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(54) Title: REAGENT STORAGE IN AN ASSAY DEVICE

(57) Abstract: The invention relates to methods for conducting binding assays in an assay device that includes one or more storage and use zone. The storage zones of the assay device are configured to house one or more reagents used in an assay conducted in the use zone of the device.

REAGENT STORAGE IN AN ASSAY DEVICE

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims benefit of U.S. Provisional Application No.

5 61/455,112, filed October 14, 2010 and reference is made to U.S. Application Serial No. 12/757,685, filed April 9, 2010, the disclosures of which are incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

10 Improved methods for conducting binding assays are provided. These methods include a pre-concentration step to improve assay performance, for example, by increasing the concentration of analyte in the sample and/or by reducing the concentration of extraneous materials that may be present in the sample which may hinder the performance of the binding assay.

BACKGROUND OF THE INVENTION

15 A substantial body of literature has been developed concerning techniques that employ binding reactions, e.g., antigen-antibody reactions, nucleic acid hybridization and receptor-ligand reactions, for the sensitive measurement of analytes of interest in samples. The high degree of specificity in many biochemical binding systems has led to 20 many assay methods and systems of value in a variety of markets including basic research, human and veterinary diagnostics, environmental monitoring and industrial testing. The presence of an analyte of interest may be measured by directly measuring the participation of the analyte in a binding reaction. In some approaches, this participation may be indicated through the measurement of an observable label attached 25 to one or more of the binding materials.

There is always a desire to improve binding assays and devices used to conduct binding assays by increasing the signal obtained from a binding event, reducing non-specific binding, and/or improving measurement accuracy and precision, especially when analyzing complex biological samples.

SUMMARY OF THE INVENTION

The invention provides an assay device including (a) a storage zone comprising a surface-reagent complex confined to the storage zone, the surface-reagent complex comprising (i) a reagent linked to a first targeting agent; and (ii) a surface linked to a second targeting agent, wherein the reagent and the surface are linked, in the surface-reagent complex, via a releasable binding interaction between the first and second targeting agents; and (b) one or more use zones each configured to use the reagent in an assay for an analyte of interest in a sample. The assay device of the invention may include one or more storage zones and/or one or more use zones. Additionally, the storage zone may also include two or more surface-reagent complexes, each including a distinct assay reagent that may be used in an assay conducted in the one or more use zones. For example, the storage zone also includes a second surface-reagent complex confined to the storage zone, the second surface-reagent complex comprising (i) a second reagent linked to a third targeting agent; and (ii) a second surface linked to a fourth targeting agent, wherein the second reagent and the second surface are linked, in the second surface-reagent complex, via a second releasable binding interaction between the third and fourth targeting agents; and the one or more use zones are further configured to use the second reagent in the assay. The use zones may each comprise two or more assay regions each configured to use the reagent(s) stored in the storage zone in one or more assays conducted with a sample in the assay device.

The device may be used to conduct a plurality of assays for one or more analytes present in the sample, e.g., a first assay region of the one or more use zones are each configured to conduct an assay for a first analyte of interest in the sample and an additional assay region in the one or more use zones is configured to conduct an assay for an additional analyte of interest in the sample. Alternatively, a first assay region of the one or more use zones is configured to conduct a first assay for the analyte of interest in the sample and an additional assay region of the one or more use zones is configured to conduct a second assay for the analyte of interest in the sample.

The invention also provides a multiplexed assay device comprising (a) a storage zone comprising a surface-reagent complex confined to the storage zone, the surface-

reagent complex comprising (i) a reagent linked to a first targeting agent; and (ii) a surface linked to a second targeting agent, wherein the reagent and the surface are linked, in the surface-reagent complex, via a releasable binding interaction between the first and second targeting agents; and (b) one or more use zones each comprising a plurality of assay regions configured to use the reagent in a multiplexed assay for a plurality of analytes in a sample. A first assay region of the one or more use zones is configured to conduct an assay for a first analyte of interest in the sample and an additional assay region in the one or more use zones is configured to conduct an assay for an additional analyte of interest in the sample. In addition, the storage zone may further comprises a second surface-reagent complex confined to the storage zone, the second surface-reagent complex comprising (i) a second reagent linked to a third targeting agent; and (ii) a second surface linked to a fourth targeting agent, wherein the second reagent and the second surface are linked, in the second surface-reagent complex, via a second releasable binding interaction between the third and fourth targeting agents; and the one or more use zones are further configured to use the second reagent in the multiplexed assay.

The invention further provides a method of conducting an assay in an assay device as described herein, including the steps: (x) introducing the sample to the one or more use zones; (y) subjecting the storage zone to a condition that releases the reagent from the surface-reagent complex; (z) transferring the reagent from the storage zone to the one or more use zones; and (xx) conducting the assay in the one or more use zones with the reagent. If the use zones are each configured to use a second reagent in an assay, the method further comprises, prior to the conducting step, subjecting the storage zone to an additional condition that releases the second reagent from the second surface-reagent complex; and transferring the second reagent from the storage zone to the one or more use zones.

A method of using such an assay device may also include the steps of (x) introducing the sample to the one or more use zones; (i) subjecting the storage zone to a condition that releases the reagent from the surface-reagent complex; (ii) subjecting the storage zone to a condition that releases the second reagent from the second surface-reagent complex; (y) transferring the reagent from the storage zone to the first assay

region; (z) transferring the second reagent from the storage zone to the second assay region; (xx) conducting an assay in the first assay region with the reagent; and (yy) conducting an assay in the second assay region with the second reagent. The transferring steps may be simultaneous or sequential. Similarly, the conducting steps may also be 5 simultaneous or sequential.

In addition, the invention provides a method of using an assay device of the invention including the steps: (x) introducing the sample to the one or more use zones; (y) subjecting the storage zone to a condition that releases the reagent from the surface-reagent complex; (z) transferring the reagent from the storage zone to the first assay 10 region and the second assay region; (xx) conducting the assays in the first and second assay regions, respectively. The assays may be conducted simultaneously or sequentially.

In another embodiment, the assay device of the invention may be used in the conduct of an assay by (x) introducing the sample to the one or more use zones via the 15 storage zone; (y) adding a diluent to the storage zone and (i) subjecting the storage zone to a condition that releases the reagent from the surface-reagent complex; (ii) subjecting the storage zone to an additional condition that releases the second reagent from the second surface-reagent complex; (z) transferring the reagent and the second reagent from the storage zone to the first and second assay regions; (xx) conducting the assays in the 20 first and second assay regions. The assays and/or transfer steps may be conducted simultaneously and/or sequentially.

Still further, the assay device may be used in an assay by (x) introducing the sample to the one or more use zones via the storage zone; (y) adding a diluent to the storage zone and (i) subjecting the storage zone to a condition that releases the reagent 25 from the surface-reagent complex; (ii) subjecting the storage zone to an additional condition that releases the second reagent from the second surface-reagent complex; (z) transferring the reagent from the storage zone to the first assay region; (xx) transferring the second reagent from the storage zone to the second assay region; (yy) conducting the assays in the first and second assay regions. The assays and/or transfer steps may be 30 conducted simultaneously and/or sequentially.

Moreover, the invention provides a multiplexed assay device comprising (a) a storage zone comprising (i) a first reagent linked to a surface in the storage zone via a first releasable binding interaction; (ii) a second reagent linked to a second surface in the storage zone via a second releasable binding interaction; (b) a first use zone configured to 5 use the first reagent in an assay for a first analyte; and (c) a second use zone configured to use the second reagent in an assay for a second analyte. The first releasable binding interaction comprises a linkage between a first targeting agent and a second targeting agent, wherein the first targeting agent is linked to the reagent and the second targeting agent is linked to the surface. Moreover, the reagent and the surface are linked to form a 10 surface-reagent complex, wherein the surface-reagent complex is confined to the storage zone. The second releasable binding interaction comprises a linkage between a third targeting agent and a fourth targeting agent, wherein the third targeting agent is linked to the second reagent and the fourth targeting agent is linked to the second surface, and the second reagent and the second surface are linked to form a second surface-reagent 15 complex, wherein the second surface-reagent complex is confined to the storage zone. Such a multiplexed assay device comprises a fluidic network, such that the storage zone and the first and second use zones are in fluidic communication, wherein the network is configured to direct fluid in the storage zone to the first use zone, the second use zone, or both. The network is configured to direct fluid to the first use zone and the second use 20 zone sequentially or simultaneously. The first and second reagents are each confined in the storage zone to distinct regions of the storage zone. The first and second releasable binding interactions require the same or different conditions to release the first and second reagents respectively, from the first and second surfaces of the storage zone, e.g., subjecting the storage zone to increased or decreased temperature, pH changes, an 25 electric potential, a change in ionic strength, competition, and combinations thereof. Moreover, each of the first and second use zones comprise a plurality of assay regions each configured to use the first and second reagents in a multiplexed assay for a plurality of different analytes in a sample.

Also provided is a method of conducting a multiplexed assay using the 30 multiplexed assay device described herein including (a) introducing a sample comprising

the first and second analytes to the first and second use zones; (b) subjecting the storage zone to a condition that releases the first reagent from the storage zone; (c) transferring the first reagent from the storage zone to at least one of the first and second use zones; and (d) conducting one or more assays for at least one of the first and second analytes.

5 The method may also include the steps of subjecting the storage zone to an additional condition that releases the second reagent from the storage zone and transferring the second reagent from the storage zone to at least one of the first and second use zones, and optionally washing at least one of the first and second use zone prior to the transferring step.

10 Also provided is a method of conducting a multiplexed assay in a multiplexed assay device including (a) introducing a sample comprising the first and second analytes to the first and second use zones; (b) subjecting the storage zone to a condition that releases the first reagent from the storage zone; (c) transferring the first reagent from the storage zone to the first use zone; (d) subjecting the storage zone to a condition that

15 releases the second reagent from the storage zone; (e) transferring the second reagent from the storage zone to the second use zone; and (f) conducting assays for the first and second analytes in the first and second use zones. The method may also include washing the first and second use zones prior to the transferring step (c), and the assays may be conducted simultaneously or sequentially.

20 BRIEF DESCRIPTION OF DRAWINGS

The accompanying drawings are provided to illustrate rather than limit the scope of the invention. Throughout the accompanying Figures, “P” refers to a particle to which one or more moieties are attached; “S” refers to a first solid phase; “A” refers to a target analyte; “C” refers to contaminants; and “*” refers to a detectable label linked to an assay 25 component.

Figs. 1(a)-1(e) illustrate various assay formats in which a particle is used as an assay component.

Figs. 2(a)-2(b) illustrate various assay formats in which a first solid phase is used as an assay component.

Figs. 3(a)-3(e) illustrate various assay formats in which a particle is used as an assay component, to which a targeting agent is linked.

Figs. 4(a)-4(b) illustrate various assay formats in which a first solid phase is used as an assay component, to which a targeting agent is linked.

5 Figs. 5(a)-5(b) illustrates one embodiment of the present invention. Fig. 5(a) shows magnetic concentration of analytes using colloids coated with anti-antibodies against the analytes and also coated with ECL labels. Multiple antibodies may be used to bind different analytes. Fig. 5(b) shows detection of the analyte-colloid complexes in a sandwich immunoassay format.

10 Figs. 6(a)-6(b) illustrate two alternative competitive immunoassays according to the methods of the present invention.

15 Fig. 7(a)-7(c) illustrate three alternative embodiments of an assay device include one or more storage zones and one or more use zones. Figs. 7(a)-(b) show an assay device including one storage zone that houses a surface-reagent complex that supplies reagent to use zones 1 and 2, while Fig. 7(c) shows an assay device including multiple storage zones that each lead to a use zone. In Fig. 7(c), sample and liquid reagent compartments in the assay device are in fluid communication with the storage and use zones.

20 Fig. 8(a)-(f) illustrate the use of an alternate assay device of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides improved solid phase binding assays that include a collection, separation and/or release step. The methods of the present invention improve assay performance by allowing for pre-concentration of an analyte in a sample and/or by 25 reducing or eliminating the amount of contaminants in a sample that may hinder the performance of the assay, e.g., by interfering with the detection step and/or by non-specifically binding with one or more of the components in the mixture.

(i) Samples/Analytes

Examples of samples that may be analyzed by the methods of the present invention include, but are not limited to food samples (including food extracts, food homogenates, beverages, etc.), environmental samples (e.g., soil samples, environmental sludges, collected environmental aerosols, environmental wipes, water filtrates, etc.), 5 industrial samples (e.g., starting materials, products or intermediates from an industrial production process), human clinical samples, veterinary samples and other samples of biological origin. Biological samples that may be analyzed include, but are not limited to, feces, mucosal swabs, physiological fluids and/or samples containing suspensions of 10 cells. Specific examples of biological samples include blood, serum, plasma, feces, mucosal swabs, tissue aspirates, tissue homogenates, cell cultures and cell culture supernatants (including cultures of eukaryotic and prokaryotic cells), urine, saliva, sputum, and cerebrospinal fluid.

Analytes that may be measured using the methods of the invention include, but 15 are not limited to proteins, toxins, nucleic acids, microorganisms, viruses, cells, fungi, spores, carbohydrates, lipids, glycoproteins, lipoproteins, polysaccharides, drugs, hormones, steroids, nutrients, metabolites and any modified derivative of the above molecules, or any complex comprising one or more of the above molecules or 20 combinations thereof. The level of an analyte of interest in a sample may be indicative of a disease or disease condition or it may simply indicate whether the patient was exposed to that analyte.

The assays of the present invention may be used to determine the concentration of 25 one or more, e.g., two or more analytes in a sample. Thus, two or more analytes may be measured in the same sample. Panels of analytes that can be measured in the same sample include, for example, panels of assays for analytes or activities associated with a disease state or physiological conditions. Certain such panels include panels of cytokines and/or 30 their receptors (e.g., one or more of TNF-alpha, TNF-beta, IL1-alpha, IL1-beta, IL2, IL4, IL6, IL-10, IL-12, IFN- γ , etc.), growth factors and/or their receptors (e.g., one or more of EGF, VGF, TGF, VEGF, etc.), drugs of abuse, therapeutic drugs, vitamins, pathogen specific antibodies, auto-antibodies (e.g., one or more antibodies directed against the Sm,

RNP, SS-A, SS-alpha, J0-1, and Scl-70 antigens), allergen-specific antibodies, tumor markers (e.g., one or more of CEA, PSA, CA-125 II, CA 15-3, CA 19-9, CA 72-4, CYFRA 21-1, NSE, AFP, etc.), markers of cardiac disease including congestive heart disease and/or acute myocardial infarction (e.g., one or more of Troponin T, Troponin I, 5 myoglobin, CKMB, myeloperoxidase, glutathione peroxidase, β -natriuretic protein (BNP), alpha-natriuretic protein (ANP), endothelin, aldosterone, C-reactive protein (CRP), etc.), markers associated with hemostasis (e.g., one or more of Fibrin monomer, D-dimer, thrombin-antithrombin complex, prothrombin fragments 1 & 2, anti-Factor Xa, etc.), markers of acute viral hepatitis infection (e.g., one or more of IgM antibody to 10 hepatitis A virus, IgM antibody to hepatitis B core antigen, hepatitis B surface antigen, antibody to hepatitis C virus, etc.), markers of Alzheimers Disease (alpha-amyloid, beta-amyloid, A β 42, A β 40, A β 38, A β 39, A β 37, A β 34, tau-protein, etc.), markers of osteoporosis (e.g., one or more of cross-linked Nor C-telopeptides, total 15 deoxypyridinoline, free deoxypyridinoline, osteocalcin, alkaline phosphatase, C-terminal propeptide of type I collagen, bone-specific alkaline phosphatase, etc.), markers of fertility state or fertility associated disorders (e.g., one or more of Estradiol, progesterone, follicle stimulating hormone (FSH), lutenizing hormone (LH), prolactin, hCG, testosterone, etc.), markers of thyroid disorders (e.g., one or more of thyroid stimulating hormone (TSH), Total T3, Free T3, Total T4, Free T4, and reverse T3), and markers of 20 prostrate cancer (e.g., one or more of total PSA, free PSA, complexed PSA, prostatic acid phosphatase, creatine kinase, etc.). Certain embodiments of invention include measuring, e.g., one or more, two or more, four or more or 10 or more analytes associated with a specific disease state or physiological condition (e.g., analytes grouped together in a panel, such as those listed above; e.g., a panel useful for the diagnosis of thyroid 25 disorders may include e.g., one or more of thyroid stimulating hormone (TSH), Total T3, Free T3, Total T4, Free T4, and reverse T3).

The methods of the present invention are designed to allow detection of a wide variety of biological and biochemical agents, as described above. In one embodiment, the methods may be used to detect pathogenic and/or potentially pathogenic virus, 30 bacteria and toxins including biological warfare agents (“BWAs”) in a variety of relevant

clinical and environmental matrices, including and without limitation, blood, sputum, stool, filters, swabs, etc. A non-limiting list of pathogens and toxins that may be analyzed (alone or in combination) using the methods of the present invention is *Bacillus anthracis* (anthrax), *Yersinia pestis* (plague), *Vibrio cholerae* (cholera), *Francisella tularensis* (tularemia), *Brucella* spp. (Brucellosis), *Coxiella burnetii* (Q fever), orthopox viruses including variola virus (smallpox), viral encephalitis, Venezuelan equine encephalitis virus (VEE), western equine encephalitis virus (WEE), eastern equine encephalitis virus (EEE), Alphavirus, viral hemorrhagic fevers, Arenaviridae, Bunyaviridae, Filoviridae, Flaviviridae, Ebola virus, staphylococcal enterotoxins, ricin, 5 botulinum toxins, *Clostridium botulinum*, mycotoxin, *Fusarium*, *Myroterium*, *Cephalosporium*, *Trichoderma*, *Verticimonosporium*, *Stachybotrys*, glanders, wheat fungus, *Bacillus globigii*, *Serratia marcescens*, yellow rain, trichothecene mycotoxins, *Salmonella typhimurium*, aflatoxin, *Xenopsylla cheopis*, *Diamanus montanus*, alastrim, monkeypox, Arenavirus, Hantavirus, Lassa fever, Argentine hemorrhagic fevers, 10 Bolivian hemorrhagic fevers, Rift Valley fever virus, Crimean-Congo virus, Hanta virus, Marburg hemorrhagic fevers, yellow fever virus, dengue fever viruses, influenza (including human and animal strains including H5N1 avian influenza), human immunodeficiency viruses I and II (HIV I and II), hepatitis A, hepatitis B, hepatitis C, hepatitis (non-A, B or C), Enterovirus, Epstein-Barr virus, Cytomegalovirus, herpes 15 simplex viruses, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, human papilloma virus, *Treponema pallidum*, *Streptococcus pneumonia*, *Haemophilus influenzae*, *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *Legionella pneumophila*, *Staphylococcus aureus*, *Moraxella catarrhalis*, *Streptococcus pyogenes*, *Clostridium difficile*, *Neisseria meningitidis*, *Klebsiella pneumoniae*, *Mycobacterium tuberculosis*, coronavirus, Coxsackie A virus, rhinovirus, parainfluenza virus, respiratory syncytial virus (RSV), metapneumovirus, and adenovirus.

20 (ii) Binding Reagents

The skilled artisan in the field of binding assays will readily appreciate the scope 25 of binding agents and companion binding partners that may be used in the present

methods. A non-limiting list of such pairs include (in either order) oligonucleotides and complements, receptor/ligand pairs, antibodies/antigens, natural or synthetic receptor/ligand pairs, amines and carbonyl compounds (i.e., binding through the formation of a Schiff's base), hapten/antibody pairs, antigen/antibody pairs, 5 epitope/antibody pairs, mimotope/antibody pairs, aptamer/target molecule pairs, hybridization partners, and intercalater/target molecule pairs.

The binding assays of the methods of the present invention may employ antibodies or other receptor proteins as binding reagents. The term "antibody" includes intact antibody molecules (including hybrid antibodies assembled by in vitro re- 10 association of antibody subunits), antibody fragments and recombinant protein constructs comprising an antigen binding domain of an antibody (as described, e.g., in Porter, R. R. and Weir, R. C. *J. Cell Physiol.*, 67 (Suppl); 51-64 (1966) and Hochman, I. Inbar, D. and Givol, D. *Biochemistry* 12: 1130 (1973)), as well as antibody constructs that have been chemically modified, e.g., by the introduction of a detectable label.

15 Binding reagents and binding partners that are linked to assay components to enable the attachment of these assay components to each other or to solid phases may be described herein as "targeting agents". For targeting agents that work in pairs, e.g., antigen-antibody, oligonucleotides-complement, etc., one targeting agent of the pair may be referred to herein as the first targeting agent, whereas the companion targeting agent 20 may be referred to as the second targeting agent. In certain embodiments, these targeting agents are selected based on the reversibility of their binding reactions. In particular, targeting agent pairs may be selected, e.g., because under a first set of conditions the pair will bind to form a binding complex which, under a second set of conditions, can be caused to dissociate to break apart the complex, e.g., by subjecting bound targeting agent 25 pairs to increased or decreased temperature, changes in chemical environment or assay buffer (e.g., ionic strength changes, pH changes, addition of denaturants, changes in light or electrical potential, etc.), adding competing binding reagents that compete with one targeting agent for binding to another targeting agent, and combinations thereof. Suitable conditions may be derived through routine experimentation. There are many well- 30 established cleavable chemical linkers that may be used that provide a covalent bond that

may be cleaved without requiring harsh conditions. For example, disulfide containing linkers may be cleaved using thiols or other reducing agents, cis-diol containing linkers may be cleaved using periodate, metal-ligand interactions (such as nickel-histidine) may be cleaved by changing pH or introducing competing ligands. The terms “cleave” or 5 “cleaving” are also used herein to refer to processes for separating linked assay components that do not require breaking covalent bonds, e.g., there are many well-established reversible binding pairs and conditions that may be employed (including those that have been identified in the art of affinity chromatography). By way of example, the binding of many antibody-ligand pairs can be reversed through changes in 10 pH, addition of protein denaturants or chaotropic agents, addition of competing ligands, etc.

The targeting agents may be pairs of oligonucleotides comprising complementary sequences. The preferred length is approximately 5 to 100 bases, preferably, approximately, 10 to 50 bases, and more preferably approximately 10 to 25 bases. In 15 addition, the targeting oligonucleotides sequences need not be identical in length and in certain embodiments it may be beneficial to provide one targeting oligonucleotide sequence that is longer than its binding partner, e.g., by up to 25 bases, or up to 15 bases, or up to 10 bases. Known methods that are commonly employed for strand separation employ i) temperatures above the melting temperature for the complex, ii) use an alkaline 20 pH of 11 (or higher) or a low pH; iii) use high ionic strength and/or iv) use nucleic acid denaturants such as formamide. Other methods for strand separation include the use of helicase enzymes such as Rep protein of *E.coli* that can catalyse the unwinding of the DNA, or binding proteins such as 32-protein of *E.coli* phage T4 that act to stabilize the single-stranded form of DNA. In specific embodiments, dissociation of complementary 25 nucleic acid strands is accomplished by exposing the strands to elevated temperature greater than 60°C.

The methods of the present invention may be used in a variety of assay devices and/or formats. The assay devices may include, e.g., assay modules, such as assay plates, cartridges, multi-well assay plates, reaction vessels, test tubes, cuvettes, flow cells, assay 30 chips, lateral flow devices, etc., having assay reagents (which may include targeting

agents or other binding reagents) added as the assay progresses or pre-loaded in the wells, chambers, or assay regions of the assay module. These devices may employ a variety of assay formats for specific binding assays, e.g., immunoassay or immunochromatographic assays. Illustrative assay devices and formats are described herein below. In certain 5 embodiments, the methods of the present invention may employ assay reagents that are stored in a dry state and the assay devices/kits may further comprise or be supplied with desiccant materials for maintaining the assay reagents in a dry state. The assay devices preloaded with the assay reagents can greatly improve the speed and reduce the complexity of assay measurements while maintaining excellent stability during storage.

10 The dried assay reagents may be any assay reagent that can be dried and then reconstituted prior to use in an assay. These include, but are not limited to, binding reagents useful in binding assays, enzymes, enzyme substrates, indicator dyes and other reactive compounds that may be used to detect an analyte of interest. The assay reagents may also include substances that are not directly involved in the mechanism of detection

15 but play an auxiliary role in an assay including, but not limited to, blocking agents, stabilizing agents, detergents, salts, pH buffers, preservatives, etc. Reagents may be present in free form or supported on solid phases including the surfaces of compartments (e.g., chambers, channels, flow cells, wells, etc.) in the assay modules or the surfaces of colloids, beads, or other particulate supports.

20 In one embodiment, assay reagents may be provided in an assay device that includes one or more regions or zones used for reagent storage. These storage zones may include the reagent bound to a surface within the storage zone, such that the reagent is confined within that zone until it is subjected to conditions sufficient to release the reagent for use elsewhere in the device. For example, the storage zone may include a

25 surface-reagent complex comprising a reagent linked to a first targeting agent and a surface linked to a second targeting agent, wherein the reagent and the surface are linked in the surface-reagent complex, via a releasable binding interaction between the first and second targeting agents. In this embodiment, the reagent is released from the surface-reagent complex and the storage zone by subjecting the storage zone to conditions

30 sufficient to disrupt the releasable binding interaction between the first and second

targeting agents. As described herein, those conditions may include but are not limited to, subjecting the storage zone to increased or decreased temperature, light, altering the pH of that zone, applying an electrical potential, changes in ionic strength, adding a competitor, and combinations thereof.

5 The surface to which the second targeting agent, and thereby, the reagent, is linked, may be any solid support that can be incorporated within or confined to the storage zone. For example, the surface may be the surface of one or more particles, as described herein, present in the storage zone. Alternatively, the surface is a surface of the storage zone, for example, a surface of a compartment, channel, conduit, well, etc., 10 within the storage zone. Preferably, the storage zone surface is roughened or includes one or more raised features or indentations that increase the relative surface area within the storage zone available to hold surface-reagent complexes. In one embodiment, the storage zone surface includes surface area-enhancing features that increase the surface area, such that the surface area accessible to a component capable of binding to that 15 surface is at least two-fold larger than the surface area of a flat surface. In a preferred embodiment, the surface area accessible for binding is at least three-fold larger than the surface area of a flat surface. The high surface area support can be provided by roughening a surface or otherwise providing three dimensional texture to a surface. A variety of established approaches for preparing roughened or textured surfaces will be 20 available to one skilled in the art. Included in these approaches is the production of surfaces with high aspect ratio features such as arrays of columns that are prepared through conventional machining, micro-machining or lithography (e.g., approaches using LIGA or other micro-fabrication technologies as described in US Patent Nos. 5,707,799 and 5,952,173) or injection molding.

25 The storage zone surface may also include a composite material comprising exposed particles distributed in a matrix. The composite material may include, but is not limited to, carbon particles, graphitic particles, or carbon nanotubes. Optionally, the composite may be etched (e.g., by chemical or plasma etching) to expose more particles and increase the surface roughness. In one specific example, the surface is provided by a 30 printed carbon ink. In another embodiment, the storage zone surface may include a

porous support that provides an enhanced surface area through the surface area available in its pores. Such porous supports include porous membranes (such as filtration membranes and lateral flow membranes) and gels. Preferred gels include hydrogels. A number of suitable hydrogels are well established as supports for reagents, as are

5 chemistries for linking reagents to hydrogels, for applications such as affinity chromatography, solid phase synthesis of biological polymers and binding assays. in applications. Examples of such hydrogels include, but are not limited to, polymers of sugars (polysaccharides), acrylic acid, acrylates, acrylamides, ethylene glycol, propylene glycol. The hydrogels may be cross-linked and/or may be co-polymers of different

10 monomer components.

An assay device that incorporates a storage zone for reagents also includes a use zone configured to use those reagents in an assay conducted in that device. Therefore, once the reagent is released from the surface-reagent complex, free reagent is available for use in an assay conducted in the use zone. Free reagent is transferred from the storage

15 zone to the use zone, wherein it can participate in an assay for an analyte of interest. That assay may involve one or more additional reagents present in the use zone or otherwise supplied to the use zone. In one embodiment, the use zone may include one or more additional reagents bound to a solid support within the use zone and/or dried on a surface of the use zone. In a specific embodiment, the reagent is a binding reagent capable of

20 binding an analyte of interest in a sample, and the use zone includes an additional reagent, bound to a solid support within the use zone, wherein that additional reagent also binds the analyte of interest. In this embodiment, the analyte present in the sample binds to the surface of the use zone via the additional reagent, as well as to the free reagent transferred from the storage zone to form a sandwich complex. The binding reagent may

25 include a detectable label, e.g., an ECL label, and the analyte may be detected in the use zone by detecting the presence or absence of the label, e.g., via measuring electrochemiluminescence emitted in the use zone. The sample may be introduced to the use zone directly or the sample is first introduced to the storage zone and thereafter, the sample flows from the storage zone to the use zone. The reagent may be released prior to

30 contacting the storage zone with sample or after the storage zone is contacted with

sample. In one embodiment, sample is introduced to the storage zone, which is then subjected to conditions required for release of the reagent from the surface-reagent complex. Thereafter, the sample and the free reagent are optionally incubated prior to transferring the sample-reagent mixture to the use zone.

5 In a preferred embodiment, the storage zone and the use zone are in fluidic communication along a fluid path. For example, the assay device may be a cartridge and the storage zone and the use zone are positioned within the cartridge along a fluid path. Examples of this embodiment are shown in Fig. 7(a)-(c). In Fig. 7(a), the assay device includes a storage zone and at least two use zones and each of the storage zones and use 10 zones are in fluid communication. The use zones may be configured in the assay device in series, as shown in Fig. 7(a) or in parallel, as shown in Fig. 7(b). Fig. 7(c) shows yet another configuration of an assay device including multiple storage and use zones. In the embodiment shown in Fig. 7(c), the storage and use zones are also in fluidic communication with sample and/or reagent compartments within the assay device.

15 Another embodiment is shown in Fig. 8. The assay device of Fig. 8 includes a storage zone including a first surface-reagent complex and a second surface-reagent complex and at least two use zones, wherein the storage zone and the use zone are in fluidic communication via a fluidic network. Sample is introduced into a compartment of the device in panel (a) and the fluidic network carries that sample to the use zones, as 20 shown in panel (b). Panels (b) and (c) also shows that diluents can be passed through the storage zone (under conditions that do not release the surface-reagent complexes) and carried through the fluidic network to the use zones to provide an optional wash of the use zones. Diluent is then passed through the storage zone while subjecting the storage zone to a condition that releases the first reagent, which is then carried to the fluidic 25 network in to use zone 1, as shown in panel (d). The second reagent is then released by a second set of conditions and carried, via flow of diluents through the microfluidic network, to use zone 2, as shown in panel (e). Finally, the use zones are optionally washed to remove excess reagent, as shown in panel (f).

In one embodiment, the storage zone and use zones are included within a fluidic 30 network further comprising one or more vent ports in fluidic communication with the

storage and use zones (directly or through vent conduits) so as to allow the equilibration of fluid in the zones with the atmosphere or to allow for the directed movement of fluid into or out of a specified zone by selectively sealing, opening (to atmospheric pressure) or applying positive or negative pressure to specific vent ports.

5 In another embodiment, the assay device is a multi-well assay plate and the use zone is positioned within a well of the plate, while the storage zone is located on a supplemental surface of the well that does not overlap with the use zone.

In a further embodiment, the assay device may include one or more surface-reagent complexes in the storage zone. In the embodiment depicted in Figure 8, for 10 example, the storage zone includes a first surface-reagent complex (as described above) and also includes a second surface-reagent complex confined to the storage zone, the second surface-reagent complex including (i) a second reagent linked to a third targeting agent; and (ii) a second surface linked to a fourth targeting agent, wherein the second reagent and the second surface are linked, in the second surface-reagent complex, via a 15 second releasable binding interaction between the third and fourth targeting agents; and the use zone is further configured to use the second reagent in the assay. The various reagents stored within the storage zone may be used in one or more assays conducted in the use zone, or each of the reagents stored within the storage zone may be used in each of the assays conducted in the use zone. The reagents stored within the storage zone may 20 be selectively released, i.e., one of the reagents may be released from the surface-reagent complex composition by a first set of conditions that differ from a second set of conditions used to release another reagent stored in the storage zone.

Additionally, the use zone may include two or more assay regions each 25 configured to use the reagents stored within the storage zone in one or more assays conducted with a single sample in the device. In one embodiment, the use zone includes a first assay region configured to conduct an assay for a first analyte of interest in a sample and the use zone may also include an additional assay region configured to conduct an assay for an additional analyte of interest that may also be present in the sample. Alternatively, the first assay region in the use zone may be used to conduct a 30 first assay for an analyte, while another assay region in the use zone may be used to

conduct a second assay for the same analyte. Still further, the assay device may include a plurality of use zones each configured to use the reagents stored within the storage zone in one or more assays conducted with a single sample in the device. Each use zone may include one or more assay regions as described above. Moreover, the assay device may 5 include a plurality of storage zones, e.g., for each use zone there is a corresponding storage zone. Various configurations of an assay device including multiple use zones and/or storage zones are shown in Fig. 7(a)-(c) and Fig. 8.

As described above, a storage zone may include a plurality of different reagents as surface-reagent complexes. In one embodiment different reagents are held in the storage 10 zone by different releasable binding reactions that are cleaved under different conditions. Therefore, by subjecting each defined region of the storage zone to the appropriate conditions, each reagent is selectively released from the storage zone. The different reagents may be in surface-reagent complexes that are inter-mixed or held in distinct regions of the storage zone. As described herein, those conditions may include but are not 15 limited to, subjecting the region to increased or decreased temperature, light, altering the pH of that region, changing the ionic strength, applying an electrical potential, adding a competitor, and combinations thereof. By using binding reactions cleaved under different conditions, it is possible to selectively release one reagent at a time from surface-reagent complexes in the storage zone. For example, one reagent may be 20 selectively released by heating while another may be selectively released by changing pH or one reagent may be selectively released using a first competitor while another may be selectively released using a second competitor. In another embodiment, different reagents may be released one at a time using different releasable binding reactions that require increasingly stringent cleavage conditions (such as increasing temperature, 25 increasing or decreasing pH, increasing competitor concentration, increasing levels of light, increasing or decreasing ionic strength, etc.). For example, a first reagent may be released at a first temperature level and a second reagent may be subsequently released at a second higher temperature level.

In another embodiment, the storage zone may include a plurality of defined 30 spatial regions, at least two of the different regions holding different reagents in surface-

reagent complexes that hold the reagents through releasable binding interactions as described above. In this embodiment, cleavage of a reagent in a specific spatial region can be carried out by applying cleavage conditions (such as applying light, temperature, electrical potential, etc.) in a manner that confines the cleavage condition to the specific 5 spatial region of interest. In this embodiment, releasable binding interactions used for holding different reagents can be the same or different, because release of individual reagents can be directed by application of the cleavage condition to defined region. In a preferred embodiment, the device is configured for a multiplexed assay measurement and the device includes (a) a storage zone comprising a surface-reagent complex confined to 10 the storage zone, the surface-reagent complex including (i) a reagent linked to a first targeting agent; and (ii) a surface linked to a second targeting agent, wherein the reagent and the surface are linked, in the surface-reagent complex, via a releasable binding interaction between the first and second targeting agents; and (b) a use zone comprising a plurality of assay regions configured to use the reagent in a multiplexed assay for a 15 plurality of analytes in a sample. The storage zone may further comprise a second surface-reagent complex confined to the storage zone, the second surface-reagent complex including (iii) a second reagent linked to a third targeting agent; and (iv) a second surface linked to a fourth targeting agent, wherein the second reagent and the second surface are linked, in the second surface-reagent complex, via a second releasable 20 binding interaction between the third and fourth targeting agents; and the use zone is further configured to use the second reagent in the multiplexed assay. In this regard, the use zone comprises two or more assay regions each configured to use the reagent and the second reagent in one or more assays conducted with the sample in the assay device, and this configuration of assay device enables the conduct of a plurality of assays in the use 25 zone with the reagent and optionally, a second reagent.

The use zone may include a first assay region configured to conduct an assay for a first analyte of interest in the sample and an additional assay region configured to conduct an assay for an additional analyte of interest in the sample, and an assay in such a device comprises the following steps:

30 (x) introducing the sample to the use zone via the storage zone;

- (y) introducing a diluent to the storage zone;
- (z) subjecting the storage zone to a condition that releases the reagent from the surface-reagent complex;
- (xx) transferring the reagent from the storage zone to the first assay region and 5 the second assay region; and
- (yy) conducting the assays in the first and second assay regions, respectively.

The conducting step of each assay may be performed simultaneously or sequentially.

Alternatively, an assay method may include an incubation step between the 10 sample and free reagent before the mixture of sample and free reagent is introduced to the use zone. Such a method would include the following steps:

- (x) introducing the sample to the storage zone;
- (y) subjecting the storage zone to a condition that releases the reagent from the surface-reagent complex, and optionally incubated the sample with the free reagent in 15 the storage zone;
- (z) transferring the mixture formed in (y) from the storage zone to the first assay region and the second assay region; and
- (xx) conducting the assays in the first and second assay regions, respectively.

Still further, the use zone may include a first assay region configured to conduct 20 an assay for a first analyte of interest in the sample and an additional assay region in the use zone configured to conduct an assay for an additional analyte of interest in the sample, and an assay in such a device may comprise:

- (x) introducing the sample to the use zone via the storage zone;
- (y) introducing a diluent to the storage zone and
- 25 i) subjecting the storage zone to a condition that releases the reagent from the surface-reagent complex;
- ii) subjecting the storage zone to an additional condition that releases a second reagent from a second surface-reagent complex;
- (z) transferring the reagent and the second reagent from the storage zone to 30 the first and second assay regions; and

(xx) conducting the assays in the first and second assay regions.

The conducting step of each assay may be performed simultaneously or sequentially. Likewise, the transfer of the reagent and the second reagent may be done simultaneously or sequentially.

5 Alternatively, an assay method using a device that includes a first assay region configured to conduct an assay for a first analyte of interest in the sample and an additional assay region in the use zone configured to conduct an assay for an additional analyte of interest in the sample may also include an incubation step, i.e.,

(x) introducing the sample to the storage zone;

10 i) subjecting the storage zone to a condition that releases the reagent from the surface-reagent complex;

ii) subjecting the storage zone to an additional condition that releases a second reagent from a second surface-reagent complex;

15 (iii) incubating the storage zone with the free reagent and free second reagent formed in steps (x)(i) and (x)(ii);

(y) transferring the mixture formed in step (x)(iii) from the storage zone to the first and second assay regions; and

(z) conducting the assays in the first and second assay regions.

In one specific embodiment, the assay device is a cartridge, such as that described 20 in copending application serial no., 61/284,276, filed December 16, 2009, the disclosure of which is incorporated herein by reference. As shown, e.g., in Fig. 9 of USSN 61/284,276, a cartridge may include various compartments, i.e., a sample chamber, an assay reagent chamber, waste chambers, and detection chambers, as well as a fluidic network that connects various compartments and/or fluid ports/vents. The storage zone 25 may be incorporated within, e.g., a reagent chamber, and likewise, the use zone may be included within, e.g., the detection chamber. Additionally or alternatively, an additional storage chamber may be incorporated within the cartridge described therein.

In another specific embodiment, the assay device is a multi-well assay plate, such as that described in co-pending application serial no. 11/642,970, filed December 21, 30 2006, the disclosure of which is incorporated herein by reference. The assay plate may

include a plate body with a plurality of wells defined therein, wherein the plurality of wells includes a binding surface having a capture reagent immobilized therein, and an additional reagent located on a surface of the plate or well that does not overlap with the binding surface. In one embodiment, the additional reagent is located on a reagent 5 storage shelf positioned on a wall of a well. Alternatively, an assay plate may include assay wells that are connected to dedicated reagent spaces located in the regions between the assay wells. In such an embodiment, a reagent space may be in fluidic communication with the surrounding wells via e.g., a notch. In addition, suitable assay plates are described in U.S. Patent Application Serial No. 11/642970, the disclosure of 10 which is incorporated herein by reference.

(iii) Solid Phases

A wide variety of solid phases are suitable for use in the methods of the present invention including conventional solid phases from the art of binding assays. Solid 15 phases may be made from a variety of different materials including polymers (e.g., polystyrene and polypropylene), ceramics, glass, composite materials (e.g., carbon-polymer composites such as carbon-based inks). Suitable solid phases include the surfaces of macroscopic objects such as an interior surface of an assay container (e.g., test tubes, cuvettes, flow cells, cartridges, wells in a multi-well plate, etc.), slides, assay 20 chips (such as those used in gene or protein chip measurements), pins or probes, beads, filtration media, lateral flow media (for example, filtration membranes used in lateral flow test strips), etc.

Suitable solid phases also include particles (including but not limited to colloids or beads) commonly used in other types of particle-based assays e.g., magnetic, 25 polypropylene, and latex particles, materials typically used in solid-phase synthesis e.g., polystyrene and polyacrylamide particles, and materials typically used in chromatographic applications e.g., silica, alumina, polyacrylamide, polystyrene. The materials may also be a fiber such as a carbon fibril. Microparticles may be inanimate or alternatively, may include animate biological entities such as cells, viruses, bacterium and 30 the like.

The particles used in the present method may be comprised of any material suitable for attachment to one or more binding partners and/or labels, and that may be collected via, e.g., centrifugation, gravity, filtration or magnetic collection. A wide variety of different types of particles that may be attached to binding reagents are sold 5 commercially for use in binding assays. These include non-magnetic particles as well as particles comprising magnetizable materials which allow the particles to be collected with a magnetic field. In one embodiment, the particles are comprised of a conductive and/or semiconductive material, e.g., colloidal gold particles.

The microparticles may have a wide variety of sizes and shapes. By way of 10 example and not limitation, microparticles may be between 5 nanometers and 100 micrometers. Preferably microparticles have sizes between 20 nm and 10 micrometers. The particles may be spherical, oblong, rod-like, etc., or they may be irregular in shape.

The particles used in the present method may be coded to allow for the 15 identification of specific particles or subpopulations of particles in a mixture of particles. The use of such coded particles has been used to enable multiplexing of assays employing particles as solid phase supports for binding assays. In one approach, particles are manufactured to include one or more fluorescent dyes and specific populations of particles are identified based on the intensity and/or relative intensity of fluorescence 20 emissions at one or more wave lengths. This approach has been used in the Luminex xMAP systems (see, e.g., US Patent No. 6,939,720) and the Becton Dickinson Cytometric Bead Array systems. Alternatively, particles may be coded through differences in other physical properties such as size, shape, imbedded optical patterns and the like.

In certain embodiments of assays of the invention, particles may have a dual role 25 as both i) a solid phase support used in an analyte concentration, collection and/or separation step and ii) as a detectable label or platform for detectable labels in a measurement step. In one example, a method of conducting a binding assay may comprise contacting a sample comprising an analyte with a particle linked to a first binding reagent that binds that analyte to form a complex comprising the analyte bound 30 to the first binding reagent. The complex is then collected by collection of the particle

(via magnetic collection, centrifugation, gravity sedimentation, etc.) and some or all of the unbound components of the sample are separated from the complex by removing some or all of the sample volume and, optionally, washing the collected particles. The complex is then released by resuspending the particles in the original or a new liquid media. The complex on the particle is then contacted with a second binding reagent bound to a solid phase, the second binding reagent binding the complex so as to bring the complex and particle to a surface of the solid phase. The amount of analyte in the sample is measured by measuring the amount of analyte bound to the solid phase, which in turn is measured by measuring the amount of particles bound to the solid phase (either by directly measuring the particles or by measuring detectable labels in or on the particles by, e.g., the measurement approaches described below).

The invention also includes assay methods that employ magnetic particles as detectable labels or as platforms for detectable labels in a binding assay. Advantageously, when using magnetic particles as a label or a label platform, a magnetic field can be applied to speed the kinetics for the binding of i) assay components linked to a magnetic particle to ii) binding reagents immobilized on a solid phase.

Accordingly, one embodiment is a method for conducting a binding assay comprising

- (a) contacting (i) a sample comprising a target analyte with (ii) a magnetic particle linked to a first binding reagent that binds the target analyte and thereby forms a complex comprising the target analyte bound to the first binding reagent;
- (b) contacting a solution comprising the complex with a second binding reagent bound to a solid phase, wherein the second binding reagent binds to the complex;
- (c) applying a magnetic field to concentrate the particles near to the solid phase and thereby accelerating the rate of binding between the complex and the second binding reagent and
- (d) measuring the amount of the analyte bound to the solid phase.

Optionally, such a method may also include, prior to step (b), collection and release steps as described elsewhere in this application so as to pre-concentrate the analyte and/or remove interferents from the sample. The magnetic particles used in such

method are, preferably, between 10 nm and 10 μ m in diameter, more preferably between 50 nm and 1 μ m. The step of applying a magnetic field may be achieved through the use of permanent or electromagnets, e.g., by placing the magnet on the opposite side of the solid phase relative to the second binding reagent. Optionally, the magnet or magnetic

5 field is translated and/or rotated along the solid phase so as to move the particles along the binding surface and allow the particles to interrogate the surface for available binding sites. Alternatively, or in conjunction with movement of the magnet/field, the magnetic field is intermittently removed and, while the magnetic field is removed, the particles are resuspended (e.g., by mixing) and then reconcentrated on the solid phase (thereby,

10 allowing for allowing the particles to change rotational orientation on the surface and allowing them to interrogate additional areas on the surface. The method may also include a washing step, prior to the measuring step, to remove unbound particles. During such a washing step, the magnetic field is removed to allow for non-bound particles to be washed away. Alternatively, a magnetic field above the surface can be used to pull

15 unbound particles away from the surface. The magnetic reaction acceleration approach may also be applied to multiplexed assay methods, as described elsewhere in this application, e.g., the solid phase may include an array of a plurality of different second binding reagents for use in array-based multiplexed measurements.

20 (iv) Collection and Release

Collection, as used herein, refers to the physical localization of a material in a mixture. Collection includes the localization of a material through binding reactions or adsorption. For example, a material in a mixture may be collected on a solid phase by adsorption of the material on the solid phase or by binding of the material to binding reagents on the solid phase. Collection is not, however, limited to localization at a solid phase and may also include techniques in the art for localizing materials at a location/volume within a larger fluid volume, for example, localization of materials through the use of optical tweezers (which use light to manipulate microscopic objects as small as a single atom, wherein the radiation pressure from a focused laser beam is able

to trap small particles), electric or magnetic fields, focused flow, density gradient centrifugation, etc.

Certain embodiments of the invention include the collection of microparticles or materials that are bound to microparticles. Suitable collection methods include the many methods known in the art of microparticle-based assays that achieve localization of microparticles from a suspension. These include sedimentation under gravity or by centrifugation, filtration onto a filter or porous membrane, localization (of magnetizable particles) by application of a magnetic field, binding or adsorption of the particles to a macroscopic solid phase, use of optical tweezers, etc.

Release, as used herein, refers to delocalization of a previously collected material. Materials that are held at a localized position through chemical bonds or through specific or non-specific binding interactions may be allowed to delocalize by breaking the bond or interaction so that the materials may diffuse or mix into the surrounding media. There are many well-established cleavable chemical linkers that may be used that provide a covalent bond that may be cleaved without requiring harsh conditions. For example, disulfide containing linkers may be cleaved using thiols or other reducing agents, cis-diol containing linkers may be cleaved using periodate, metal-ligand interactions (such as nickel-histidine) may be cleaved by changing pH or introducing competing ligands. Similarly, there are many well-established reversible binding pairs that may be employed (including those that have been identified in the art of affinity chromatography). By way of example, the binding of many antibody-ligand pairs can be reversed through changes in pH, addition of protein denaturants or chaotropic agents, addition of competing ligands, etc. Other suitable reversible binding pairs include complementary nucleic acid sequences, the hybridization of which may be reversed under a variety of conditions including changing pH, increasing salt concentration, increasing temperature above the melting temperature for the pair and/or adding nucleic acid denaturants (such as formamide). Such reversible binding pairs may be used as targeting agents (as described above), e.g., a first targeting agent may be linked to a first binding reagent that binds an analyte, a second targeting agent may be linked to a solid phase, and a binding interaction

of the first and second targeting agents may be used to reversibly immobilize the first binding reagent on the solid phase.

Release also includes physical delocalization of materials by, for example, mixing, shaking, vortexing, convective fluid flow, mixing by application of magnetic, 5 electrical or optical forces and the like. Where microparticles or materials bound to microparticles have been collected, such physical methods may be used to resuspend the particles in a surrounding matrix. Release may simply be the reverse of a previous collection step (e.g., by any of the mechanisms described above) or collection and release could proceed by two different mechanisms. In one such example, collection of materials 10 (such as an analyte or a complex comprising an analyte) bound to a particle can be achieved by physical collection of the particle. The materials are then released by cleaving a bond or reversing a binding reaction holding the material on the particle. In a second such example, materials (such as an analyte of a complex comprising an analyte are collected on a surface through a binding interaction with a binding reagent that is 15 linked to the surface. The material is then released by breaking a bond or a second binding interaction linking the binding reagent to the surface.

Collection followed by release may be used to concentrate and/or purify analytes in a sample. By collecting in a first volume and releasing into a second smaller volume, an analyte in a sample may be concentrated. Through concentration, it is often possible 20 to significantly improve the sensitivity of a subsequent measurement step. By collecting from a sample and removing some or all of the uncollected sample, potential assay interferents in the sample may be reduced or eliminated. Optionally, removal of the unbound sample may include washing a collected material with and releasing the collected material into defined liquid reagents (e.g., assay or wash buffers) so as to 25 provide a uniform matrix for subsequent assay steps.

(iv) Measurement Methods

The methods of the invention can be used with a variety of methods for measuring the amount of an analyte and, in particular, measuring the amount of an analyte bound to 30 a solid phase. Techniques that may be used include, but are not limited to, techniques

known in the art such as cell culture-based assays, binding assays (including agglutination tests, immunoassays, nucleic acid hybridization assays, etc.), enzymatic assays, colorometric assays, etc. Other suitable techniques will be readily apparent to one of average skill in the art. Some measurement techniques allow for measurements to be 5 made by visual inspection, others may require or benefit from the use of an instrument to conduct the measurement.

Methods for measuring the amount of an analyte include label free techniques, which include but are not limited to i) techniques that measure changes in mass or refractive index at a surface after binding of an analyte to a surface (e.g., surface acoustic 10 wave techniques, surface plasmon resonance sensors, ellipsometric techniques, etc.), ii) mass spectrometric techniques (including techniques like MALDI, SELDI, etc. that can measure analytes on a surface), iii) chromatographic or electrophoretic techniques, iv) fluorescence techniques (which may be based on the inherent fluorescence of an analyte), etc.

15 Methods for measuring the amount of an analyte also include techniques that measure analytes through the detection of labels which may be attached directly or indirectly (e.g., through the use of labeled binding partners of an analyte) to an analyte. Suitable labels include labels that can be directly visualized (e.g., particles that may be seen visually and labels that generate an measurable signal such as light scattering, 20 optical absorbance, fluorescence, chemiluminescence, electrochemiluminescence, radioactivity, magnetic fields, etc). Labels that may be used also include enzymes or other chemically reactive species that have a chemical activity that leads to a measurable signal such as light scattering, absorbance, fluorescence, etc. The use of enzymes as labels has been well established in in Enzyme-Linked ImmunoSorbent Assays, also 25 called ELISAs, Enzyme ImmunoAssays or EIAs. In the ELISA format, an unknown amount of antigen is affixed to a surface and then a specific antibody is washed over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substance is added that the enzyme converts to a product that provides a change in a detectable signal. The formation of product may be detectable, e.g., due a 30 difference, relative to the substrate, in a measurable property such as absorbance,

fluorescence, chemiluminescence, light scattering, etc. Certain (but not all) measurement methods that may be used with solid phase binding methods according to the invention may benefit from or require a wash step to remove unbound components (e.g., labels) from the solid phase. Accordingly, the methods of the invention may comprise such a 5 wash step.

In one embodiment, an analyte(s) of interest in the sample may be measured using electrochemiluminescence-based assay formats, e.g. electrochemiluminescence (ECL) based immunoassays. The high sensitivity, broad dynamic range and selectivity of ECL are important factors for medical diagnostics. Commercially available ECL instruments 10 have demonstrated exceptional performance and they have become widely used for reasons including their excellent sensitivity, dynamic range, precision, and tolerance of complex sample matrices. Species that can be induced to emit ECL (ECL-active species) have been used as ECL labels, e.g., i) organometallic compounds where the metal is from, for example, the noble metals of group VIII, including Ru-containing and Os-containing 15 organometallic compounds such as the tris-bipyridyl-ruthenium (RuBpy) moiety and ii) luminol and related compounds. Species that participate with the ECL label in the ECL process are referred to herein as ECL coreactants. Commonly used coreactants include tertiary amines (e.g., see U.S. Patent No. 5,846,485), oxalate, and persulfate for ECL from RuBpy and hydrogen peroxide for ECL from luminol (see, e.g., 20 U.S. Patent No. 5,240,863). The light generated by ECL labels can be used as a reporter signal in diagnostic procedures (Bard et al., U.S. Patent No. 5,238,808, herein incorporated by reference). For instance, an ECL label can be covalently coupled to a binding agent such as an antibody, nucleic acid probe, receptor or ligand; the participation of the binding reagent in a binding interaction can be monitored by 25 measuring ECL emitted from the ECL label. Alternatively, the ECL signal from an ECL-active compound may be indicative of the chemical environment (see, e.g., U.S. Patent No. 5,641,623 which describes ECL assays that monitor the formation or destruction of ECL coreactants). For more background on ECL, ECL labels, ECL assays and instrumentation for conducting ECL assays see U.S. Patents Nos. 5,093,268; 5,147,806; 30 5,324,457; 5,591,581; 5,597,910; 5,641,623; 5,643,713; 5,679,519; 5,705,402; 5,846,485;

5,866,434; 5,786,141; 5,731,147; 6,066,448; 6,136,268; 5,776,672; 5,308,754; 5,240,863; 6,207,369; 6,214,552 and 5,589,136 and Published PCT Nos. WO 99/63347; WO 00/03233; WO 99/58962; WO 99/32662; WO 99/14599; WO 98/12539; WO 97/36931 and WO 98/57154, all of which are incorporated herein by reference.

5 The capture/collection and release methods of the invention may be applied to singleplex or multiplex formats where multiple assay measurements are performed on a single sample. Multiplex measurements that can be used with the invention include, but are not limited to, multiplex measurements i) that involve the use of multiple sensors; ii) that use discrete assay domains on a surface (e.g., an array) that are distinguishable based 10 on location on the surface; iii) that involve the use of reagents coated on particles that are distinguishable based on a particle property such as size, shape, color, etc.; iv) that produce assay signals that are distinguishable based on optical properties (e.g., absorbance or emission spectrum) or v) that are based on temporal properties of assay signal (e.g., time, frequency or phase of a signal).

15

(v) Assay Formats

One embodiment of the present invention employs a specific binding assay, e.g., an immunoassay, immunochromatographic assay or other assay that uses a binding reagent. The immunoassay or specific binding assay according to one embodiment of the 20 invention can involve a number of formats available in the art. The antibodies and/or specific binding partners can be labeled with a label or immobilized on a surface. Thus, in one embodiment, the detection method is a binding assay, e.g., an immunoassay, receptor-ligand binding assay or hybridization assay, and the detection is performed by contacting an assay composition with one or more detection molecules capable of 25 specifically binding with an analyte(s) of interest in the sample.

In one embodiment, the assay uses a direct binding assay format. An analyte is bound to a binding partner of the analyte, which may be immobilized on a solid phase. The bound analyte is measured by direct detection of the analyte or through a label attached to the analyte (e.g., by the measurements described above).

In one embodiment, the assay uses a sandwich or competitive binding assay format. Examples of sandwich immunoassays performed on test strips are described in U.S. Pat. No. 4,168,146 to Grubb et al. and U.S. Pat. No. 4,366,241 to Tom et al., both of which are incorporated herein by reference. Examples of competitive immunoassay 5 devices suitable for use with the present methods include those disclosed in U.S. Pat. No. 4,235,601 to Deutsch et al., U.S. Pat. No. 4,442,204 to Liotta, and U.S. Pat. No. 5,208,535 to Buechler et al., all of which are incorporated herein by reference.

In a sandwich assay, analyte in the sample is bound to a first binding reagent and a second labeled binding reagent and the formation of this “sandwich” complex is 10 measured. In a solid phase sandwich assay, the first binding reagent is immobilized on a solid phase and the amount of labeled antibody on the solid phase, due to formation of the sandwich complex, is then measured. The signal generated in a sandwich assay will generally have a positive correlation with the concentration of the analyte. Various configurations of sandwich assays that use the methods of the present invention are 15 shown in Figs. 1-4. In one embodiment, e.g., in Fig. 1(a), the assay includes contacting a sample comprising a target analyte with a particle or solid phase linked to a first binding reagent that binds the target analyte, thereby forming a complex comprising the target analyte bound to the first binding reagent. The complex is collected, separated and released, as described herein, and then a sandwich is formed by contacting the complex 20 with an additional binding reagent (e.g., a second binding reagent). As shown in Fig. 1(a) and Fig. 1(b), the particle or solid phase may or may not be cleaved from the complex prior to contacting the complex with an additional binding reagent.

In a competitive assay, unlabelled analyte in the test sample is measured by its 25 ability to compete with labeled or immobilized analyte. In the example of competitive assays employing labeled analytes, the unlabeled analyte in a sample blocks the ability of the labeled analyte to bind a binding reagent by occupying the binding site. Thus, in a competitive assay, the signal generated has an inverse correlation with the concentration of analyte in a sample. Figs. 6(a) and 6(b) show the use of the methods of the present invention in a two step competitive format. As in Fig. 1(a), the analyte of interest in the 30 sample is pre-concentrated. Labeled analyte bound to a solid support is incubated with

the pre-concentrated analyte complex. Figs. 6(a) and 6(b) serve to illustrate how the methods of the present invention may be used in a competitive assay format. The skilled artisan will understand that alternate configurations of a competitive immunoassay may be achieved using the methods of the present invention without undue experimentation.

5

(vi) Specific Embodiments

In one embodiment, a method is provided for conducting a binding assay comprising contacting a sample comprising a target analyte, A, and which may also contain various sample contaminants as shown in Fig. 1(a), with a particle linked to a 10 first binding reagent that binds the target analyte and thereby forms a complex comprising the target analyte bound to the first binding reagent. Once the sample is mixed with the particle to form the complex, the complex is collected. This collection step may involve accumulation of the complex at a surface, e.g., by centrifugation of the particles, allowing the particles to rise or settle under gravity, filtering the particles onto a 15 filtration media, magnetically collecting the particles (in the case of magnetic particles), etc. Alternatively, the collection step may involve accumulation of the complex within a defined volume within the sample, e.g., by holding the particles in this defined volume through the use of optical tweezers or focused flow. Optionally, the unbound components of the sample are then separated from the complex, e.g., by removing all or 20 part of the non-collected components and/or by washing the collected complex with an additional assay medium or wash buffer. Thereafter, the complex is released, e.g., resuspended into the assay medium, and the complex is contacted with a second binding reagent bound to a solid phase, wherein the second binding reagent binds to the complex. The amount of analyte is detected by measuring the amount of a detectable label linked to 25 an assay component bound to the solid phase. The detectable label may be linked to the first binding reagent, an optional third binding reagent, if one is used in the assay format, the particle or an additional assay component that is comprised within or bound to the complex.

A variety of approaches are provided for conducting the collection and release 30 steps described above and for providing the labeled reagent. Fig 1(a) shows a method

with the following steps: (i) a first binding reagent linked to a particle binds to the analyte to form a complex, (ii and iii) the complex is collected and released by collection and resuspension of the particle during which steps the analyte may be concentrated and/or separated from contaminants in the sample, (iv) the complex binds to a second binding 5 reagent on a solid phase and (v) the complex is contacted with a labeled third binding reagent that binds the analyte in the complex such that it can be detected. Fig 1(b) shows a method similar to the one in Fig 1(a), except that the complex is released in step (iii) by cleaving the first binding reagent from the particle instead of simply resuspending the particle. Figs 1(c) and 1(d) show methods similar to the one in Fig 1(a) except that 10 that the label is attached to (or incorporated within) the particle (Fig 1(c)) or attached to the first binding reagent (Fig 1(d)) and the step of contacted the complex with a labeled third binding reagent is omitted. Alternatively, if the particle is measured directly (e.g., by direct visual observation of the particle), the label may be omitted. Fig 1(e) shows a method similar to the one in Fig 1(b) except that the label is attached to the first binding 15 reagent and the step of contacting the complex with a labeled third binding reagent is omitted.

The measuring step may comprise any suitable method of measuring the presence of a detectable label in a sample (see the Measurement Methods section), e.g., optical absorbance, fluorescence, phosphorescence, chemiluminescence, light scattering or 20 magnetism. In one embodiment, the detectable label is an electrochemiluminescent label and the measuring step comprises measuring an ECL signal and correlating that signal with an amount of analyte in the sample. Thus, the measuring step may further comprise contacting the complex with an electrode and applying a voltage waveform to the electrode to generate ECL.

25 The methods described in Figs 1(a)-1(e) may be applied to multiplex measurements for multiple analytes in a sample. In such methods, the first, second and third binding reagents (if present) may be selected to bind multiple analytes (e.g., the use of poly-dT as a binding reagent to capture multiple mRNAs in a sample through the common poly-dA tail sequence) or, alternatively, the methods may employ a plurality of 30 different first binding reagents, second binding reagents and/or third binding reagents to

bind to the multiple analytes. To allow for independent measurement of different analytes, such multiplex methods employs at least one of the group consisting of i) a plurality of different first binding reagents, ii) a plurality of second binding reagents and iii) a plurality of third binding reagents (the different reagents within (i), (ii) or (iii) being selected for their ability to preferentially bind a target analyte relative to other target analytes). Where a plurality of first binding reagents are used, individual particles may be attached to mixtures of the different first binding reagents or, alternatively, the particles may be prepared so that individual particles are attached to only one type of first binding reagent (e.g., such that an individual particle preferentially binds one of the target analytes relative to other target analytes).

The multiplex methods may use a variety of approaches for independently measuring different analytes. In one embodiment, a plurality of labeled binding reagents with different preferences for target analytes may be used (e.g., a plurality of different labeled third binding reagents as in Figs 1(a) and 1(b), a plurality of different labeled first binding reagents as in Fig 1(e) or a plurality of different labeled first binding reagent-particle conjugates as in Figs 1(c) and 1(d)). The labels on the different labeled reagents (or, alternatively, the particles in the particle conjugates) are selected to provide distinguishable assay signals such that the different labeled reagents and, therefore, the different target analytes, can be measured independently. In another embodiment, a plurality of second binding reagents with different preferences for target analytes may be used. The different second binding reagents may be patterned into different discrete binding domains on one or more solid phases (e.g., as in a binding array) such that assay signals generated on the different binding domains and, therefore, the different analytes, can be measured independently (e.g., by independently addressing binding domains on electrode arrays or by independently measuring light emitted from different binding domains in a luminescence assay). Alternatively, the different second binding reagents may be coupled to different coded beads (as described in the Solid Phases section) to allow for the different analytes to be measured independently.

In an alternative embodiment, a method of conducting a binding assay is provided as shown in Figs. 2(a)-2(b), which comprises contacting a sample comprising a target

analyte with a first solid phase, S, linked to a first binding reagent that binds the target analyte and forms a complex comprising the target analyte bound to the first binding reagent. Once the sample is contacted with the first solid phase, the unbound components of the sample are separated from the complex, the complex is released from the solid

5 phase into the assay medium and the first solid phase is removed from the first binding reagent. Thereafter, the released complex is contacted with a second solid phase comprising a second binding reagent that binds to the complex, and the amount of analyte bound to the second solid phase is quantified. The detectable label may be linked to the first binding reagent, an optional third binding reagent, if one is used in the assay format,

10 the particle or an additional assay component that is comprised within or bound to the complex. In Fig. 2(a), the label is attached to a third binding reagent (and the method includes the step of contacting the complex with the third binding reagent), whereas the label is attached to the first binding reagent in Fig. 2(b).

As described for Fig 1, the methods described in Fig 2 may also be extended to

15 multiplex measurements, e.g., by employing at least one of the group consisting of i) a plurality of different first binding reagents, ii) a plurality of second binding reagents and iii) a plurality of third binding reagents (the different reagents within (i), (ii) or (iii) being selected for their ability to preferentially bind a target analyte relative to other target analytes).

20 The invention also provides a method of conducting a multiplexed binding assay for a plurality of analytes that includes contacting (i) a sample with (ii) one or more first solid phases linked to one or more first binding reagents that bind the analytes to form complexes comprising the analytes bound to the first binding reagents. The unbound components of the sample are, optionally, separated from the complexes. The complexes

25 are released and then contacted with a plurality of binding domains comprising second binding reagents that bind to the complexes, wherein each binding domain comprises a second binding reagent that binds to a complex comprising a secondary target analyte. Thereafter, the amount of analyte bound to the binding domains is measured.

According to another embodiment, a multiplexed assay may comprise the acts of

30 contacting at least a portion of a sample with one or more binding surfaces comprising a

plurality of binding domains, immobilizing one or more analytes on the domains and measuring the analytes immobilized on the domains. In certain embodiments, at least two of the binding domains differ in their specificity for analytes of interest. In one example of such an embodiment, the binding domains are prepared by immobilizing, on one or 5 more surfaces, discrete domains of binding reagents that bind analytes of interest.

Optionally, the sample is exposed to a binding surface that comprises an array of binding reagents. Optionally, the surface(s) may define, in part, one or more boundaries of a container (e.g., a flow cell, well, cuvette, etc.) which holds the sample or through which the sample is passed. The method may also comprise generating assay signals that are 10 indicative of the amount of the analytes in the different binding domains, e.g., changes in optical absorbance, changes in fluorescence, the generation of chemiluminescence or electrochemiluminescence, changes in reflectivity, refractive index or light scattering, the accumulation or release of detectable labels from the domains, oxidation or reduction or redox species, electrical currents or potentials, changes in magnetic fields, etc.

15 Assays of certain embodiments of the invention may employ targeting agents to link the target analyte with a binding reagent in the assay medium. Such assay formats are illustrated in Figs. 3(a)-3(e) and Figs. 4(a)-4(b), which are analogous to Figs 1(a)-1(e) and Figs 2(a)-2(b), except that the binding of analyte to a first binding reagent on a solid phase/particle takes place through two steps: (i(a)) contacting the first binding reagent 20 linked to a first targeting agent to a particle (or other solid phase) linked to a second targeting agent that binds to the first targeting agent (thus attaching the first binding reagent to the particle or other solid phase) and (i(b)) contacting the first binding reagent with a sample comprising a target analyte that binds the first binding reagent. Step i(a) may occur before step i(b) (as shown in the figures) or the two steps may occur in the 25 reverse order or concurrently. Steps i(a) and i(b) may both be carried out during the conduct of an assay or, alternatively, the first binding reagent may be supplied to the user pre-bound to the solid phase through the targeting agents (e.g., if the targeting agents were pre-bound during manufacturing), in which case step i(a) may be omitted.

Thus, in one embodiment, the method includes contacting a sample comprising a 30 target analyte with a particle linked to a first binding reagent that binds the target analyte,

wherein the first binding reagent is linked to a first targeting agent and the particle is linked to a second targeting agent, and the first binding reagent and the particle are linked via a binding reaction between the first and second targeting agents to form a complex comprising the target analyte bound to the first binding reagent (see e.g., Fig. 3(a)). The 5 complex is then collected and unbound components in the sample are separated from the complex. The complex is released and the released complex is contacted with a second binding reagent bound to a solid phase, wherein the second binding reagent binds to the complex. The amount of analyte bound to the solid phase is measured. As in the embodiments described above and illustrated in Figs. 1(a)-1(e), the detectable label may 10 be attached to various assay components in the medium, e.g., to a third binding reagent, as in Figs. 3(a)-3(b), to the particle, as in Fig. 3(c), or to the first binding reagent, as in Figs. 3(d)-3(e). Moreover, the complex is optionally cleaved from the particle prior to 15 the detection step, as in Figs. 3(b) and 3(d).

In one embodiment, the assay may include (a) contacting a sample comprising a 15 target analyte with a first solid phase linked to a first binding reagent that binds the target analyte, wherein the first binding reagent is linked to a first targeting agent and the first solid phase is linked to a second targeting agent, and the first binding reagent and the first solid phase are linked via a binding reaction between the first and second targeting agents to form a complex comprising the target analyte bound to the first binding reagent (see e.g., Figs. 4(a)-4(b)). The complex is then collected and unbound components in the 20 sample are separated from the complex. The complex is released, e.g., resolubilized, and the first solid phase is removed. The released complex is contacted with a second binding reagent bound to a second solid phase, wherein the second binding reagent binds to the complex. The amount of analyte bound to the second solid phase is measured. 25 The detectable label may be attached to any suitable assay component, e.g., the first binding reagent, as in Fig. 4(b), or the third binding reagent, as in Fig. 4(a).

The releasing step in the various assay formats described herein may comprise cleaving a binding reagent from the particle (e.g., as shown in Fig. 1(b)). This may be accomplished by any suitable method, e.g., subjecting the complex to increased

temperature, pH changes, altering the ionic strength of the solution, competition, and combinations thereof.

If a targeting agent is employed in the assay format, the releasing step comprises disassociating the first and second targeting agents, e.g., by subjecting the complex to increased temperature, pH changes, altering the ionic strength of the solution, competition, and combinations thereof as discussed above.

The measuring step in the various assay formats described herein may comprise any suitable method of measuring the presence of a detectable label in a sample, e.g., optical absorbance, fluorescence, phosphorescence, chemiluminescence, light scattering or magnetism. In one embodiment, the detectable label is an electrochemiluminescent label and the measuring step comprises measuring an ECL signal and correlating that signal with an amount of analyte in the sample. Thus, the measuring step may further comprise contacting the complex with an electrode and applying a voltage waveform to the electrode to generate ECL.

By analogy to the description of Figures 1 and 2, the methods in Figures 3 and 4 may also be extended to multiplex measurements, e.g., by employing at least one of the group consisting of i) a plurality of different first binding reagents, ii) a plurality of second binding reagents and iii) a plurality of third binding reagents (the different reagents within (i), (ii) or (iii) being selected for their ability to preferentially bind a target analyte relative to other target analytes). In such multiplex methods, a common targeting reagent pair may be used to link a plurality of different first binding reagents to the corresponding particles or other solid phases. Alternatively, a unique targeting reagent pair may be used for each different first binding reagent (e.g., a different set of complementary oligonucleotides may be used to target each of the different first binding reagents). Such an approach may be used to i) target different first binding reagents to different distinguishable particles (e.g., particles bearing distinguishable labels) or ii) enable multiplexing through the use of a plurality of different second binding reagents, each of which binds preferentially to a different first targeting agent (thus preferentially binding complexes comprising one of the plurality of analytes).

EXAMPLES

Example 1 – Dual use of labeled magnetic particle to concentrate and detect analytes of interest

As shown in Figure 5, magnetic particles are coated with antibodies against the 5 analytes of interest and a large number (e.g., greater than 100) ECL labels. By attachment of the ECL labels to the antibodies (either before or after coating the antibodies on the particles), very high numbers of labels can be easily achieved. A particle of only 60 nm in diameter can support roughly 160 antibody molecules, assuming about 50 nm² of surface area per antibody. Thus, attachment of only 1 label per antibody 10 allows labeling ratios of greater than 100 labels per particle to be achieved for 60 nm particles. Labeling ratios of greater than 1000 labels per particle are achieved by increasing the number of labels per antibody and/or increasing the particle size).

A 1 mL or greater volume of sample is combined with the particles in a container and after incubating the mixture to allow the antibodies to bind their respective targets, a 15 magnetic field is applied such that the magnetic particles collect on a surface in the container (a variety of commercial magnetic tube holders or probes are available for carrying out this step). The complexes are washed with buffered saline to remove unbound components of the sample. The magnetic field is removed and the particles are then re-suspended in 100 uL of a suitable assay diluent, thus providing a 10-fold or 20 greater increase in concentration relative to the original sample. The particle-analyte complexes are transferred to an assay plate (e.g., a MULTI-ARRAY® 96-well assay plate, Meso Scale Diagnostics, LLC, Gaithersburg, MD) that includes a binding surface comprising an array of antibody binding reagents directed against the analytes of interest. Complexes that bind the array are measured by ECL on a SECTOR® Imager instrument 25 (Meso Scale Diagnostics, LLC). The magnetic collection step provides for improvements in assay performance by allowing for pre-concentration of analyte into a small volume and removal of potential interferents in the sample.

Example 2 – Assay using antibodies coupled to magnetic particles through oligonucleotide hybridization reactions

Magnetic particles are coated with oligonucleotides and a large number (greater than 100) ECL labels. Conjugates are formed comprising antibodies against analytes of interest and oligonucleotides complementary to the oligonucleotides on the particles. The antibody conjugates and particles are subjected to conditions sufficient to hybridize the complementary oligonucleotide sequences (e.g., appropriate temperature, ionic strength and denaturing conditions, as described hereinabove) and thereby coat the antibodies on the particles. These particles are then used to assay for analytes of interest as described in Example 1.

Example 3 – Demonstration of the release of antibodies coupled to magnetic particles through oligonucleotide hybridization reactions

Magnetic beads (Dynalbeads® MyOneTM-Streptavidin C1 beads, Invitrogen Corporation) were coated with a biotinylated oligonucleotide by the following procedure: The beads (3 mg) were washed three times at 60°C in hybridization buffer (20 mM Tris, 1 mM EDTA, 250 mM NaCl, 0.01% Triton-X at pH=8 and 0.1% BSA). The beads were then coated at room temperature with 750 pmoles of a 19-mer biotinylated oligonucleotide (Oligo 1, Tm = 40°C), in 1 mL of hybridization buffer, for one hour with gentle mixing. The coated beads were washed 5x with hybridization buffer at 60°C and then resuspended in hybridization buffer at a final concentration of 10 ug/mL. The magnetic beads were then coated with labeled mouse immunoglobulin by the following procedure: Mouse immunoglobulin (mIgG) was labeled with Sulfo-TAGTM ECL labels (Meso Scale Diagnostics, LLC.) according to the manufacturer's instructions. The protein was also labeled with an oligonucleotide having a terminal thiol group (Oligo 2, the complement of Oligo 1) using a bifunctional coupling reagent (sulfosuccinimidyl 4-(*N*-maleimidométhyl)-1-cyclohexane carboxylate ("SMCC")) and conventional coupling protocols, e.g., protein is reacted with the NHS-ester in SMCC to label the protein and the resulting complex is reacted with thiolated oligonucleotides which reacts with the maleimide group in SMCC. The labeled mIgG-oligo conjugate (0.1 pmol) was

then mixed with the oligo-coated magnetic beads (500 ug of beads) in hybridization buffer for 1 hour at room temperature to hybridize the complementary oligonucleotide sequences and thereby immobilize the mIgG onto the beads. The resulting antibody-coated beads were washed and resuspended in hybridization buffer.

5 The beads were incubated under different conditions, including incubating the suspension at room temperature for one hour (with or without the presence of free Oligo2 as a competitor) and incubating the suspension at 60°C for 10 min. (with or without the presence of free Oligo2 as a competitor). The beads were then magnetically collected and the supernatant analyzed by ECL assay to measure the amount of labeled mIgG that
10 was released from the beads. To measure the labeled mIgG, the supernatant was transferred to the well of a MULTI-ARRAY plate in which the electrode is coated with goat anti-mouse antibodies (MULTI-ARRAY GAM Plate, Meso Scale Diagnostics, LLC.). The plate was incubated with shaking during which time labeled mIgG in the solution bound to the immobilized goat anti-mouse antibodies. The wells were washed
15 with PBS, filled with 150 uL of Read Buffer T (Meso Scale Diagnostics) and analyzed on a SECTOR Imager instrument.

Table 1 shows that, in the absence of competing oligonucleotides, the linkage of the mIgG to the beads was stable at room temperature. The mIgG could be efficiently released from the beads by exposure to short periods of time above the melting
20 temperature of the Oligo1-Oligo2 pair. The efficiency of release could be further enhanced by addition of free Oligo2 as a competitor.

Table 1. Efficiency of different release techniques.

Release Technique	% of Released Material
1 H at RT	6%
1H at RT with free Oligo	23%
10 min 60C	50%
10 min 60 C with Free Oligo	57%

Example 4 – Assay including capture of analyte through collection of magnetic particles and release by denaturation of a linkage comprising an oligonucleotide pair

Magnetic beads (Dynalbeads® MyOneTM-Streptavidin C1 beads, Invitrogen Corporation) were coated with biotinylated oligonucleotides as described in Example 3. The magnetic beads were then coated with antibodies against human TNF-alpha and IL-5 using i) antibodies that were labeled with Sulfo-TAG and Oligo1 and ii) the coating procedure of Example 3.

Assay Procedure with Pre-Concentration. Sample containing human TNF-alpha or IL-5 (1 mL of sample) was combined with 200 ng of antibody-coated beads (prepared as described above) and incubated for 1 hr at room temperature. The beads were magnetically collected and washed with hybridization buffer. The antibody on the beads (including any labeled-antibody-analyte complexes that were formed during the incubation) were released into 100 uL of a 1:20 dilution of hybridization buffer (~10 mM salt) at elevated temperature (60°C), i.e., by denaturing the oligonucleotide pairs linking the antibodies to the beads. The resulting solution was transferred to a well of a MULTI-ARRAY 96-well plate, each well of which included an array of capture antibodies including an anti-TNF-alpha spot and an anti-IL-5 spot. The plate was incubated with shaking for 1 hr at room temperature to allow the labeled-antibody-analyte complexes to bind to the appropriate capture antibody spots. The wells were then washed three times with PBS and then filled with 125 uL of Read Buffer T (Meso Scale Diagnostics) and read on a SECTOR Imager instrument. The instrument measures and reports the ECL intensity from each array element (or “spot”) in the antibody array.

Conventional Immunoassay Protocol without Pre-Concentration. Sample containing human TNF-alpha or IL-5 (30 uL) was combined with 20 uL of a solution containing labeled (Sulfo-TAG) detection antibodies at a concentration of 1 ug/mL. The resulting solution was incubated for 1 hr in a well of a MULTI-ARRAY plate having anti-TNF-alpha and anti-IL-5 spots. The wells were washed, filled with Read Buffer T and analyzed in a SECTOR Imager instrument as described for the protocol with collection and release.

Results. The results presented in Table 2 show that the protocol with collection and release provided specific assay signals for both TNF-alpha and IL-5 (signal in the presence of analyte – signal in the absence of analyte) that were substantially higher than those obtained using the conventional protocol, without any substantial change in the 5 background signal in the absence of analyte. The enhancement in specific signal for 10 pg/mL samples was greater than 5-fold for TNF-alpha and greater than 10-fold for IL-5.

Table 2.

Assay	TNF		IL-5	
	Conventional	Pre-Concentration	Conventional	Pre-Concentration
0	371	398	22	27
1	530	1,095	95	451
10	3,301	18,323	723	9,831
100	31,005	75,864	8,057	48,895

10

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the method in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the 15 scope of the claims. Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

CLAIMS:

1. An assay device comprising
 - (a) a storage zone comprising a surface-reagent complex confined to said storage zone, said surface-reagent complex comprising
 - (i) a reagent linked to a first targeting agent; and
 - (ii) a surface linked to a second targeting agent, wherein said reagent and said surface are linked, in said surface-reagent complex, via a releasable binding interaction between said first and second targeting agents; and
 - (b) one or more use zones each configured to use said reagent in an assay for an analyte of interest in a sample.
2. The assay device of claim 1 wherein said one or more use zones each comprise an additional reagent used in said assay.
3. The assay device of claim 1 wherein said reagent is a binding reagent that binds a component of said assay conducted in said use zone.
4. The assay device of claim 3 wherein said binding reagent binds said analyte.
5. The assay device of claim 2 wherein said additional reagent binds said analyte.
6. The assay device of claim 4 wherein said binding reagent binds a complex formed between said additional reagent and said analyte.
7. The assay device of claim 6 wherein said one or more use zones each comprise a solid support and said additional reagent is bound to said solid support.
8. The assay device of claim 1 wherein said reagent comprises a detectable label.
9. The assay device of claim 8 wherein said detectable label is an ECL label.

10. The assay device of claim 1 wherein said storage zone and said one or more use zones are in fluidic communication along a fluid path.
11. The assay device of claim 1 wherein said one or more use zones each comprise two or more assay regions each configured to use said reagent in one or more assays conducted with said sample in said assay device.
12. The assay device of claim 11 wherein a first assay region of said one or more use zones is configured to conduct an assay for a first analyte of interest in said sample and an additional assay region in said one or more use zones is configured to conduct an assay for an additional analyte of interest in said sample.
13. The assay device of claim 11 wherein a first assay region of said one or more use zones is configured to conduct a first assay for said analyte of interest in said sample and an additional assay region of said one or more use zones is configured to conduct a second assay for said analyte of interest in said sample.
14. The assay device of claim 11 wherein each of said two or more assay regions comprise an additional reagent used in said assay.
15. The assay device of claim 14 wherein said additional reagent is an additional binding reagent.
16. The assay device of claim 11 wherein said one or more use zones each comprise an array of said two or more assay regions.

17. The assay device of claim 1 wherein said surface is a particle.
18. The assay device of claim 1 wherein said surface is roughened such that the surface area accessible to a component capable of binding to said surface is at least three-fold larger than the surface area of a flat surface.
19. The assay device of claim 1 wherein said surface is roughened such that the surface area accessible to a component capable of binding to said surface is at least two-fold larger than the surface area of a flat surface.
20. The assay device of claim 1 wherein said surface comprises a composite material including exposed particles distributed in a matrix.
21. The assay device of claim 20 wherein said composite material comprises carbon particles, graphitic particles, or carbon nanotubes.
22. The assay device of claim 20 wherein said composite is etched.
23. The assay device of claim 20 wherein said surface comprises one or more indentations and/or raised features.
24. The assay device of claim 1 wherein said surface comprises a hydrogel.
25. The assay device of claim 1 wherein said reagent is released from said surface-reagent complex by subjecting said storage zone to increased or decreased temperature, pH changes, an electric potential, a change in ionic strength, competition, and combinations thereof.
26. The assay device of claim 25 wherein said reagent is released by subjecting said storage zone to increased temperature.

27. The assay device of claim 26 wherein said increased temperature exceeds the melting temperature of said binding interaction.

28. The assay device of claim 1 wherein said assay device is a cartridge.

29. The assay device of claim 1 wherein said assay device is a multi-well assay plate and said use zone is positioned within a well of said assay plate.

30. The assay device of claim 29 wherein said storage zone is located on a supplemental surface of said well that does not overlap with said use zone.

31. The assay device of claim 1 wherein said device is configured to conduct a multiplexed measurement.

32. A method of conducting an assay for an analyte of interest in a sample, wherein said method is conducted in an assay device comprising:

(a) a storage zone comprising a surface-reagent complex confined to said storage zone, said surface-reagent complex comprising

- (i) a reagent linked to a first targeting agent; and
- (ii) a surface linked to a second targeting agent,

wherein said reagent and said surface are linked, in said surface-reagent complex, via a releasable binding interaction between said first and second targeting agents; and

(b) one or more use zones each configured to use said reagent in an assay for an analyte of interest in a sample;

said method comprising:

- (x) introducing said sample to said one or more use zones ;
- (y) subjecting said storage zone to a condition that releases said reagent from said surface-reagent complex;
- (z) transferring said reagent from said storage zone to said one or more use zones; and

(xx) conducting said assay in said one or more use zones with said reagent.

33. The method of claim 32 wherein said condition is selected from increased or decreased temperature, pH changes, applying an electrical potential, a change in ionic strength, competition, and combinations thereof.

34. The method of claim 32 wherein said condition comprises increasing the temperature of said storage zone.

35. The method of claim 34 wherein said increased temperature exceeds the melting temperature of said binding interaction.

36. The method of claim 32 wherein said reagent is a binding reagent that binds said analyte and said method further comprises detecting the amount of analyte bound to said binding reagent in said one or more use zones.

37. The method of claim 36 wherein said reagent comprises a detectable label and said detecting step comprises detecting the presence or absence of said detectable label in said one or more use zones.

38. The method of claim 37 wherein said detectable label is an ECL label and said detecting step comprises detecting electrochemiluminescence emitted in said one or more use zones.

39. The method of claim 32 wherein said storage zone and said one or more use zones are in fluidic communication along a fluid path and said transferring step (z) comprises transferring said reagent from said storage zone via said fluid path to said one or more use zones.

40. The method of claim 32 wherein said use zone comprises two or more assay regions each configured to use said reagent in one or more assays conducted with said sample in said assay device, said method further comprising the step of conducting a plurality of assays in said one or more use zones with said reagent.

41. The method of claim 40 wherein said one or more use zones each comprise a first assay region configured to conduct an assay for a first analyte of interest in said sample and an additional assay region configured to conduct an assay for an additional analyte of interest in said sample, said method comprising:

- (x) introducing said sample to said one or more use zones;
- (y) subjecting said storage zone to a condition that releases said reagent from said surface-reagent complex;
- (z) transferring said reagent from said storage zone to said first assay region and said second assay region;
- (xx) conducting said assays in said first and second assay regions, respectively.

42. The method of claim 41 wherein said assays are conducted simultaneously.

43. The method of claim 41 wherein said assays are conducted sequentially.

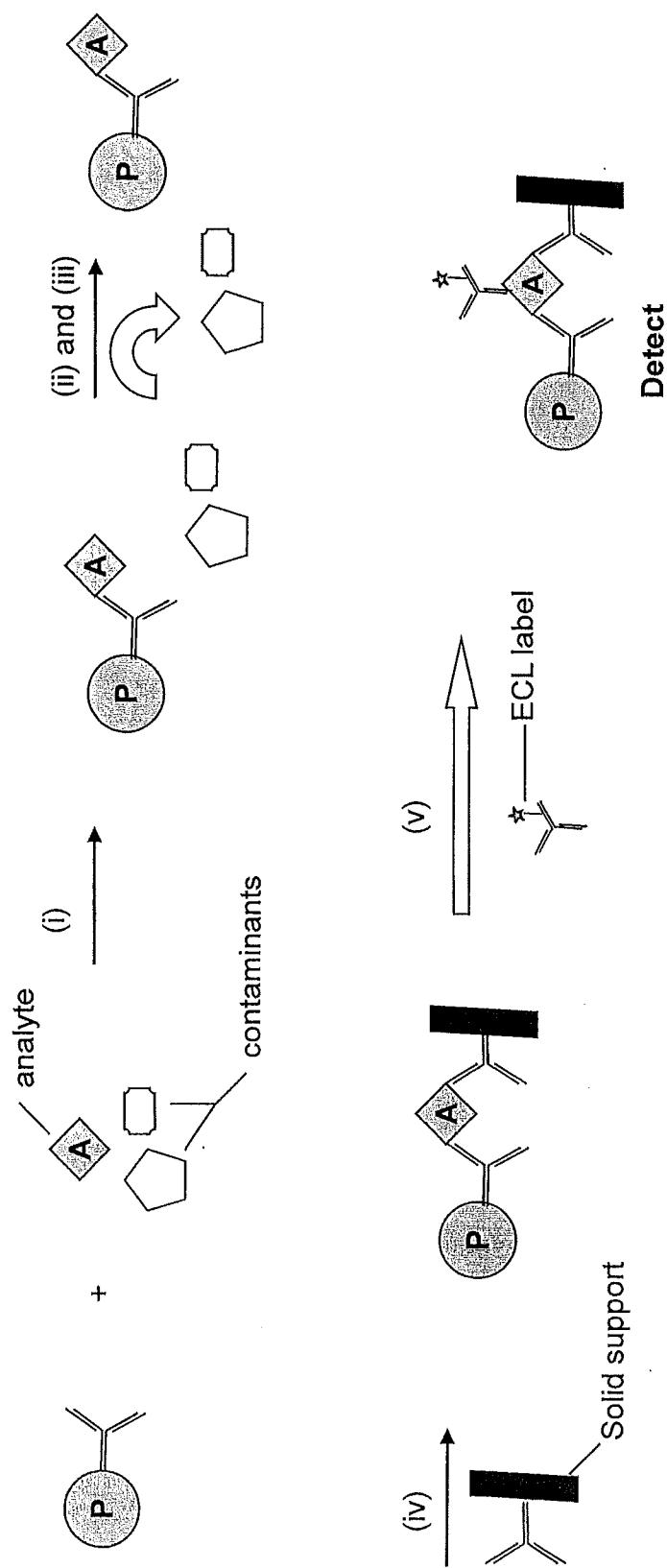


Fig. 1(a)

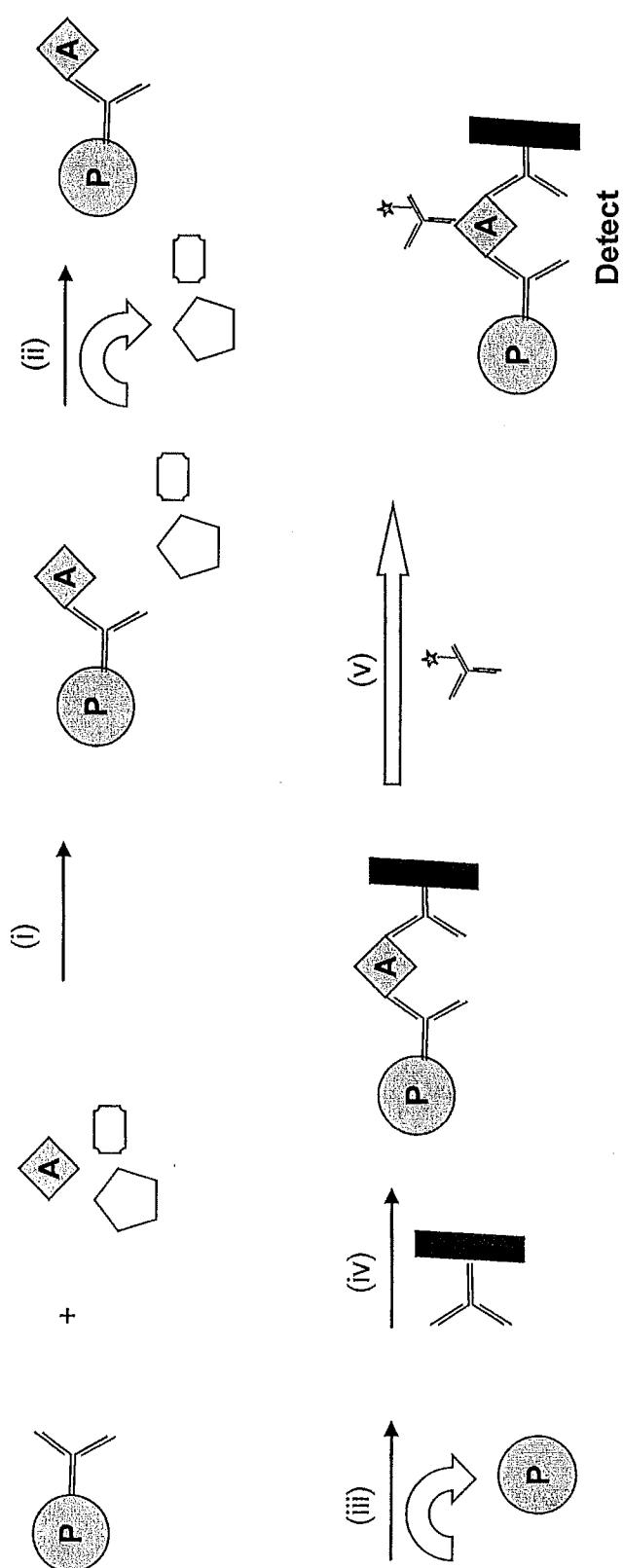


Fig. 1(b)

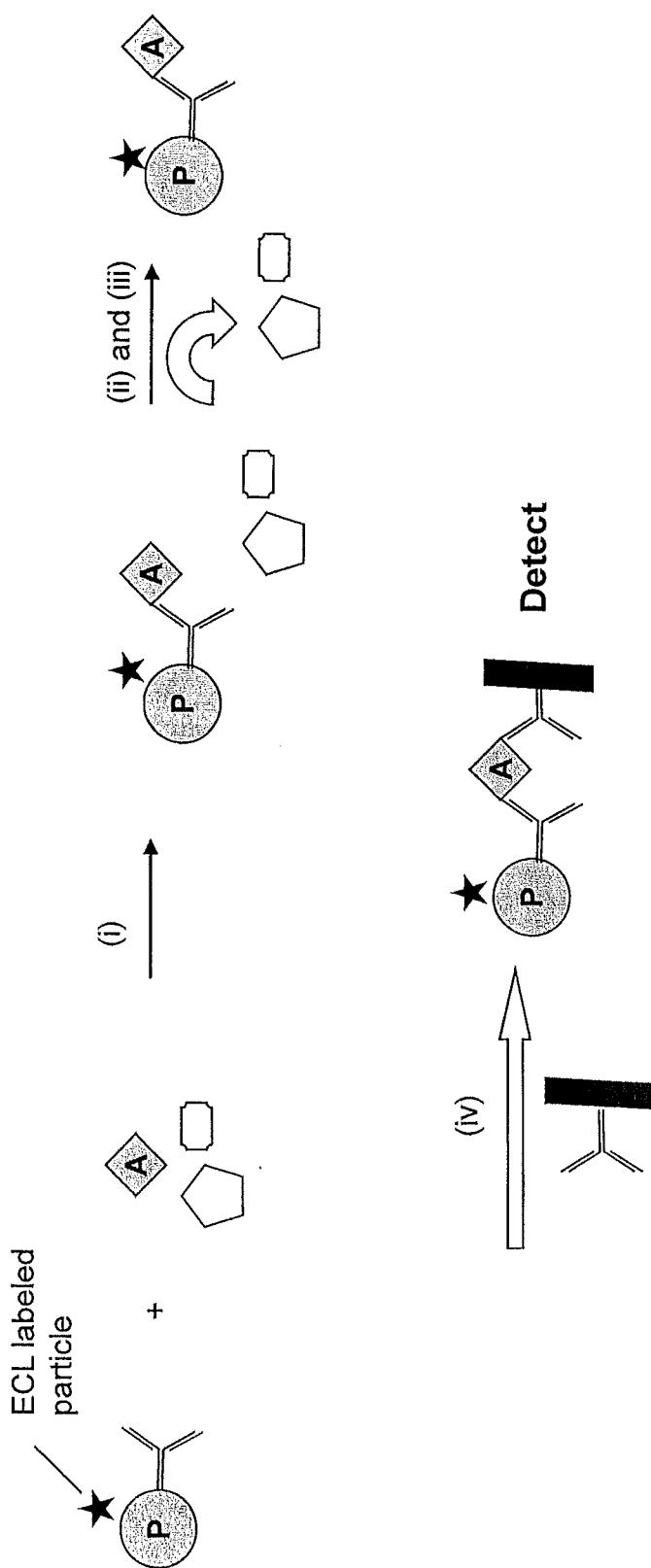


Fig. 1(c)

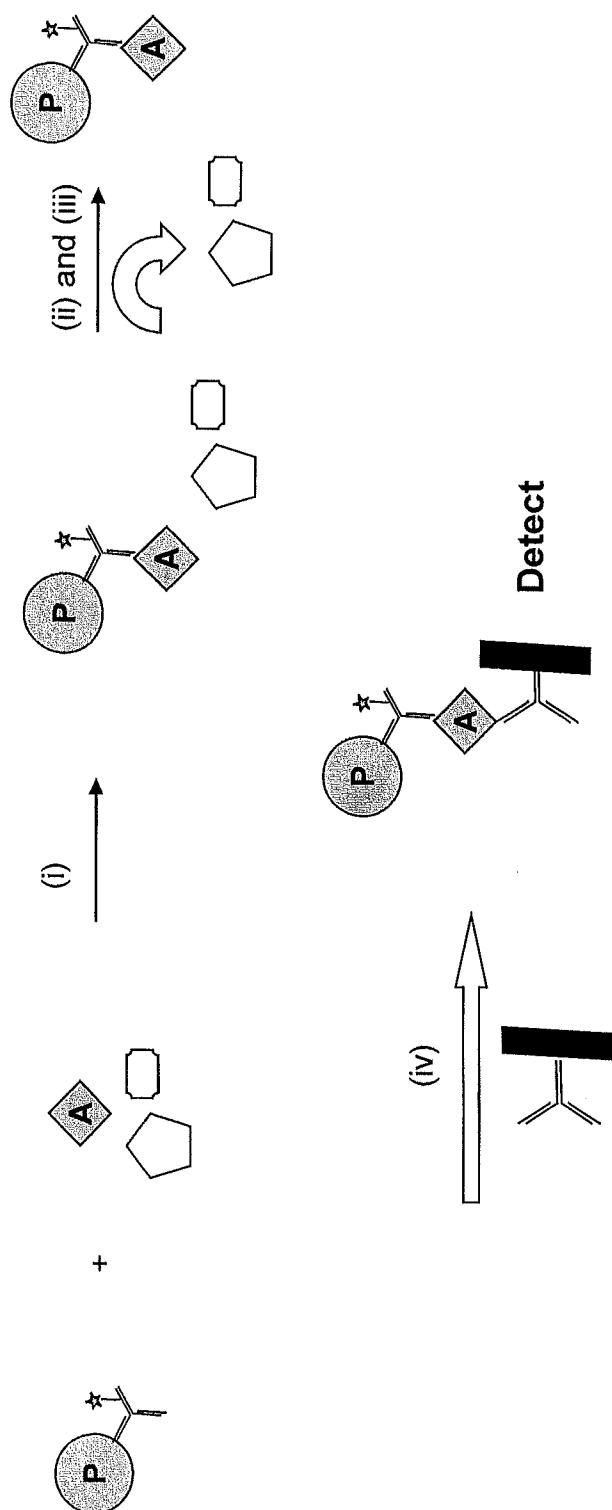


Fig. 1(d)

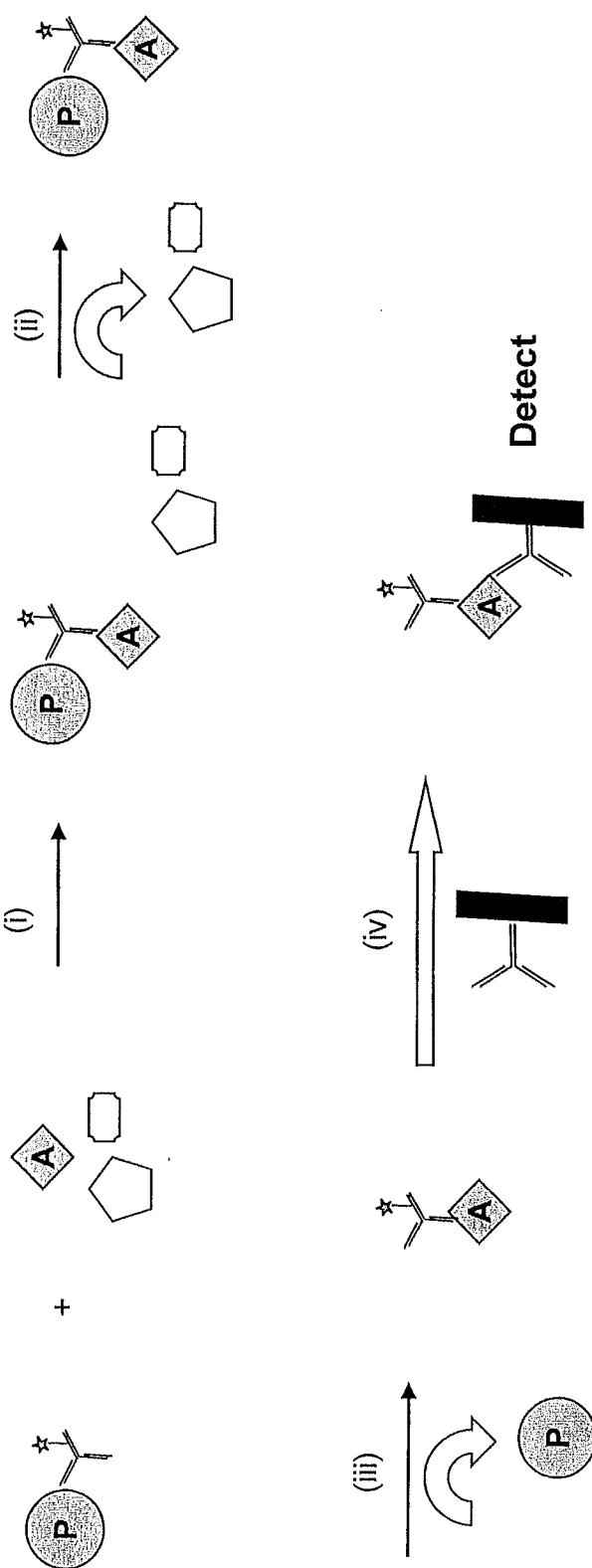


Fig. 1(e)

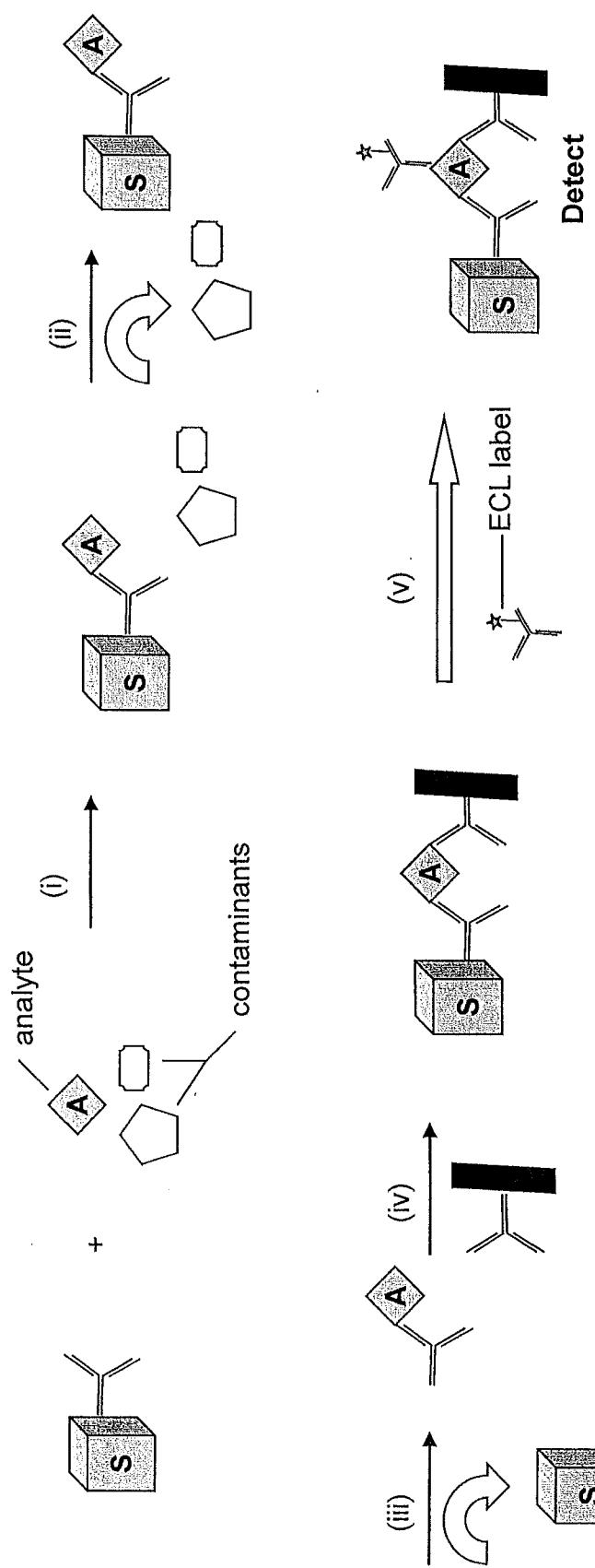


Fig. 2(a)

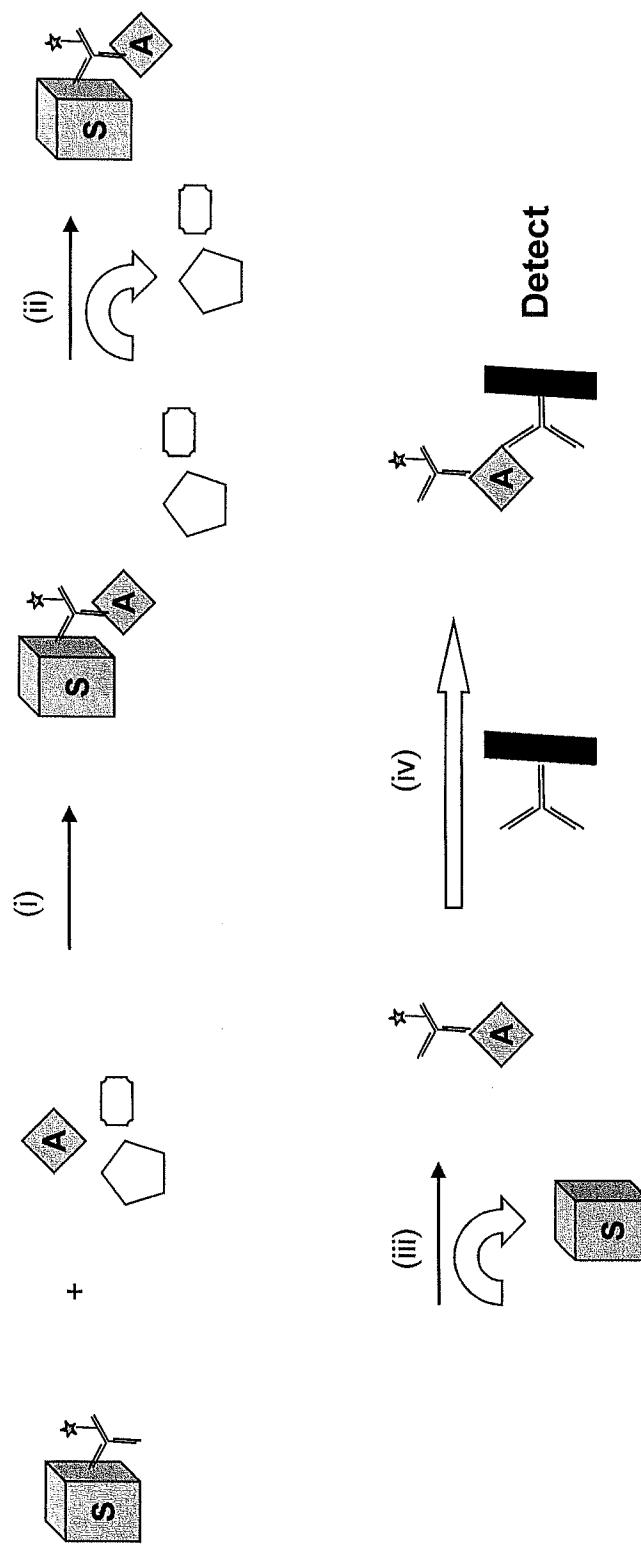


Fig. 2(b)

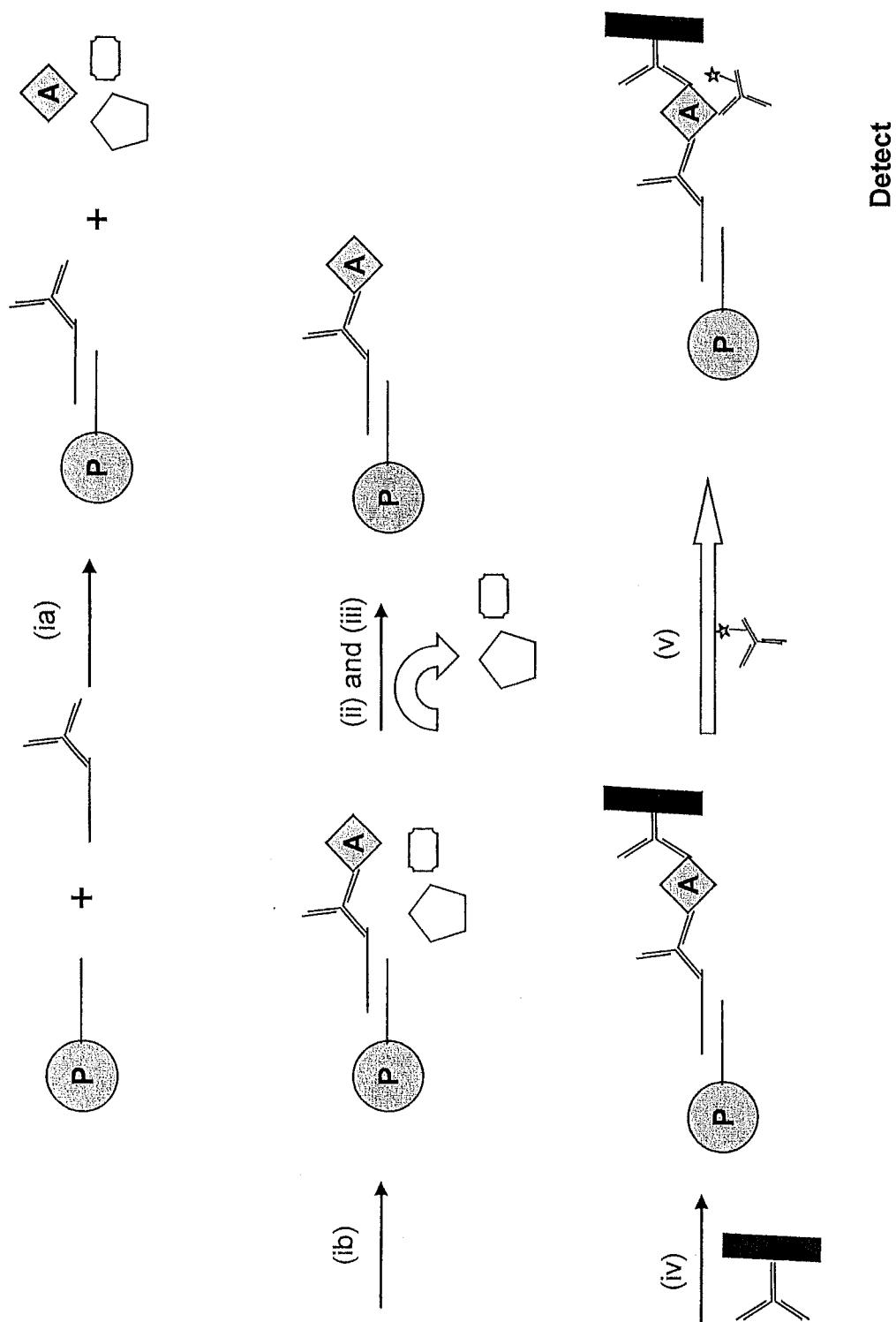


Fig. 3(a)

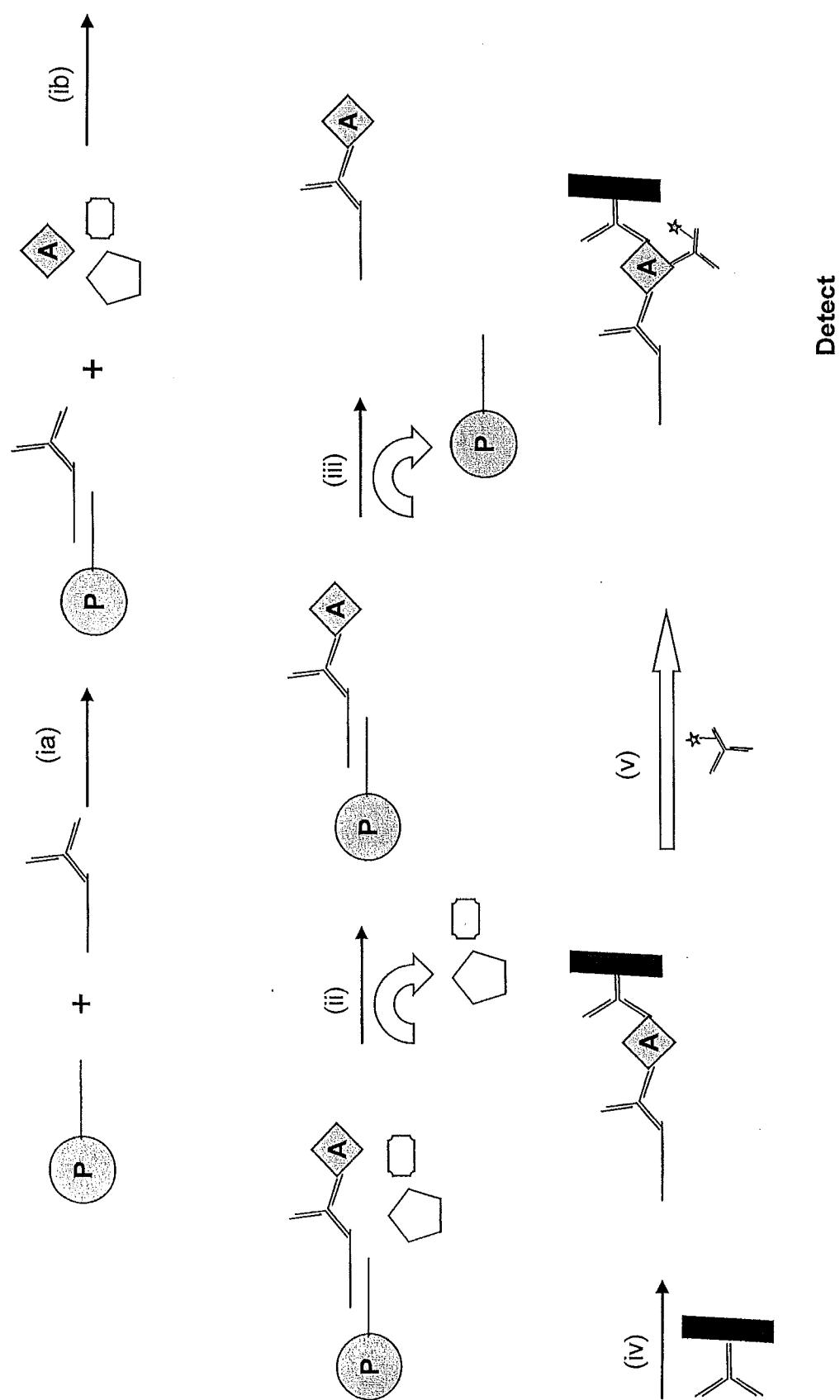


Fig. 3(b)

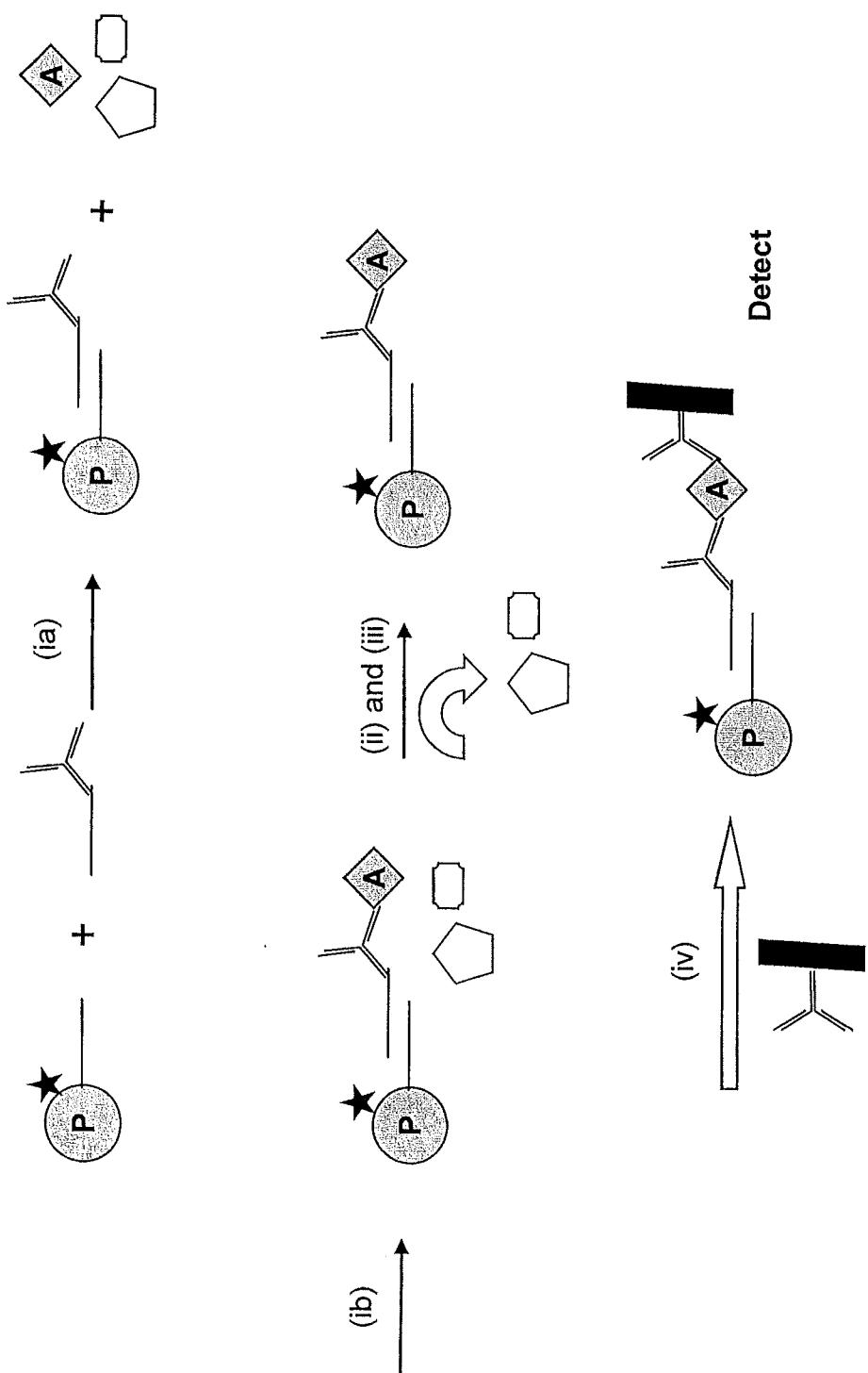


Fig. 3(c)

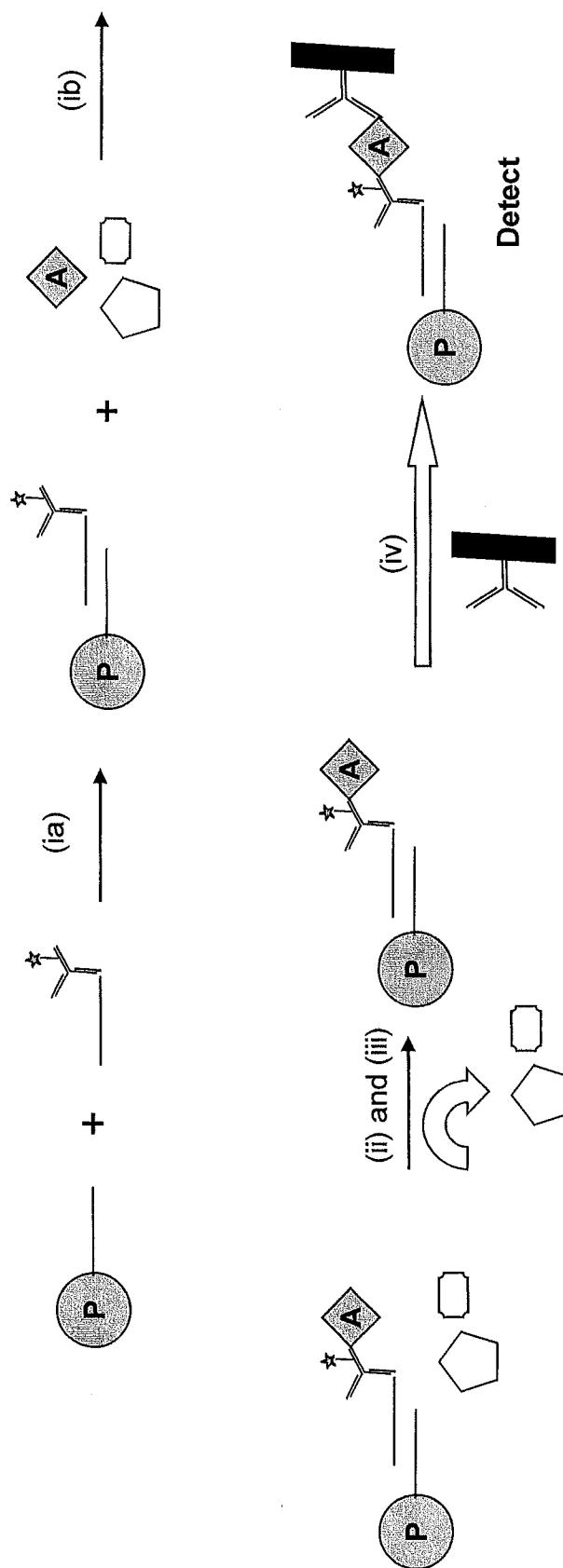


Fig. 3(d)

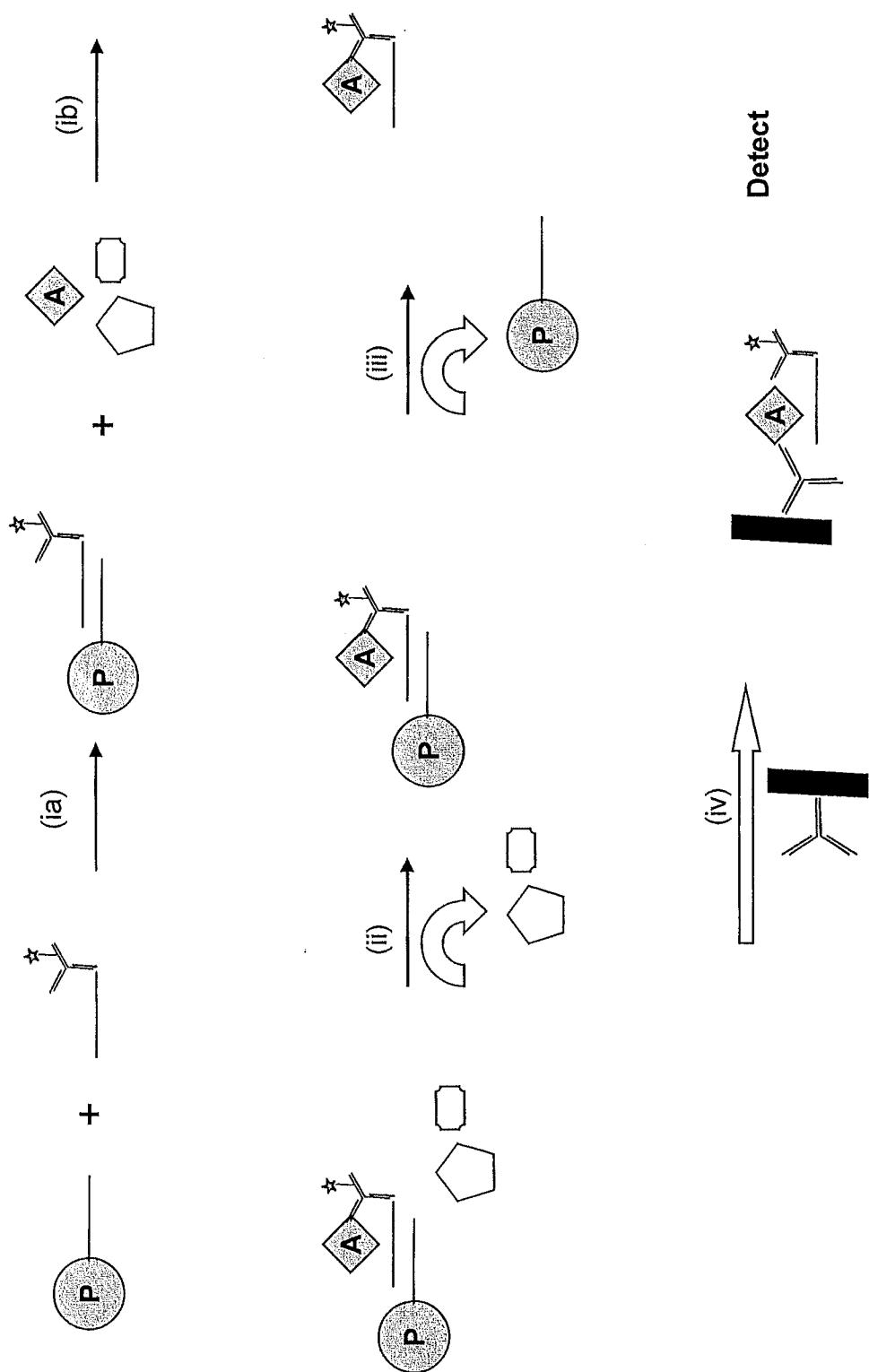


Fig. 3(e)

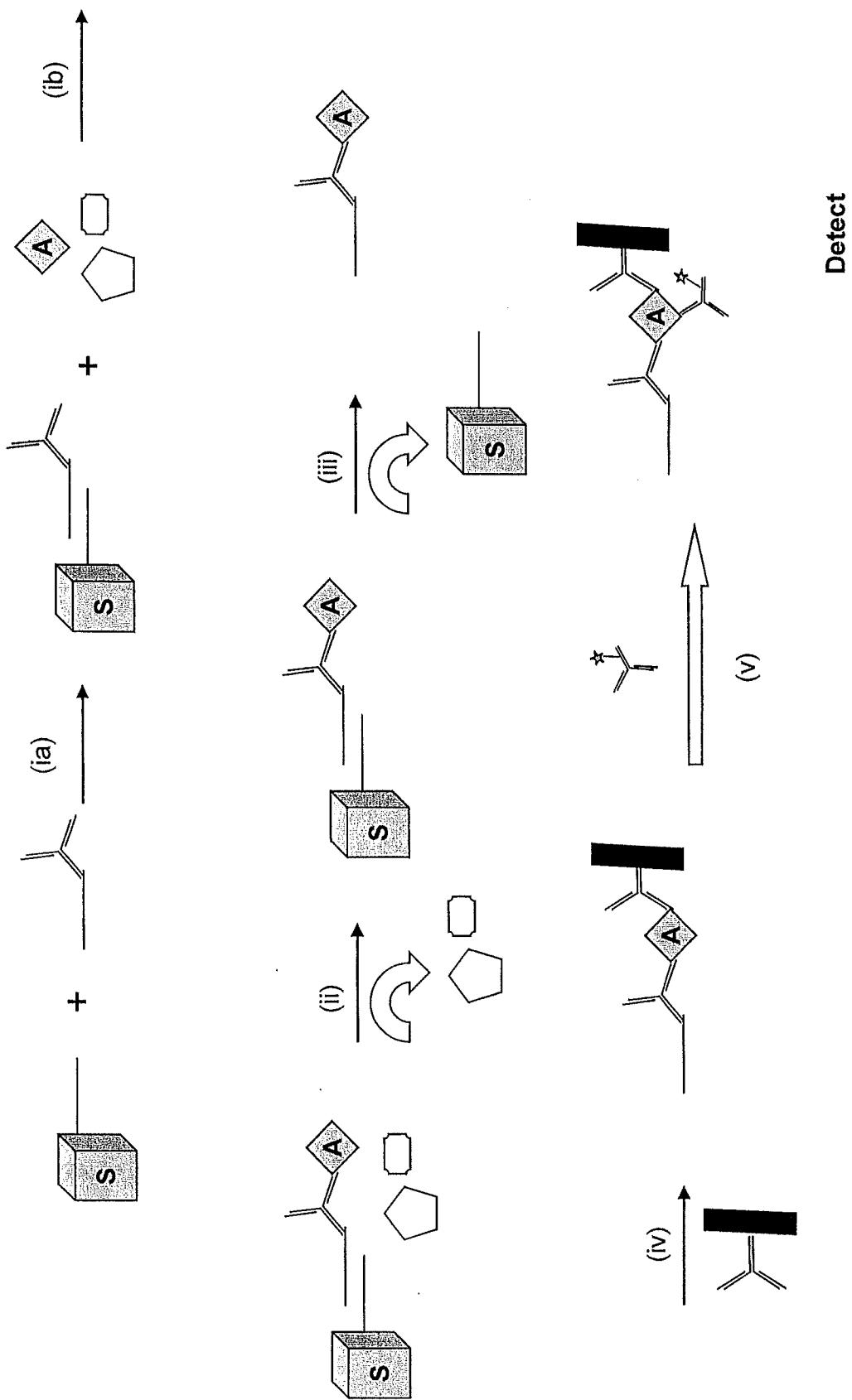


Fig. 4(a)

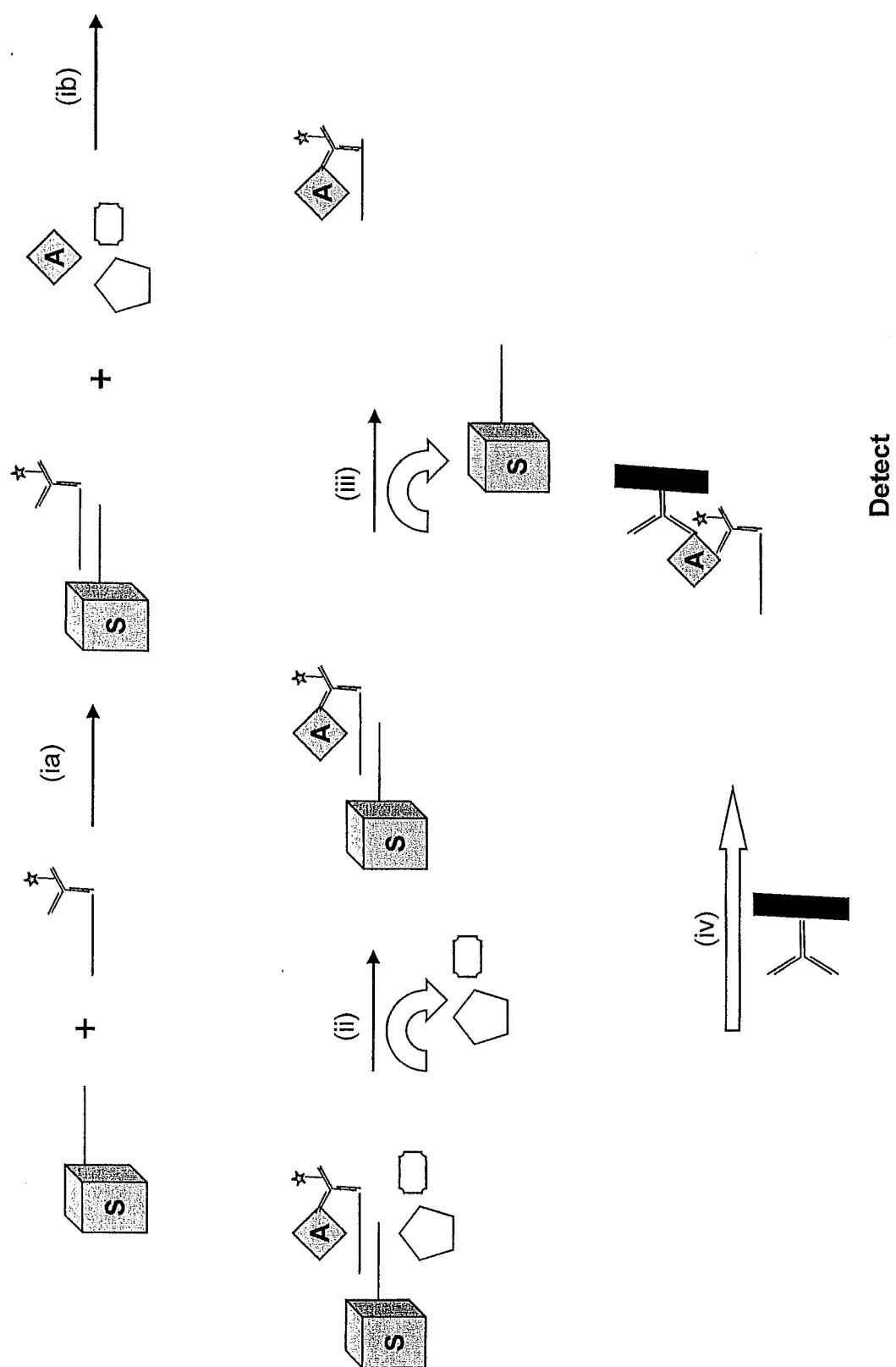


Fig. 4(b)

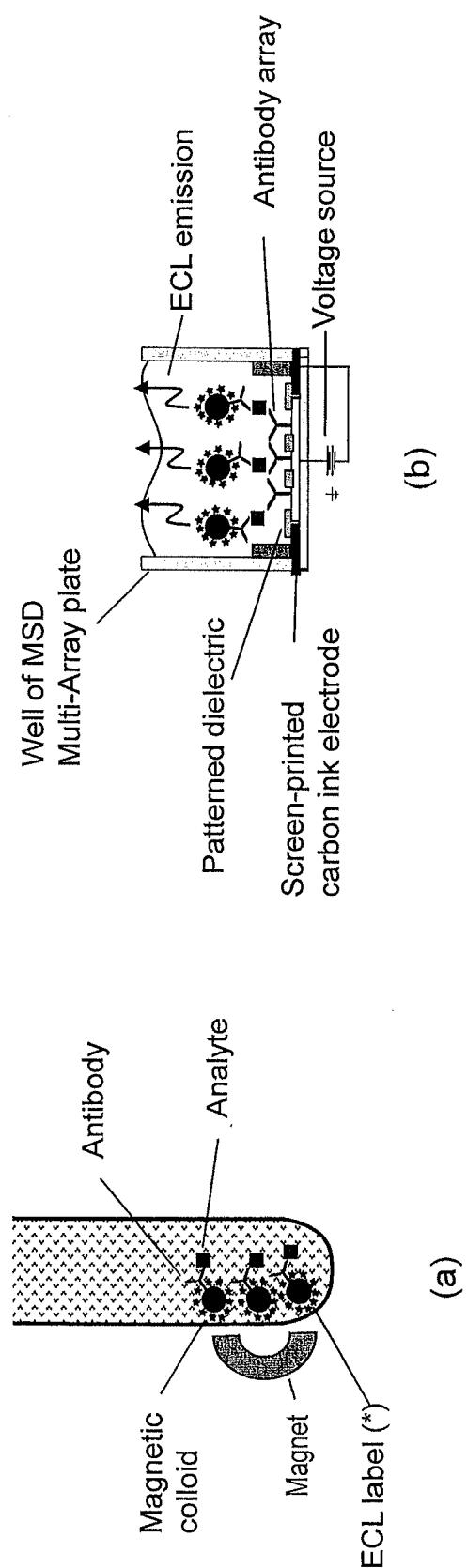


Fig. 5

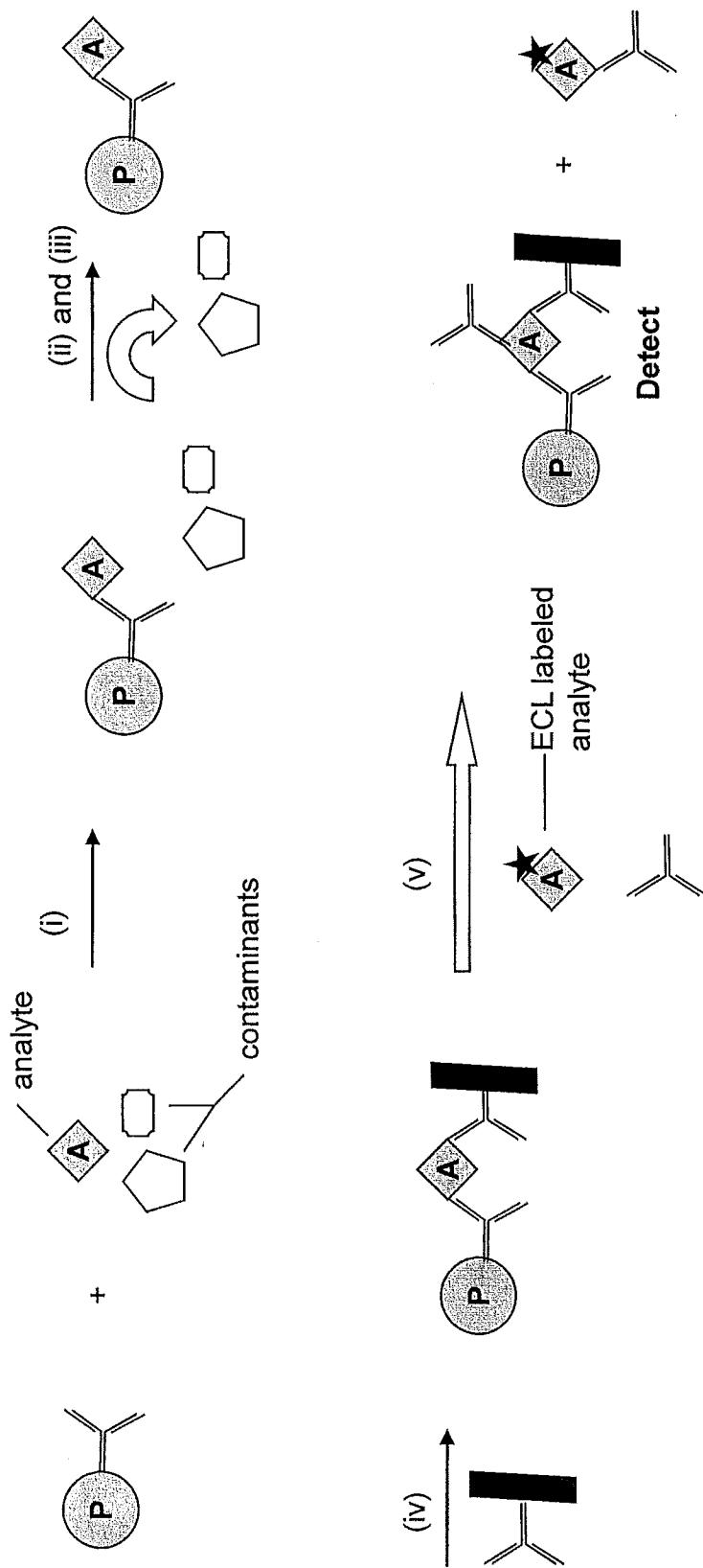
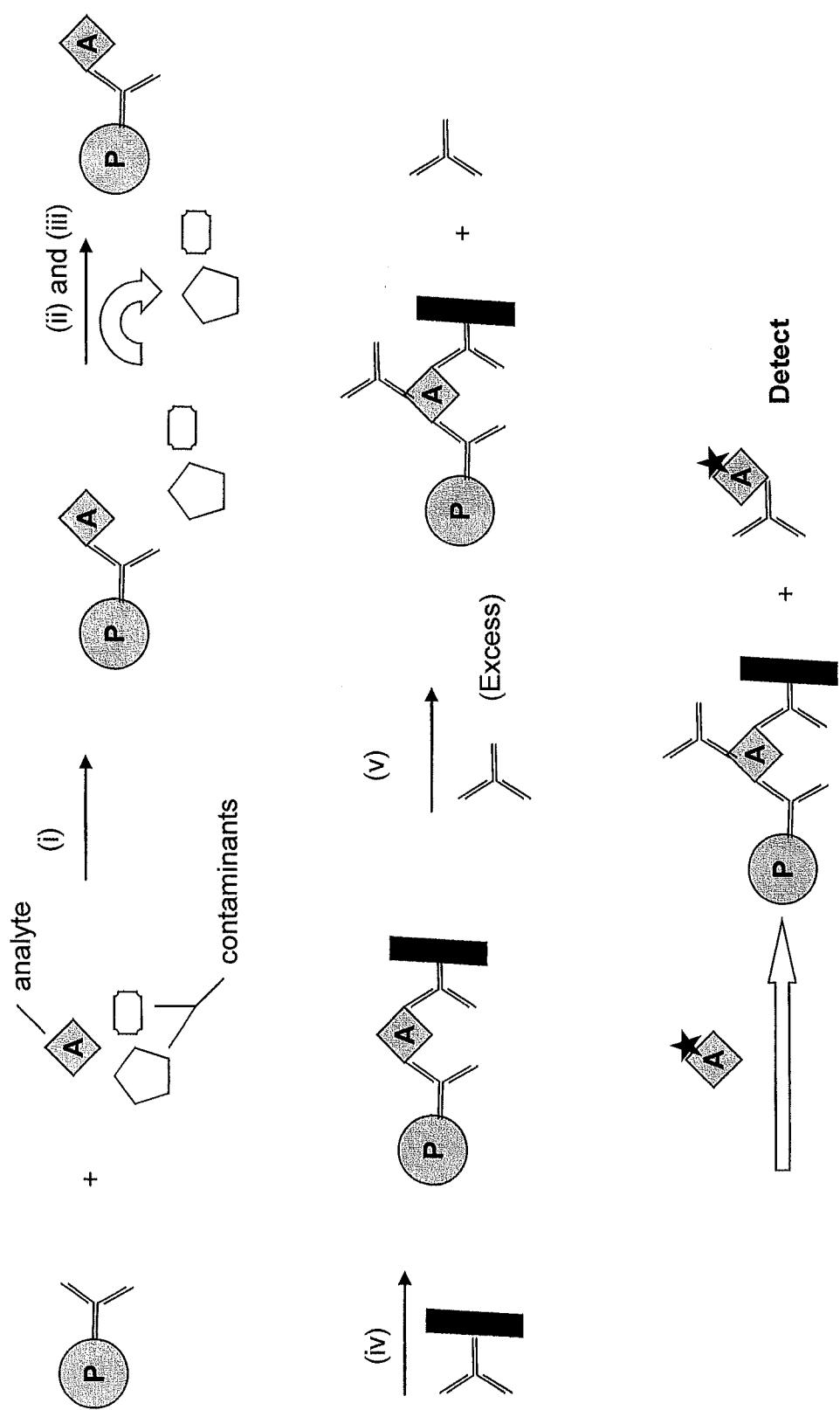


Fig. 6(a)

**Fig. 6(b)**

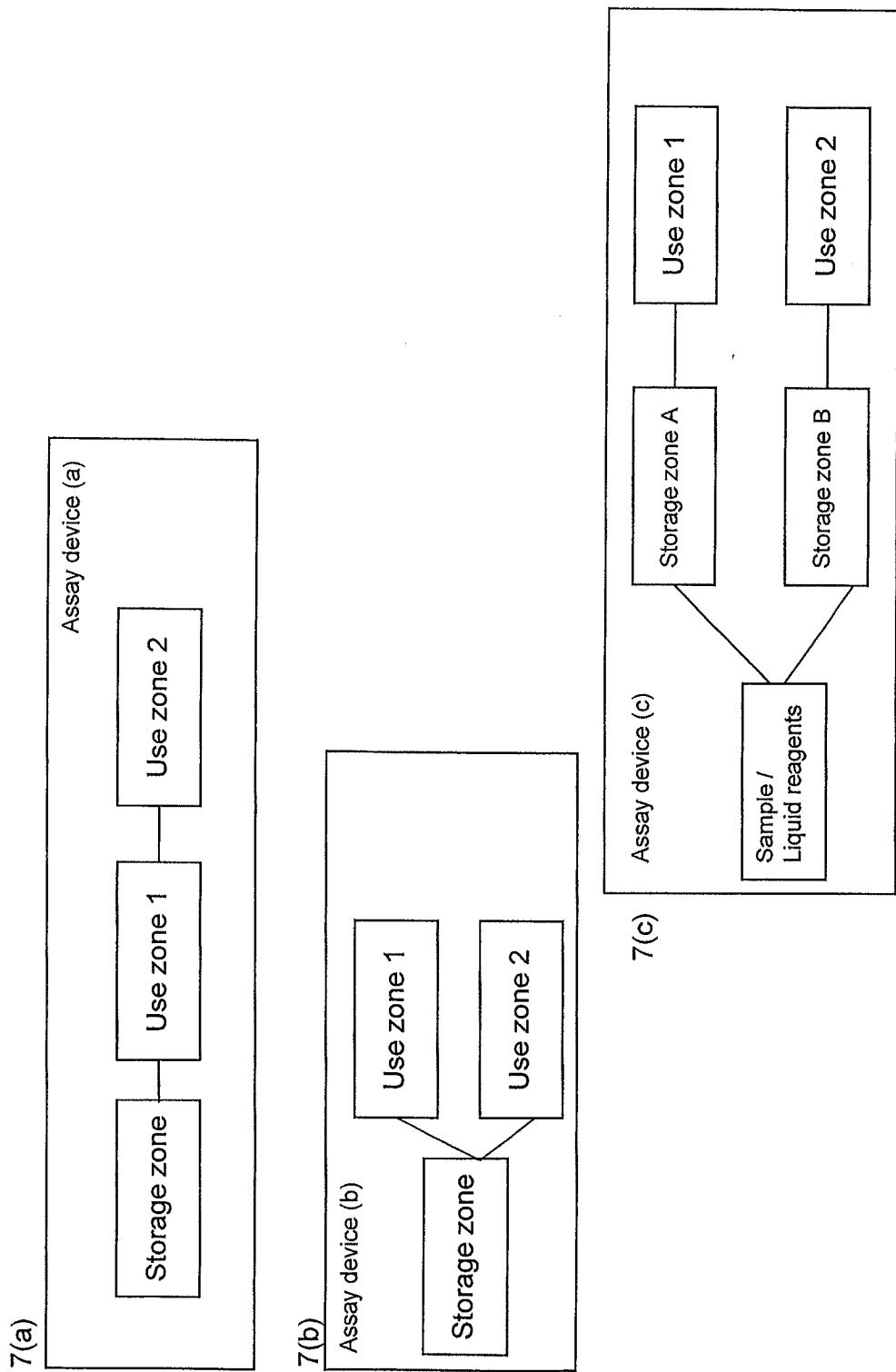
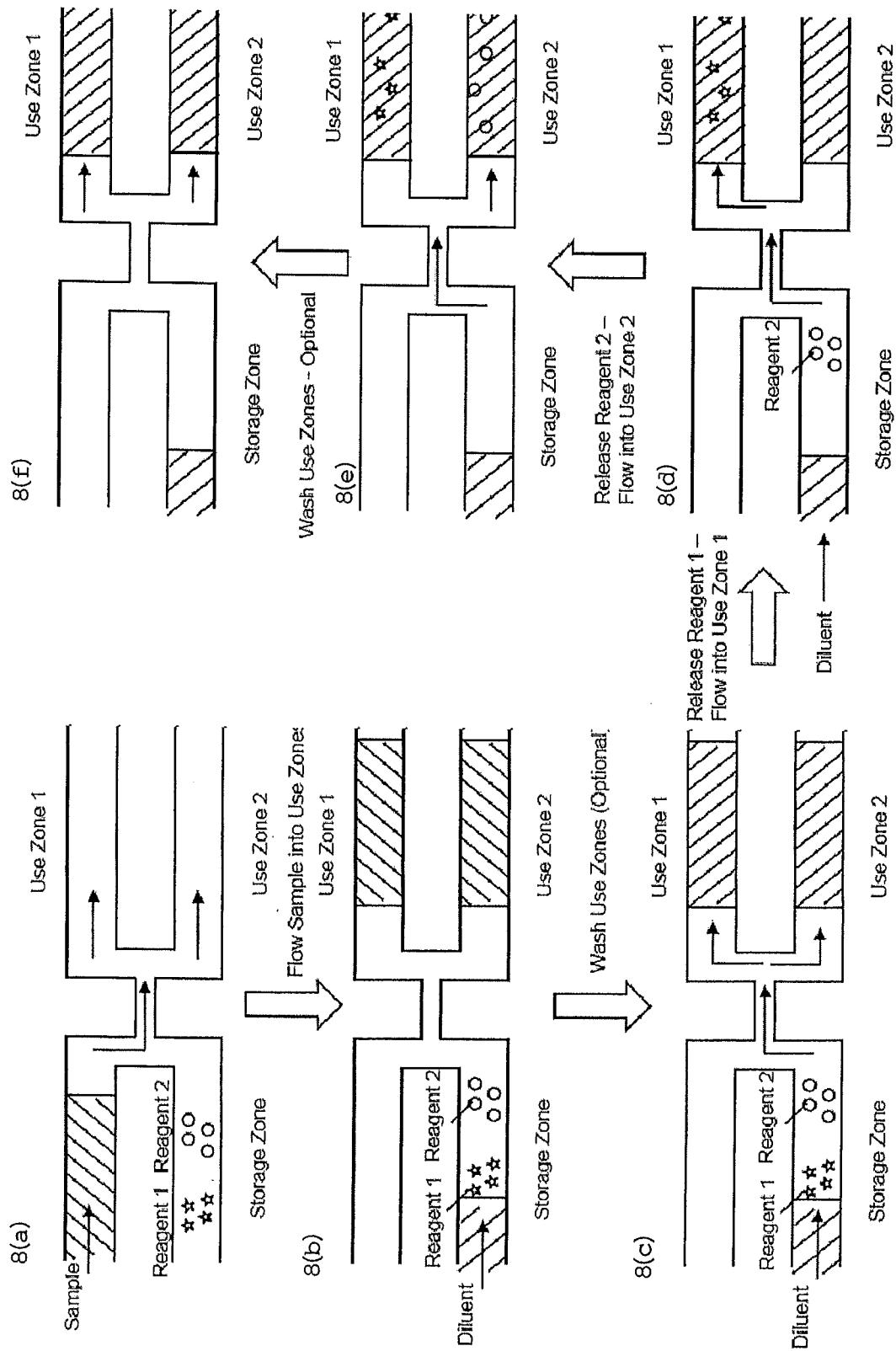


Fig. 7



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