiently in the present invention is the capillary conduit that passes through the lens means described in copending U.S. patent application Ser. No. 07/924,720.

The system described in the aforementioned copending application is disclosed to be particularly adapted to the use of fluorescent tags and to optical detection of the fluorescence as it develops throughout an assay. The present invention contemplates that the tags employed can be of the fluorescent, luminescent, or calorimetric type; indeed, the well-known radioactive tags, which are not preferred for environmental and health reasons, are also useful. Indeed, any tag that will produce a detectable signal, preferably by electrical or electronic means can be utilized.

In this regard, it is noted that the aforementioned co-pending patent application Ser. No. 07/924,720 employs an optical detection system that conveys a signal to an electrical detector. According to the co-pending application, the total fluorescence is optically detected continuously as the immunoassay reaction proceeds.

It should be noted that the monitoring of total fluorescence throughout an immunoassay is not necessarily the same as monitoring immunological binding of analyte to immobilized binding partner. At certain stages of the assay, total fluorescence developed will greatly exceed fluorescence attributable to immunologic binding for various reasons, including the presence of unbound tagged primary or secondary antibody, the occurrence of nonspecific binding, etc. In addition, the electrical signal, which includes that from the total optically detected fluorescence, does not separate the latter from its own system noise or from baseline scattering of fluorescence that may be detected when no analyte is present.

Despite these caveats, it has been found that monitoring the total fluorescent signal developed during an assay, and in particular monitoring the rate of accumulation of signal during an assay, yields a highly satisfactory (in terms of sensitivity, reproducibility etc.) measure of analyte concentration, e.g. see Examples 5 and 6 below. This ability to monitor signal in real time is also critical for implementation of the quality assurance invention described below.

A single point calibrator form of assay can be successfully utilized to perform both sandwich and competition type assays of the present invention. Use of the single point calibrator assay has particular appeal, e.g., in reference laboratories. Among the known advantages of single point calibrator assays is that they avoid the need to conduct separate assays on reference standard samples essentially contemporaneously with the assay of samples of unknown analyte concentration; they afford a direct indication with the same solid substrate of the comparative reference standard/unknown sample binding in the assay. As is also known, if the immobilized binding partner on the solid substrate is, e.g., partly inactive for some reason, the single point calibrator result is valid nonetheless because it is performed on the same solid substrate and with the same reagents with samples of both known and unknown concentration.

As indicated, the assays of this invention can readily be automated in whole or in part. They can be designed to be run in a defined apparatus under circumstances wherein the assay reactants, including the solid substrate impregnated with the immunological binding partner of the desired analyte, the tag or tagged antibody, the buffer and analyte reference standards are packaged in kit form. Such kits may include, e.g., disposable reaction chamber devices such as cassettes, receptacles for beads, coated capillaries, etc.

Ability to monitor the signal in real time has the additional advantage of permitting the establishment of "quality assurance" curves for the assays of given analytes with specified reagents and process sequences. Construction of quality assurance curves is effected by:

(1) Fitting raw output data to a mathematical function. This will depend in part upon the data parameters selected to be utilized and in part upon the process sequence, since a mathematical function for a given assay may consist of several functions, each covering a specified time interval of the assay. Once the function is established, a standard fitting algorithm (such as that for least squares regression) is used to vary the function parameters and achieve a statistical "best fit" curve. The algorithm will be utilized so as to minimize "residual error" where the latter is defined as the difference between the fitted function and the existing data.

(2) Comparing each residual error with a pre-programmed threshold value. A computer can perform this task.

(3) Thereafter using the computer to flag runs wherein the residual error exceeds the threshold value and depending upon the extent of assay automation, to either cause the assay to be automatically rerun or to discard it as flawed.

The threshold values will be determined by fitting the function to accumulate sets of both good and bad data and will be subject to reevaluation as further data is collected.

By fitting, insofar as is feasible, to equations based on binding kinetics, the use of quality assurance may enable rejection of false positives and of false negatives due to abnormally high nonspecific binding. In any event, it will reject values due to assay process malfunction, e.g., such as that caused by air bubbles, discussed in some of the Examples that follow.

Various kinetic explanations based on known first and second order phenomena have been proposed for the assays of this invention and have met with considerable success in describing the outcome of various experiments. Nevertheless, these explanations are regarded as tentative and are included here purely as an aid to understanding and are in no way to be interpreted as limiting the scope of the invention, as set forth in the claims below.

In one embodiment of the present invention, we perform a competition-like assay in which we pre-react the sample containing analyte with an analyte-specific antibody and then flow the pre-reacted mixture past the solid phase, which is also capable of binding the analyte-specific antibody.

A tentative explanation for the excellent results we obtain is that the relatively brief (much less than a second) time of contact of each increment of volume of the pre-reacted mixture with the solid phase substantially inhibits the establishment of a classical three-way competitive equilibrium, as occurs if pre-formed analyte/antibody complexes are dissociating while antibody/solid phase complexes are forming. Instead, we hypothesize that the observed signal is due almost entirely to the binding of the residual free (i.e. not complexed to sample antigen) antibody left in the pre-reacted mixture. This effect is thought to arise because of the relatively slow rate of dissociation of analyte/antibody complex. In essence, very few of the complexed antibody/antigen pairs will dissociate during the short contact time.

As a test of this theory, we fit it to two sets of data. One set of data was obtained using a prototype immunoassay system, as described in copending application Ser. No. 07/924,270. The other set of data was obtained using standard ELISA technology.

The following Examples are presented as illustrative of the invention rather than limiting.

In the assays presented in the Examples, the emission from the fluorescent indicator tags was optically directed in accordance with the disclosure of the aforementioned copending application to an electrical detector comprising a computer programmed to calculate each of (1) "delta" defined as the difference between the detected baseline and the detected endpoint, (2) the slope of the signal accumulation, as monitored in real time, (3) "sigma", the standard deviation among multiple identical sample runs, and (4) "C.V.", the coefficient of variation of these identical runs.

The invention further comprises means for performing a "quality assurance" analysis of the assay either during performance of the assay or at a later time. The analysis provides a determination of whether some of the assay results should be rejected due to errors or deficiencies.

The invention further comprises a calibration liquid which can be used to check the performance of the assay solid phase material and other associated analytical ligands within a few seconds of the time that these materials are used to assay an unknown sample. While use of a calibrator of this sort is well known in sandwich immunoassay art, its use with a competition assay format has never been accomplished or disclosed.

In the preferred embodiment, the solid phase consists of a fluid permeable porous mass which typically has a substantial excess of a first binding ligand immobilized on its surface. This porous mass may consist of a multitude of particles contained in a flow cell and held in place by a support means such as a fluid permeable screen. The screen may either be fixed permanently in the flow cell, or may be formed temporarily as described in U.S. patent application Ser. No. 08/026,507, also incorporated in its entirety by reference herein.

In accordance with the present invention the analyte containing sample will be brought into intimate contact with the solid phase material for a relatively short period of time. In the preferred embodiment of the competition-like assay of the present invention, the sample will be flowed through the porous mass of the solid phase material. In this case, the contact time is understood to refer to the time during which each increment of volume is in contact with the solid phase.

It will be appreciated that a label or tag is necessary in order to detect the amount of second binding ligand immobilized on the solid phase. Any radiation emitting tag can be used with the present invention, including, but not limited to, fluorescent tags, chemiluminescent tags, bioluminescent tags, radioisotope tags, calorimetric tags, etc. It will be further appreciated that the label or tag may be coupled directly to one of the primary binding ligands utilized in the assay, or may be coupled to the primary binding ligand through an intermediary third binding ligand capable of forming complex with the appropriate primary binding ligand. Techniques utilizing an intermediary third binding ligand are, in the specific case of antibody-based assays, typically referred to as "second antibody techniques" by those skilled in the art.

Preferably, a competition-like assay of the present invention is performed in the following steps: A substantial excess of a first binding ligand, typically either the sample analyte or an analog of the sample analyte, is bound to the porous solid phase material. The sample, containing an unknown quantity of analyte, is mixed with a typically limiting quantity of labeled second binding ligand. The mix of second binding ligand and sample is then flowed past the solid phase material.

As is known in the art, standard competition assays are typically utilized to determine the presence or amount of an analyte in a sample. This determination typically involves a comparison of the level of first binding ligand/second binding ligand complex formation (typically assayed by detecting the presence and/or amount of a second-binding-ligand-associated detectable tag that is retained on the solid phase) that is achieved in the absence of analyte, with the level of first binding ligand/second binding ligand complex formation that is achieved in the presence of a sample that contains an unknown amount of analyte (for example, when the sample and second binding ligand have been pre-reacted; or when the sample, first binding ligand, and second binding ligand are all reacted simultaneously).

The competition-like assays of the present invention can be utilized in the same way. For such assays, it is important that formation of analyte/second binding ligand complexes inhibits formation of first binding ligand/second binding ligand complexes.

The mixing of second binding ligand and sample analyte may be performed prior to introduction of the mixture to the flow system, or it may be accomplished automatically, e.g. by injecting a stream containing second binding ligand into a flowing stream of analyte containing sample.

It is an object of the present invention to enable quantitation of the sample analyte using a substantial excess of first

binding ligand on the solid phase by limiting the contact time of the premixed sample and labeled second binding ligand with the first binding ligand on the solid phase. This will be understood by those skilled in the art to represent a departure from previously understood practice in which the concentration of solid phase binding ligand must be roughly equal to the concentration of analyte to be detected.

Preferably, a single point calibration can be performed in the case of the competition-like format by incorporating the following steps: Either immediately before, or immediately after performing a competition-like assay as described above, a known reference liquid, typically a known concentration of analyte compound or analog thereof is mixed with another aliquot of labeled second binding ligand drawn from the same source as that used with the sample analyte. After combining, which may be accomplished automatically as described above, the mixture of reference liquid and labeled second binding ligand is again brought into intimate but temporary contact with the solid phase. Two signals are measured, one arising from the reference liquid and one arising from the sample. Since the reference liquid is of known properties, the signal arising from it can be used as a reference to compensate for variations in labeled second binding ligand activity, first binding ligand activity, and the like. It will be appreciated that this single point calibration is enabled by the excess solid phase binding capacity, effective utilization of which is a primary object of the present invention.

Preferably, a sandwich assay of the present invention is performed in the following steps: A substantial excess of a first binding ligand, capable of binding the analyte of interest, is immobilized on the solid phase material. The sample is brought into intimate but temporary contact with the solid phase material, as by flowing. A second labeled binding ligand, capable of forming a complex with the sample analyte/first binding ligand complex is then brought into intimate but temporary contact with the solid phase material, again, as by flowing. The amount of analyte is then quantified by measuring the amount of labeled binding ligand retained on the solid phase material. Those skilled in the art will appreciate that the referred to quantitation of analyte will require subtraction of a non-specific binding (i.e. binding of the labeled second ligand in the absence of analyte) term which is always found to be present in sandwich assays. It is an object of the present invention to increase the ratio of specific binding over non-specific binding by limiting the contact time between the labeled second binding ligand and the solid phase material.

Preferably, a quality assurance method can be implemented on either the competition-like assay or the sandwich assay of the present invention by monitoring and recording, typically on a computer, the course of the signal generation during the assay. By comparing the shape of the resultant signal curve versus time to a pre-determined standard curve, errors due to air bubbles, other mechanical problems, and the like will be readily detectable.

An important element of the invention is that the contact time of the analyte and/or reagent containing solutions with the solid phase material be relatively short. For example, in a preferred embodiment utilizing the capillary flow cell and porous solid phase, as described fully in copending U.S. patent application Ser. No. 07/924,720, only a relatively small volume of the analyte (about 2 microliters) is in contact with the solid phase material at any given time.

Typical competition-like assays of the present invention were performed at flow rates of approximately 1000 ul/min. For such assays, each increment of analyte is in contact with the solid phase for an average of only approximately one five-hundredth of a minute, or slightly over a tenth of a second. While the optimum contact time has not yet been precisely determined, and while it is believed that the optimum time will vary with other assay parameters including the binding kinetics of the particular analyte being studied, it is certainly anticipated that the optimum contact time in all cases will be relatively short.

By "relatively short" or "relatively brief" as used herein, it is meant that contact time of the sample and reagent containing solutions with the solid phase, depending on the binding kinetics of the analyte, will be less than about one minute, preferably less than about ten seconds and most preferably less than about 1 second.

In various assays, flow rates ranging from about 250 ul/min to about 2000 ul/min have been investigated. Flow rates in this range have been found to be suitable for a wide variety of analysis and sample matrices, but the invention is in no way limited to this range of flow rates.

Relatively simple models based on first order binding kinetics have been proposed and have met with considerable success in describing the outcome of various experiments. These models, and explanations based on them, are regarded as tentative and are included here purely as an aid to understanding and are in no way to be interpreted as limiting the scope of the invention, as set forth in the claims below.

In one embodiment of the present invention, we perform a competition-like assay in which we pre-react the sample that contains an unknown amount of analyte with a specific antibody and then flow the pre-reacted mixture past the solid phase, which has a substantial excess of the analyte material immobilized on it and is thus also capable of binding the specific antibody. In a competition-like assay the important parameter is the mean contact time of any portion of the sample with the solid phase, not the contact time of the solid phase with the total sample.

While not wishing to be bound by any particular theory, it is believed that a tentative explanation for the excellent (e.g. highly sensitive, highly reproducible, exhibiting a large linear range) results we obtain is that the relatively brief (less than a second) time of contact of each increment of volume of the pre-reacted mixture with the solid phase essentially prohibits the establishment of a classical three way competitive equilibria. Instead we hypothesize that the observed signal is due almost entirely to the binding of the residual free (i.e. not complexed to sample antigen) antibody left in the pre-reacted mixture. This effect is thought to arise because of the relatively slow rate of dissociation of antibody-antigen complex. In essence, very few of the complexed antibody antigen pairs will dissociate during the short contact time. In other words, by limiting contact time between the solid phase and increments of pre-reacted mixture, we are able to avoid the problems associated with competing reactions.

As a test of this theory, we used two assay methods, one described in copending U.S. patent application Ser. No. 07/924,720, and one using standard ELISA technique and plate readers. In both cases the coating of the solid phase material was as nearly identical as possible to facilitate comparison. Equations were derived to approximate both the model described above and a standard three way competitive equilibria. The equations used to approximate the "competitive like" model assumed that the labeled antibody reached equilibrium with the antigen in the sample, and that the residual free antibody then reached equilibrium with the

solid phase. This model is thought to be a gross simplification of the actual process and is in no way intended to limit the scope of the Invention. Unknown parameters in this assay were the antibody affinity and the effective concentration of the solid phase antigen. As shown in FIG. 1, reasonable values (antibody affinity=4×10^{3} ^{9} and effective solid phase antigen concentration=1×10^{3} ^{8} molar) were found for these parameters which still allowed the models to simultaneously fit the two sets of data.

An alternate way to present this theory is embodied in the graph presented as FIG. 12. FIG. 12 shows a plot of complex formation versus contact time (where "contact time" refers to the time that a single increment of pre-reacted mixture is in contact with the solid phase) for (i) an "idealized" competition-like assay in which there is no dissociation of pre-formed analyte-second binding ligand complexes ("without dissociation"); and (ii) a typical competition-like assay ("with dissociation"). The curves presented in FIG. 12 were calculated using standard mathematical modelling techniques (see, for example, *Chemical Kinetics and Dynamics*. J. I. Steinfeld et al., Prentiss Hall Press, 1989). One of ordinary skill in the art would readily be able to generate similar curves for any binding reactions of interest.

With reference to FIG. 12, it is clear that, if the contact time of the pre-reacted mixture with the solid phase is less than about 5 seconds (for the particular association rate constant shown), then the actual signal observed is virtually identical to the hypothetical case of no dissociation. In preferred competition-like assays of the invention, therefore, the step of contacting the pre-reacted mixture (containing sample and second binding ligand) with the solid phase is performed under conditions and for a time that is sufficiently limited that the binding reaction between the first binding ligand on the solid phase and the second binding ligand has not progressed to a point that a plot of complex formation versus time (such as that presented in FIG. 12, "with dissociation" curve) has diverged substantially from an idealized plot of the same parameters that is generated assuming that no dissociation of the preformed analyte/second binding ligand complex occurs.

The binding of a relatively low concentration of free antibody to a substantially excessive solid phase antigen is an inherently linear process over a fairly large range of free antibody concentrations which is believed to be the cause of the relatively large linear range observed in our examples.

In a preferred embodiment of the single point calibrator, constructed to work in conjunction with the competition-like assay above, we mix a known concentration of antigen (the calibrator liquid) with an aliquot of the labeled antibody drawn from the same location as the labeled antibody mixed with the sample. This mixture is then flowed past the solid phase, typically at the same rate and for the same time as the unknown sample. Using the preferred embodiment of a translucent solid phase deployed in a transparent capillary (see Ser. No. 07/924,720) it is possible to observe the signal generation in real time. In this case, the slope of the signal generation response is found to be a reliable indicator of the analyte concentration. In the case of the single point calibrator we calculate the slope in two regions, one corresponding to the calibrator liquid, the other corresponding to the unknown sample. The slope of the response from the (known) calibrator liquid provides a reference for other possible assay variables, such as antibody activity, temperature, and the like.

Alternatively, the single point calibrator can be implemented using pure labeled antibody. This is similar to using a calibrator liquid with zero antigen but different because there is no dilution of the antibody into the sample or calibrator. Nevertheless, this method can also provide an effective reference.

In the absence of capability to measure signal generation in real time, the calibrator can be implemented by measuring the change in signal level at discrete times, such as before and after passage of the calibrator liquid and before and after passage of the sample antibody mixture.

As described in general terms above, the preferred embodiment of the sandwich immunoassay again utilizes the apparatus described in Ser. No. 07/924,720. The preferred embodiment of the sandwich assay again incorporates the fundamental concept of a limited contact time of labeled reagent with the solid phase. The model here, which again must be regarded as tentative, not limiting, and included for purposes of explanation only, is somewhat different.

As described in the background section above, a common difficulty in implementing high sensitivity sandwich immunoassays is the presence of non-specific binding of the labeled antibody to the solid phase. Although it is not guaranteed to hold for all systems, it is often observed that the non-specific binding signal is slower to reach equilibrium than the specific binding signal. In general terms, we believe that by limiting the contact time of the labeled antibody with the solid phase (i.e. limiting the total time during which labeled antibody is in contact with the solid phase) we are able to effect an increase in the ratio of specific binding over non-specific binding. This effect can be tentatively understood with reference to FIG. 2.

FIG. 2 shows simplified binding curves representing specific (B) and non-specific (B_0) binding reactions. These simplified binding curves are representative of actual binding curves that could readily be plotted by one of ordinary skill in the art using standard mathematical modelling techniques. Consistent with observation of actual binding reactions, the curves presented in FIG. 2 depict a situation in which the rate of non-specific binding, B_0, is lower than that of specific binding, B.

As is known in the art and shown in FIG. 2, binding curves consist of three basic regions: an approximately linear region that increases from zero (region 1 for B curve; regions 1–3 for B_0 curve); a curved region representing a levelling off of the binding reaction (region 2 for B curve; region 4 for B_0 curve); and a substantially constant region that is achieved after longer times, as the binding reaction reaches equilibrium (regions 3–5 for B curve; region 5 for B_0 curve).

For any particular binding curve, the slope of the approximately linear region is given by:

$$ka*[Ab]*[S]$$

where ka is the association rate constant and [Ab] and [S] are the concentrations of the two reactants. The slope of this region is linear only to the extent that [Ab] and [S] are constant.

At t=0, the slope of the binding curve is given by:

$$d[AbS]/dt=ka*[Ab_o]*[S_o] \quad (1)$$

When t approaches infinity, the value approached by the constant region of the binding curve is given by:

$$[AbS]=K*[AB]*[S] \quad (2)$$

where K is the equilibrium binding constant.

Thus, when comparing the two curves presented in FIG. 2, the B/B_0 ratios can be determined exactly for t=0 and for t approaching infinity:

$$\text{For } t=0,\ B/B_0=k_{ns}*[S_o]/k_{nn}*[S_{on}] \quad (3)$$

$$\text{For } t\sim\infty,\ B/B_0=K_s*[S]/K_n*[S_n] \quad (4)$$

where s represents specific binding and n represents non-specific binding.

Referring to FIG. 2, during the time period indicated as region 1, both curves are linear and the ratio B/B_0 is constant and of maximal value. In region 2, specific binding B levels off so that during time period of region 3, the ratio B/B_0 decreases as B_0 continues to increase. During the time period of region 4, B_0 levels off and the B/B_0 ratio assumes its lower, constant, equilibrium level.

It is apparent from reference to FIG. 2 that sandwich assays of the invention are preferably performed so that the step of contacting the second labeled binding ligand with the solid phase material is time-limited within the upper bound of region 3. That is, this step is performed such that non-specific binding levels do not have a chance to level off and the B/B_0 ratio does not assume its lower, constant equilibrium value.

In particularly preferred sandwich assays of the invention, this step is time-limited within the upper bound of region 2 of the graph presented in FIG. 2. Most preferably, the sandwich assays of the invention are time-limited in this step within the upper bound of region 1 of the graph of FIG. 2.

In other words, it is most desirable to perform sandwich assays of the present invention under conditions and for a time that is sufficiently limited that both the specific (i.e. second binding partner/analyte/first binding partner complex) and non-specific (i.e. second binding partner/solid phase) binding reactions are within the linear range of their respective binding curves, and have not reached equilibrium. Under such circumstances, the ratio, B/B_0, of specific to non-specific binding is constant.

It will also be apparent that, having temporally limited this step of the sandwich assays of the present invention within the upper bound of region 1 of the graph of FIG. 2, there is no benefit of further limiting the time of the reaction step. In fact, further decreases in reaction time are not desirable as they decrease the total amount of signal produced.

In standard sandwich assays, both the specific and non-specific binding reactions are allowed to go to equilibrium (as in region 4 and beyond of FIG. 2). Non-specific binding can contribute significantly to background reaction "noise" in such assays. Sandwich assays performed according to the methods of the invention, in which the step of contacting the second labeled binding ligand with the solid phase material is time-limited within the upper bound of region 1, 2, and/or 3 of the graph of FIG. 2, show improved sensitivity over such standard sandwich assays in which non-specific binding significantly contributes to background reaction noise.

An added benefit of the ability to monitor signal generation in real time is that it permits the establishment of "quality assurance" curves which may then be used to detect certain problems in specific assays. Although not yet fully implemented and tested, construction of quality assurance curves will be effected with the following steps:

1. The raw output data (as represented in FIG. 3 with and without air bubbles will be fit to a mathematical function, as indicated by the dashed lines. It is anticipated that the function used will depend on the particular assay and the specific timing and flow rate used. It is further anticipated that the function may consist of several functions with each covering a specified time interval. The fitting of the data to the function will be accomplished with a standard fitting algorithm such as the least squares best fit algorithm, and will vary the function parameters and get an optimal fit. The fitting algorithm will minimize the difference between the fitted function and the data set.

2. After fitting, the computer will compare the residual difference between the fitted data and function with a pre-programmed threshold value.

3. If the residual error exceeds the threshold value the computer will flag the run as flawed, or possibly, depending on the level of automation, automatically re-run it.

In the data shown, the large spike at approximately 65 on the x axis, is expected to increase the residual error enough to raise it over the threshold. The threshold value will be determined by fitting the function to large sets of both good and bad data.

Fitting to equations derived from binding kinetics should enable use of the quality assurance method to reject false positive and false negatives due to abnormally high non-specific binding. The expectation is that in the data fit the affinity constant parameter would fall outside acceptable limits.

EXAMPLE 1

In this example, a competition-like assay is performed in the method of this invention.

For the competition-like assay, the solid phase used was PMMA particles of 90 to 125 um in diameter. These particles were obtained from Bangs Laboratories, Inc., Carmel, Ind. They were adsorption coated by mixing 200 mg of particles with 1 ml of phosphate buffered saline solution (PBS) from Sigma Chemical, St. Louis, Mo., Part No. 1000-3, containing 1 mg/ml gentamicin-BSA conjugate (OEM Concepts, Toms River, N.J., Lot No. 135-68749). This mixture was incubated for 1 hour while being continuously rocked. After the 1 hour incubation, the particles were allowed to settle and the supernatant was drawn off. Next, 1 ml of a blocking solution of 10% normal goat serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa., Item No. 005-000-1210) was added and the particles were again rocked for 1 hour. The particles were then diluted to 30 ml in PBS and were maintained in a container.

The reaction chamber was a glass capillary with an ID of approximately 1.7 mm (Fisher Scientific, Catalog No. 21-16402J) with a 48 micrometer nylon mesh affixed approximately in the center. The reaction chamber is connected, through tubing, to computer controlled syringe pumps and valves, to accurately control the sequence and timing of the assay steps.

To perform an assay the reaction chamber is first loaded with particles in the following manner: the reaction chamber is backflushed for 30 seconds to remove any material from a prior assay. Next, 500 ul PBS is flowed forward through the reaction chamber in a period of 20 seconds. During this time a mechanical stirrer agitates the container of particles to uniformly suspend the particles. Over the next 30 seconds 750 ul of the particle containing solution is flowed through the reaction chamber, which packs about 5 mg of particles on top of the nylon mesh. This is followed by another forward PBS flow of 600 ul over 24 seconds. Next is a gentle backflush of 60 ul over 10 seconds followed by 20 seconds of no flow. This is done to allow the beads to settle by gravity to ensure a uniform bead packing. Finally a forward flow of PBS of 160 ul over 6 seconds prepares the reaction chamber for the assay.

An antibody solution is prepared as follows: mouse anti-gentamicin antibody (ImmunoPharmaceutics, Inc. San Diego, Calif. Item number 16102) is diluted to 0.1 ug/ml in PBS. 1 mg/ml BSA and 2 ug/ml FITC labeled goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc. West Grove, Pa. Item number 115-095-0710) is added and the solution is incubated to allow the antibodies to reach equilibrium (about 30 to 60 minutes).

In this example the samples were whole milk spiked with gentamicin at concentrations of 0, 1, 3 and 10 ng/ml. These samples were placed on the instrument and the instrument would flow 1.5 ml of sample, while injecting an additional 1.5 ml of antibody mixture into the flow stream, through the reaction chamber over a period of 120 seconds. This was followed by 3 ml of wash over 120 seconds. The fluorescent output was monitored during the 240 seconds of sample/antibody flow and wash time. Measurements of the four samples were done in triplicate and the raw output is shown in FIG. 4.

As demonstrated in this example the preincubation time of approximately 15 seconds (the time needed to flow from the point of injection to the reaction chamber) is adequate, giving excellent sensitivity and reproducibility.

EXAMPLE 2

This example demonstrates the single point calibrator concept using undiluted label as the reference liquid. The particles are prepared using the same procedure as in example 1. After the reaction chamber is loaded with particles, antibody mixed with sample at one of three concentrations (0, 1, or 10 ng/ml) is flowed over and through the particles for 1 min at a rate of 1.5 ml/min. This is followed by a wash for 1 minute at the same rate. Next there is 40 seconds of flow of pure undiluted antibody at the same rate, giving rise to a second binding signal, which in this case is the reference. This is followed by another 1 minute wash with buffer.

FIG. 5 shows that it is possible to generate two sequential binding signals using the same solid phase. Since the second binding slopes, occurring from approximately 140 to 170 seconds and representing the reference are both reproducible and not significantly affected by the varying sample binding, occurring from approximately 25 to 70 seconds, they provide a direct measure of overall system performance.

EXAMPLE 3

A series of competition-like assay runs were made, using known concentrations of digoxin as the analyte, for the purpose of gaining a fundamental understanding of the factors that may affect sensitivity and precision using the assay methodology of this invention.

In these examples the solid phase particles were coated with digoxin-BSA (Immunotech Corp. Boston, Mass. item number 685) using a coating solution concentration of 1 mg/ml, with identical procedures as in the gentamicin example above.

A series of digoxin serum standards which are included in the commercial digoxin radioimmunoassay kits heretofore sold under the trademark "Phase II" by Binax Corporation, Portland, Me., in which the digoxin concentrations are, respectively, 0.0, 0.5, 1.0, 2.0 and 4.0 ng per ml of human serum was used as the analyte. In each instance it was diluted in a 1:4 ratio with a 1:40,000 solution in PBS of rabbit anti-digoxin unless otherwise stated below.

The primary antibody in this series of experiments was rabbit anti-digoxin at a 1:40,000 dilution in PBS unless otherwise stated below.

The secondary antibody was goat anti-rabbit immunoglobulin conjugated to fluorescein isothiocyanate at a concentration of one microgram per ml of PBS containing 0.1 percent of bovine serum albumin.

The approach adopted was to vary each of the flow rate, primary antibody concentration and analyte dilution factor while keeping the other two factors constant to the extent possible.

A set of standard conditions was chosen from which the variations were made. These conditions are:

flow rate=750 microliters per minute

primary antibody concentration ("[AB]")=1:40,000 dilution

analyte concentration ("[analyte]")=1:4 dilution

In all of the runs the coated beads were introduced into the system in an identical manner to the gentamicin example above. After each assay the bead pack was flushed out of the tube and a new one was inserted in the same manner.

For each of these assay runs, the analyte is diluted with primary antibody solution, thereby enabling analyte and primary antibody to pre-react. The analyte dilution was introduced to the capillary conduit over a span of one minute in a volume equal to the flow rate chosen. An aliquot of 1,500 ul of secondary antibody solution was then introduced over a 120-second period and the beads were then washed with 3000 ul PBS for 120 seconds.

In the first series of experiments, sample flow rate was varied as shown in Table 1 below. It should be understood for the Table 1 runs that the total sample volume exposed to the beads is in each instance the volume introduced in one minute.

TABLE 1

Average delta values and C.V.'s for digoxin assay variable flow rate

		Digoxin Conc. (ng/ml)				
Flow Rate		0	0.5	1	2	4
250 ul/min	Delta	2.48	2.18	1.87	1.05	0.40
	% CV	5.0	4.7	2.9	3.7	1.5
750 ul/min	Delta	6.09	5.39	4.63	3.23	0.75
	% CV	4.5	3.9	2.2	3.7	1.3
1500 ul/min	Delta	6.29	5.37	4.78	2.58	0.80
	% CV	0.9	1.4	0.2	0.5	1.2

While the Table shows the C.V. data to be best at the 1500 ul/minute flow rate, all of the C.V. data is within the 5.0% range generally considered acceptable in the art.[1] FIG. 6 shows that standard curves plotted from the variable flow rate data. As expected the change in flow rate does not affect the % inhibition curve (FIG. 7), but instead only changes the overall level, mostly due to a change in total sample/antibody used.

In the second series of experiments the dilution of primary antibody was varied as shown in Table 2 below while holding the flow rate at 750 ul per minute and the volume of sample actually utilized at 750 ul. Again, triplicate runs were made and the values given in Table 2 are the average of these.

TABLE 2

Average delta values and C.V.'s for digoxin assay variable [AB]

Dilution of Primary Antibody		Digoxin Conc. (ng/ml) 0	0.5	1	2	4
1:20,000	Delta	6.35	6.31	6.10	5.28	3.42
	% CV	3.3	2.7	1.6	1.0	6.5
1:40,000	Delta	6.09	5.39	4.63	3.23	0.75
	% CV	4.6	3.9	2.2	3.7	1.3
1:100,000	Delta	2.87	1.50	0.86	0.49	0.37
	% CV	2.4	3.4	10.4	0.8	20.7

Table 2 shows that poor coefficients of variation are obtained as complete signal inhibition is approached (as in the 1:100,000 dilution runs). FIG. 8 is a plot of the standard curves for variable primary antibody dilution and it demonstrates that, as expected, sensitivity increases as the primary antibody dilution factor increases. This is made clear by calculating the concentration of digoxin which would inhibit the signal an amount equal to 2 standard deviations from the zero concentration. For this example, 1:20,000 gives 0.494 ng/ml, 1:40,000 gives 0.392 ng/ml, and 1:100,000 gives 0.050 ng/ml.

The third series of runs, again performed in triplicate, was made to test the effect of varying the analyte dilution. It should be noted that since analyte is diluted with primary antibody solution, each change in analyte dilution factor results in a change in both analyte and primary antibody dilution even though this change has not been calculated. In all cases, the analyte solution was diluted with 1:40,000 primary antibody solution. Table 3 shows the average delta and C.V. values for each of this series of assay runs:

TABLE 3

Average delta values and C.V.'s for digoxin assay variable [Analyte]

Analyte Dilution		Digoxin Conc. (ng/ml) 0	0.5	1	2	4
1:2	Delta	5.66	4.22	2.73	0.90	0.59
	% CV	7.0	2.3	2.5	5.2	1.8
1:4	Delta	6.09	5.39	4.63	3.23	0.75
	% CV	4.6	3.9	2.2	3.7	1.3
1:10	Delta	5.11	4.86	4.77	4.18	3.4
	% CV	3.3	0.6	3.9	1.9	2.9

These data exhibit C.V.'s within generally acceptable limits. FIG. 9 hereof is a plot of signal versus digoxin concentration for each of the dilution factors. It again follows our understanding in that the sensitivity increases with increasing primary antibody dilution—i.e., with decreasing free primary antibody concentration.

EXAMPLE 4

A sandwich type assay was performed with the same system as in the previous examples. The same beads identified in Example 1 were coated with sheep anti-horse ferritin of concentration 0.1 mg/ml in PBS in the manner described in Example 1. The coated beads were blocked with 10% sheep serum in PBS in the manner set forth in Example 1.

For each determination, a bead pack was established in the capillary conduit of the lens means as described in Example 1. Analyte samples were prepared by spiking a known amount of ferritin (from horse spleen) into PBS at concentration levels of 0.0 ng, 5.0 ng and 50 ng per ml of PBS.

Sheep anti-horse ferritin labelled with FITC was dissolved at 1 microgram per ml concentration in PBS containing 0.1% BSA and used as the "sandwich" antibody.

The assays were run as follows: 400 ul of analyte solution was flowed through the bead pack for 20 seconds, 750 ul of the labelled sheep anti-horse ferritin was then flowed through the bead pack for 30 seconds, followed by washing of the bead pack with 1500 ul of PBS for 60 seconds. Each of the analyte concentrations was run in duplicate. Table 4 below shows delta and slope values plus the related sigma and C.V. values for each, at all three ferritin concentrations.

TABLE 4

Ferritin Concentration	Delta	Sigma	C.V.	Slope	Sigma	C.V.
0.0 ng	0.0754	.0075	9.9%	.0513	.0018	3.4%
5.0 ng	0.1475	.0003	0.2%	.0539	.0017	3.2%
50.0 ng	0.0348	.0025	0.3%	.0775	.0015	2.0%

FIGS. 10A and 10B, respectively, are plot of delta signals against ferritin concentration in ng/ml and a plot of slope against ferritin concentration in ng/ml for these assays.

Example 5

A competition-like assay was run for varying concentrations of gentamicin in milk.

Beads were prepared in the manner described in Example 1. The bead pack for each assay was established using the flow rate and timing described in Example 1.

The primary and secondary antibodies were prepared by incubation for one hour at room temperature. The primary antibody, antigentamicin at a concentration of 1 microgram per ml in PBS containing 0.1% BSA was combined with secondary antibody at a concentration of 4 micrograms per ml in the same buffer solution and the incubation was allowed to proceed.

The analyte solutions were prepared by serial dilution of whole milk containing 28 ng gentamicin per ml of milk to obtain samples of concentration levels 0.0, 0.11, 0.22, 0.44, 0.88, 1.75, 3.5, 7 and 14 ng/ul gentamicin in milk. Each of these serial dilutions was combined with an equal volume of the pre-incubated primary/secondary antibody solution to perform the assay determinations.

In each assay, 1500 ul of the combined sample/antibody solution was flowed through the bead pack for 60 seconds and 3000 ul of PBS was then flowed through the beads for 120 seconds as a wash. Table 5 shows the mean delta and slope for duplicate assays, as calculated, with the sigma and C.V. of each, at all gentamicin concentration levels.

TABLE 5

Gentamicin Concentration	Delta	Sigma	C.V.	Slope	Sigma	C.V.
0.0 ng/ml	3.0695	0.0991	3.2%	0.0405	0.0011	2.8%
0.11 ng/ml	3.0359	0.0676	2.2%	0.0399	0.0009	2.4%
0.44 ng/ml	2.9448	0.0774	2.6%	0.0411	0.0039	9.4%
1.75 ng/ml	2.0841	0.0175	0.6%	0.0371	0.0002	0.5%
7.00 ng/ml	1.4761	0.0200	1.4%	0.0192	0.0003	1.3%
0.00 ng/ml	3.1631	0.0382	1.2%	0.0404	0.0002	0.5%
0.22 ng/ml	3.0420	0.0650	2.1%	0.0389	0.0013	3.3%

TABLE 5-continued

Gentamicin Concentration	Delta	Sigma	C.V.	Slope	Sigma	C.V.
0.88 ng/ml	2.9419	0.0617	2.1%	0.0370	0.0009	2.3%
3.50 ng/ml	2.4352	0.0266	1.1%	0.0305	0.0003	0.9%
14.0 ng/ml	0.3838	0.0050	1.3%	0.0050	0.0001	23%

The first set of 0.0 ng, 0.11 ng, 0.44 ng, 1.75 ng and 7.0 ng determinations was made on a different day from the remaining assays—hence the repeat of the 0.0 ng assays.

FIGS. **11**A and **11**B are plots, respectively, of delta signal against gentamicin in ng/ml and slope as calculated versus gentamicin in ng/ml. FIGS. **11**A and **11**B also provide a graphical indication of the large linear range of the "competition-like" assay system of the present invention.

EXAMPLE 6

A gentamicin single point calibrator study was made. In this study the beads were coated and blocked as described in Example 1. For each assay, a bead pack was established in the capillary conduit as described in Example 1.

The primary/secondary antibody reagent was prepared as in Example 1.

The analyte samples were prepared by serial dilution of gentamicin in whole milk to final concentrations of 0.0, 1.25, 2.5, 5.0 and 10.0 ng/ml of milk. Each was then mixed with an equal volume of the primary/secondary antibody reagent.

The reference material or calibrator was 5 ng gentamicin/ml of milk prepared in the same manner as the analyte samples.

The calibrator and analyte samples were run in the same manner which entailed flowing 1500 ul of calibrator through the beads for 60 seconds followed by flowing 1500 ul of sample containing analyte through the bead pack for 120 seconds, followed by 1500 ul of PBS for 60 seconds and then 3000 ul of PBS for 120 seconds.

Each sample was run in triplicate with calibrator and unknown in tandem each time. The mean results for slope values with associated sigma and C.V. appear in Table 6:

TABLE 6

Sample	Slope	Sigma	C.V
Calibrator Set 1	0.0446	0.0004	0.9%
0.0 ng/ml gentamicin	0.0526	0.0308	1.6%
Calibrator Set 2	0.0445	0.0006	1.3%
1.25 ng/ml gentamicin	0.0512	0.0011	2.2%
Calibrator Set 3	0.0440	0.0009	2.0%
2.5 ng/ml gentamicin	0.0436	0.0019	4.4%
Calibrator Set 4	0.0446	0.0008	1.7%
5.0 ng/ml gentamicin	0.0328	0.0007	2.2%
Calibrator Set 5	0.0445	0.0008	1.7%
10.0 ng/ml gentamicin	0.0119	0.0005	3.8%

This table again shows excellent reproducibility of the slope, independent of the sample concentration.

The preceding examples are merely illustrative of the present invention and are not intended to be limiting. Furthermore, it should be understood that the preceding is merely a detailed description of certain preferred embodiments of the present invention. It therefore should be apparent to those skilled in the art that various modifications and equivalents can be made without departing from the spirit or scope of the present invention, as set forth in the following claims.

What is claimed is:

1. A method of detecting the presence or level of an analyte in a sample, the method comprising steps of:

(a) providing a sample containing an analyte;

(b) mixing said sample with a second ligand, which second ligand binds to said analyte when incubated therewith, so that analyte/second ligand complexes are formed;

(c) providing a solid phase having bound thereto a first ligand, which first ligand is characterized by an ability to bind to said second ligand in such a way that, were said first ligand and analyte exposed simultaneously to unbound second ligand, said first ligand would compete with said analyte for binding to said second ligand;

(d) contacting the mixture produced in step (a) with said solid phase so that unbound second ligand in said mixture binds to said first ligand on said solid phase, said contacting being performed for a time sufficiently limited that substantially no dissociation of said analyte/second ligand complexes occurs while said mixture is in contact with said solid phase;

(e) binding a detectable tag to said second ligand either prior to or after step (a), step (b), step (c), or step (d) so that a portion of said tag is retained on said solid phase upon formation of said first ligand/second ligand complex; and

(f) detecting said portion of said tag and comparing it to an amount of tag retained on said solid phase in the presence of a known amount of analyte to determine the presence or level of said analyte in said sample.

2. The method of claim **1** wherein the step of contacting is performed for a time sufficiently limited that a plot of first ligand/second ligand complex formation versus time does not diverge substantially from an idealized plot of the same parameters, said idealized plot representing a reaction in which no dissociation of said analyte/second ligand complex formed in step (a) occurs.

3. The method of claim **1** wherein the step of contacting is performed by serially exposing increments of mixture to the solid phase, each increment being a volume of mixture that, on average, contains a single analyte/second ligand complex, the mean contact time for any one increment being limited to less than about 10 seconds.

4. The method of claim **3** wherein the step of contacting is performed so that the mean contact time for any one increment is limited to less than about 1 second.

5. The method of claim **3** wherein the step of contacting is performed so that the mean contact time for any one increment is limited to approximately one five-hundredth of a minute.

6. The method of claim **1** wherein said first ligand and said analyte are identical.

7. The method of claim **1** wherein said first ligand and said analyte are different.

8. The method of claim **7** wherein said first ligand comprises an analog of said analyte.

9. The method of claim **1** wherein the step of mixing comprises combining said sample with said second ligand in such a way that said analyte and said second ligand are present at approximately similar concentrations in said mixture.

10. The method of claim **1** wherein the step of contacting comprises contacting said mixture with a solid phase having bound thereto an amount of said first ligand in substantial excess to the amount of unbound second ligand in said mixture.

11. The method of claim 1 wherein:

the step of mixing comprises incubating said sample and second ligand together so that analyte/second ligand complexes form and the amount of unbound second ligand in said mixture is thereby reduced; and

the step of contacting comprises forming a number of first ligand/second ligand complexes on the solid phase, the number of first ligand/second ligand complexes being smaller than it would have been had said analyte/second ligand complexes not been formed in said mixture prior to said contacting step.

12. The method of claim 1 wherein:

the step of providing a solid phase comprises providing a column including a solid phase to which said first ligand is bound;

the step of contacting comprises flowing said mixture through said column; and

the step of detecting comprises detecting accumulation of said tag on said column, above a background level of tag in solution.

13. A method of detecting the presence or level of an analyte in a sample, the method comprising steps of:

(a) contacting said sample with:

(i) a solid phase having bound thereto a first ligand that binds said analyte when incubated therewith; and

(ii) a second ligand that binds said analyte when incubated therewith, the result of the two contacting steps being that a first ligand/analyte/second ligand complex is formed on said solid phase,

(b) limiting the contact time between said second ligand and said solid phase so that:

(i) non-specific binding between said second ligand and said solid phase is not allowed to reach equilibrium; and

(ii) a first binding curve, in which formation of a non-specific second ligand/solid phase complex is plotted versus time, does not level off;

(c) binding a detectable tag to said second ligand either prior to or after formation of said first ligand/analyte/second ligand complex so that a portion of said tag is retained on said solid phase upon formation of said first ligand/analyte/second ligand complex;

(d) detecting said retained tag to determine the presence or level of said analyte in said sample.

14. The method of claim 13 wherein the step of contacting comprises steps of:

(a') contacting said sample with said solid phase so that a first ligand/analyte complex is formed on said solid phase; and

(a") contacting said first ligand/analyte complex with said second ligand so that said first ligand/analyte/second ligand complex is formed on said solid phase.

15. The method of claim 13 wherein the step of contacting comprises steps of:

(a') contacting said sample with a second ligand so that an analyte/second ligand complex is formed; and

(a") contacting said solid phase with said analyte/second ligand complex so that said first ligand/analyte/second ligand complex is formed on said solid phase.

16. The method of claim 13 wherein the step of contacting comprises contacting said sample with said solid phase and with said second ligand simultaneously so that said first ligand/analyte/second ligand complex is formed on said solid phase.

17. The method of claim 13 wherein the step of limiting further comprises limiting the time of contact between said second ligand and said solid phase so that:

(iii) binding said second ligand to said analyte or to said first ligand/analyte complex does not reach equilibrium; and

(iv) a second binding curve, in which formation of said first ligand/analyte/second ligand complex is plotted versus time, does not level off.

18. The method of claim 17 wherein the step of limiting comprises limiting the time of contact between said second ligand and said solid phase so that both said first binding curve and said second binding curve are approximately linear during the period of contact.

19. The method of claim 13 wherein the step of contacting comprises contacting said sample with a solid phase having bound thereto a substantial excess of said first ligand relative to said analyte.

20. The method of claim 13 wherein the step of contacting comprises contacting said sample with a substantial excess of said second ligand relative to said first ligand/analyte complex.

21. A method of detecting formation of a first ligand/second ligand complex on a solid phase, the method comprising steps of:

(a) contacting an analyte that binds said second ligand with said second ligand so that analyte/second ligand complexes formed;

(b) providing a solid phase having bound thereto a first ligand, which first ligand is characterized by an ability to bind to said second ligand in such a way that, were said first ligand and analyte exposed simultaneously to unbound second ligand, said first ligand would compete with said analyte for binding to said second ligand;

(c) contacting said analyte/second ligand complex with said solid phase so that a first ligand/second ligand complex is formed;

(d) limiting the time of contact between said analyte/second ligand complex and said solid phase so that substantially all of the second ligand participating in analyte/second ligand complexes remains in analyte/second ligand complexes and does not participate in interactions with said first ligand;

(e) binding a detectable tag to said second ligand either prior to or after step (a), step (b), step (c), or step (d) so that a portion of said tag is retained on said solid phase upon formation of said first ligand/second ligand complex; and

(f) detecting said portion of said tag and thereby detecting the formation of said first ligand/second ligand complex on said solid phase.

22. The method of claim 21 further comprising the step of quantifying the level of said first ligand/second ligand complex on said solid phase.

23. The method of claim 22 further comprising comparing said level with a level of first ligand/second ligand complex that is formed on said solid phase in a comparison reaction wherein said second ligand is not contacted with said analyte prior to being contacted with said solid phase.

24. A method of detecting the presence or level of an analyte in a sample, the method comprising steps of:

(a) contacting said sample with:

(i) a solid phase having bound thereto a first ligand, which first ligand is characterized in that it binds to said analyte when incubated therewith, the contacting being performed so that a first ligand/analyte complex is formed on said solid phase; and

(ii) a second ligand that binds said first ligand/analyte complex when incubated therewith, so that a first

ligand/analyte/second ligand complex is formed on said solid phase, said contacting being performed under conditions and for a time sufficiently limited that substantially no non-specific binding between said second ligand and said solid phase occurs;

(b) binding a detectable tag to said second ligand either prior to or after formation of said first ligand/analyte/second ligand complex so that a portion of said tag is retained on said solid phase upon formation of said first ligand/analyte/second ligand complex;

(c) detecting said retained tag to determine the presence or level of said analyte in said sample.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,664,114 B1
DATED : December 16, 2003
INVENTOR(S) : Lackie et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,
Item [*] Notice, delete "0" and insert -- 1017 --.

Signed and Sealed this

Twenty-seventh Day of April, 2004

JON W. DUDAS
Acting Director of the United States Patent and Trademark Office