US 20090148847A1

(19) **United States**

(12) **Patent Application Publication**

Kokoris et al.

(10) **Pub. No.: US 2009/0148847 A1**

(43) **Pub. Date: Jun. 11, 2009**

(54) **RAPID MAGNETIC FLOW ASSAYS**

(75) Inventors: **Mark Kokoris**, Bothell, WA (US); **Melud Nabavi**, Seattle, WA (US); **Wayne L. Breidford**, Seattle, WA (US); **John Gerdes**, Columbine Valley, CO (US); **Stephen Mordue**, Kirkland, WA (US); **C. Frederick Battrell**, Redmond, WA (US)

Correspondence Address:
SEED INTELLECTUAL PROPERTY LAW GROUP PLLC
701 FIFTH AVE, SUITE 5400
SEATTLE, WA 98104 (US)

(73) Assignee: **MICRONICS, INC.**, Redmond, WA (US)

(21) Appl. No.: **12/203,779**

(22) Filed: **Sep. 3, 2008**

Related U.S. Application Data

(63) Continuation of application No. PCT/US2007/006585, filed on Mar. 15, 2007.

(60) Provisional application No. 60/782,649, filed on Mar. 15, 2006, provisional application No. 60/844,811, filed on Sep. 14, 2006.

Publication Classification

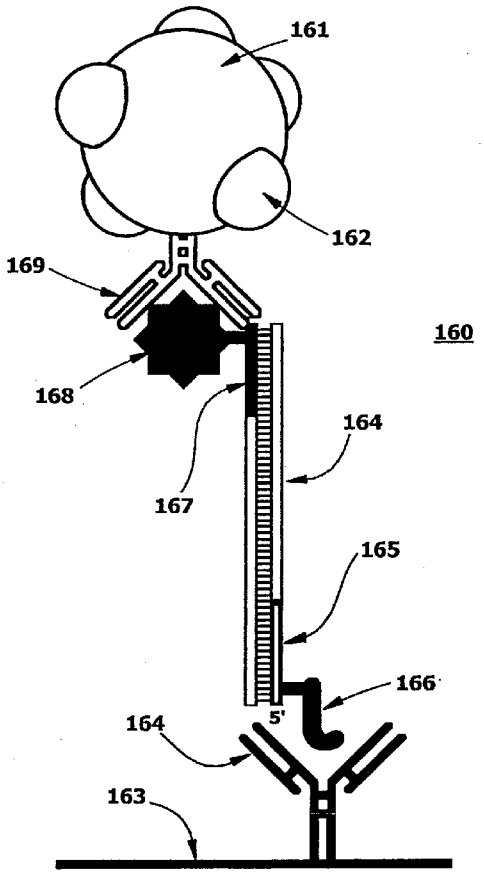
(51) **Int. Cl.**

C12Q 1/68	(2006.01)
C07H 21/04	(2006.01)
B01J 19/00	(2006.01)

(52) **U.S. Cl.** **435/6**; 536/24.33; 422/186.01

(57) **ABSTRACT**

Disclosed is an improvement in methods for nucleic acid and immunological bioassays. The methods comprise a step for "sweeping" paramagnetic bead: target molecule complexes so as to capture them with an affinity capture agent on a test pad by moving a magnetic force field from outside to inside the test pad area so as to bring into contact the paramagnetic complexes with the capture agent, while sweeping any unbound paramagnetic material off the test pad by moving the magnetic field from inside to outside the test pad area. Surprisingly, the paramagnetic complexes are rapidly affinity-extracted from the moving magnetic field.



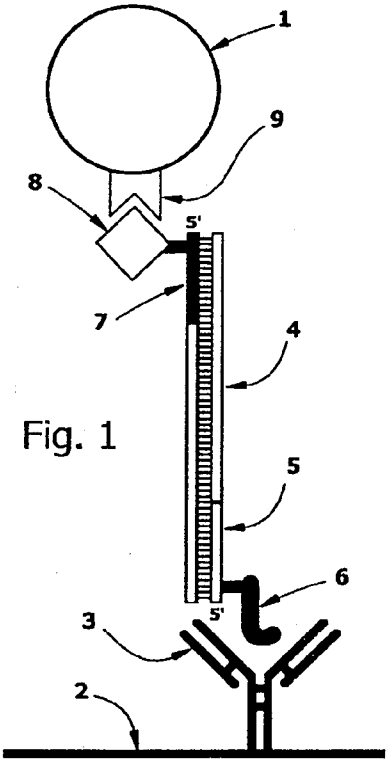


Fig. 1

Fig. 2

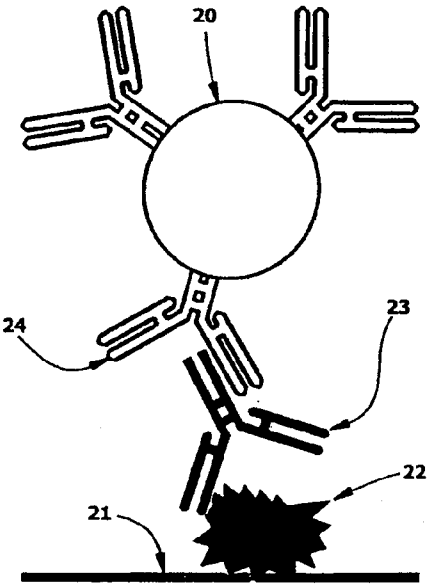


Fig. 3

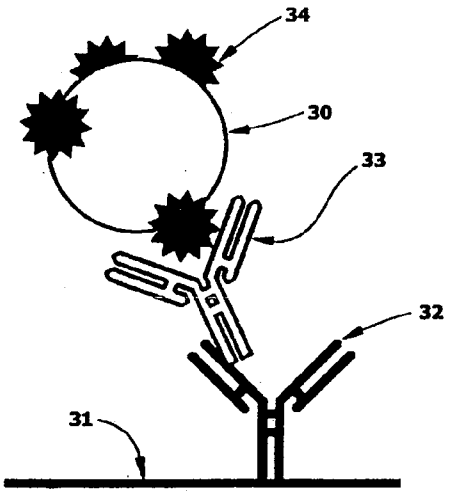
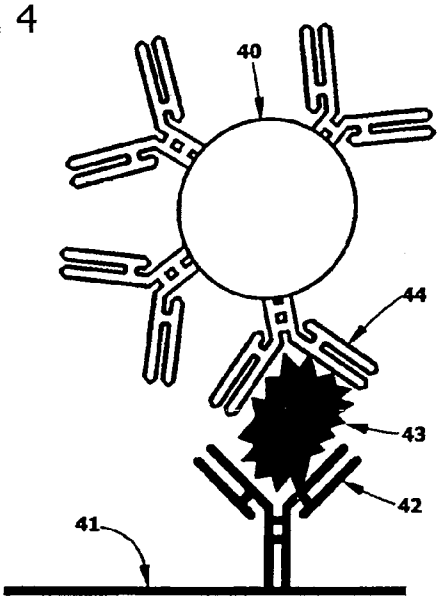


Fig. 4



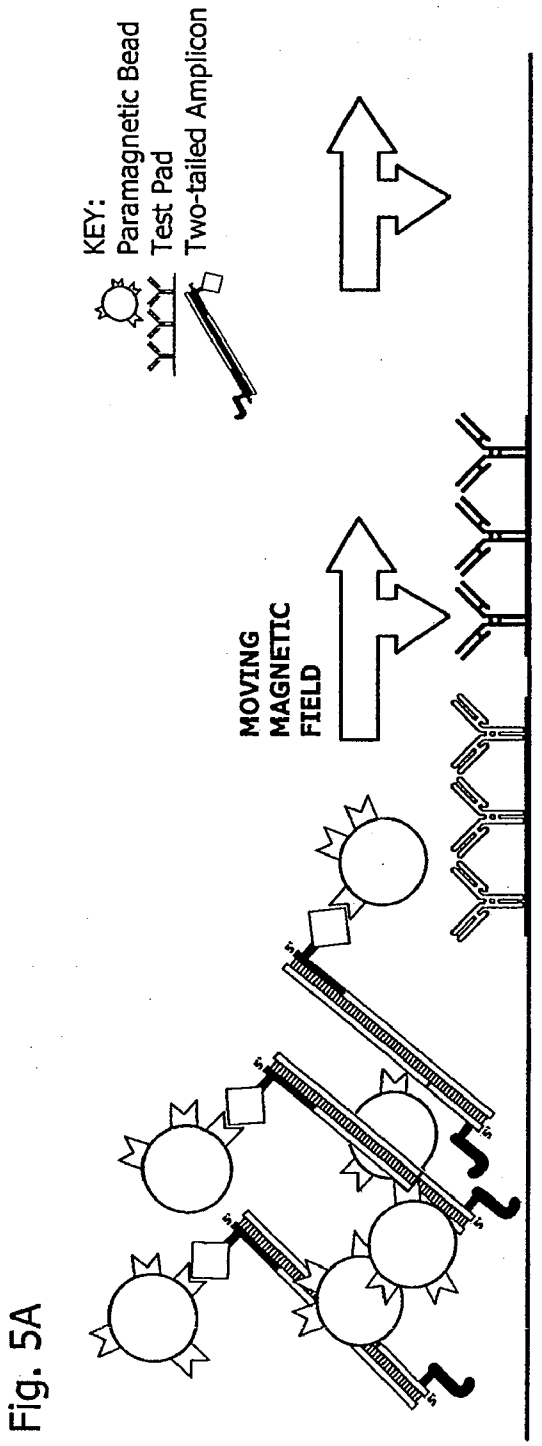

KEY:
Paramagnetic Bead
Test Pad
Two-tailed Amplicon
MOVING MAGNETIC FIELD

Fig. 5A

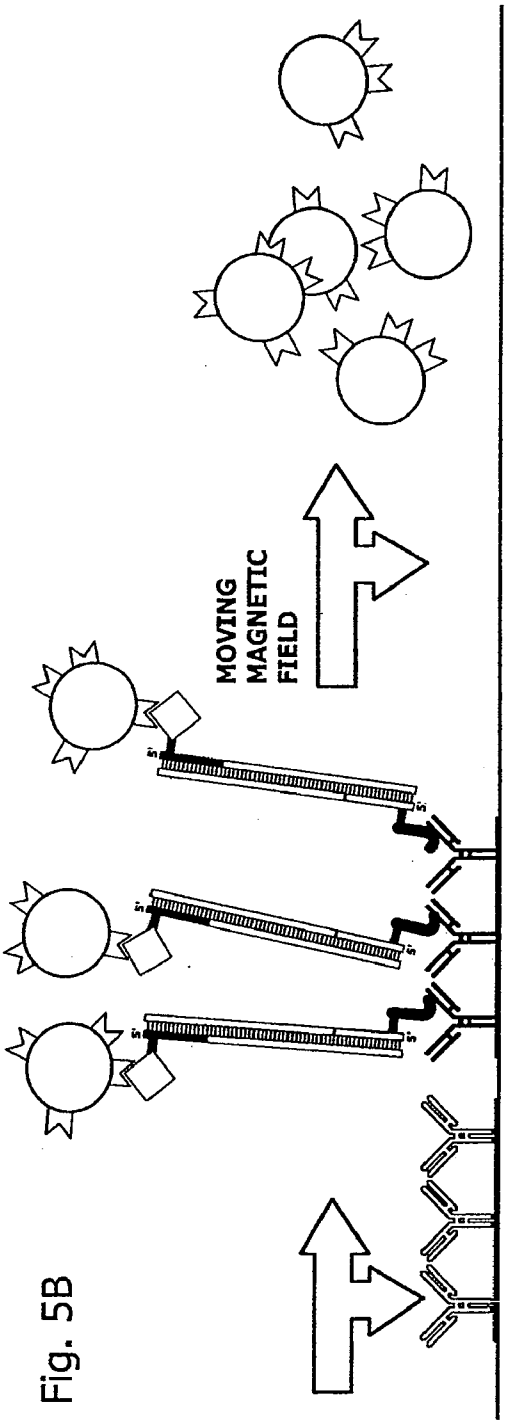

MOVING MAGNETIC FIELD

Fig. 5B

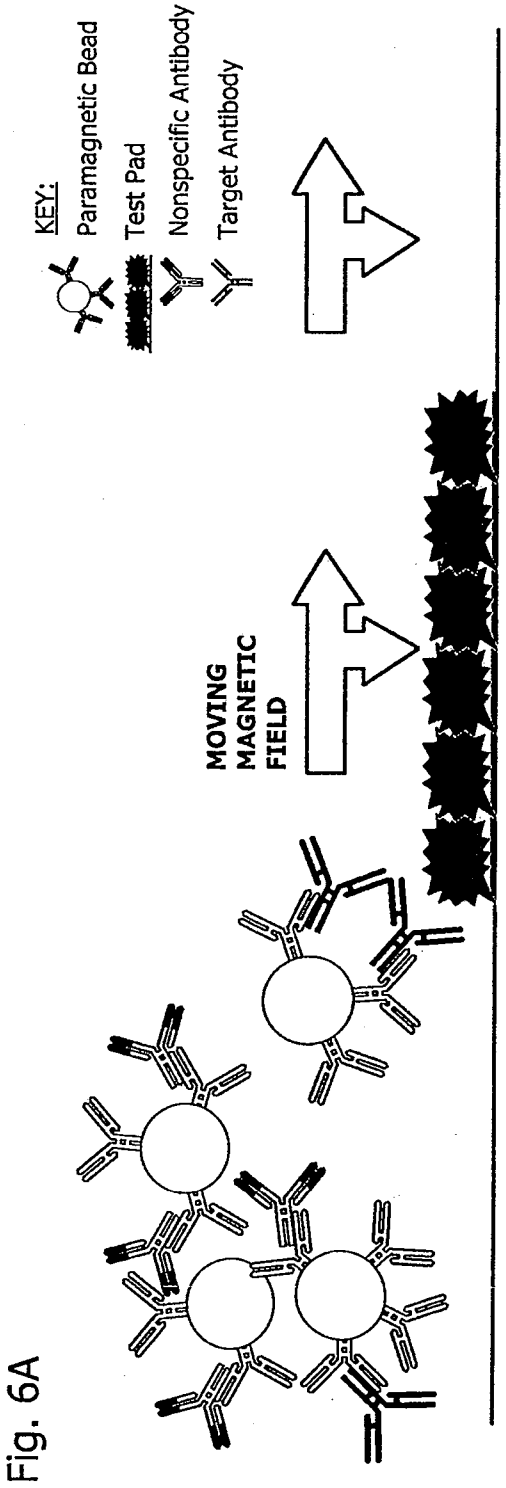

KEY:
Paramagnetic Bead
Test Pad
Nonspecific Antibody
Target Antibody
MOVING MAGNETIC FIELD

Fig. 6A

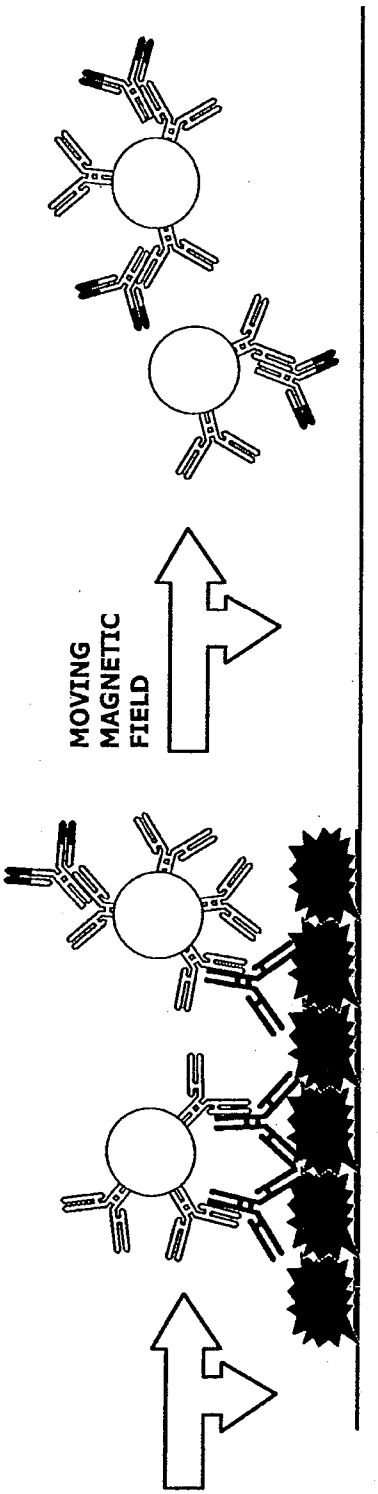

MOVING MAGNETIC FIELD

Fig. 6B

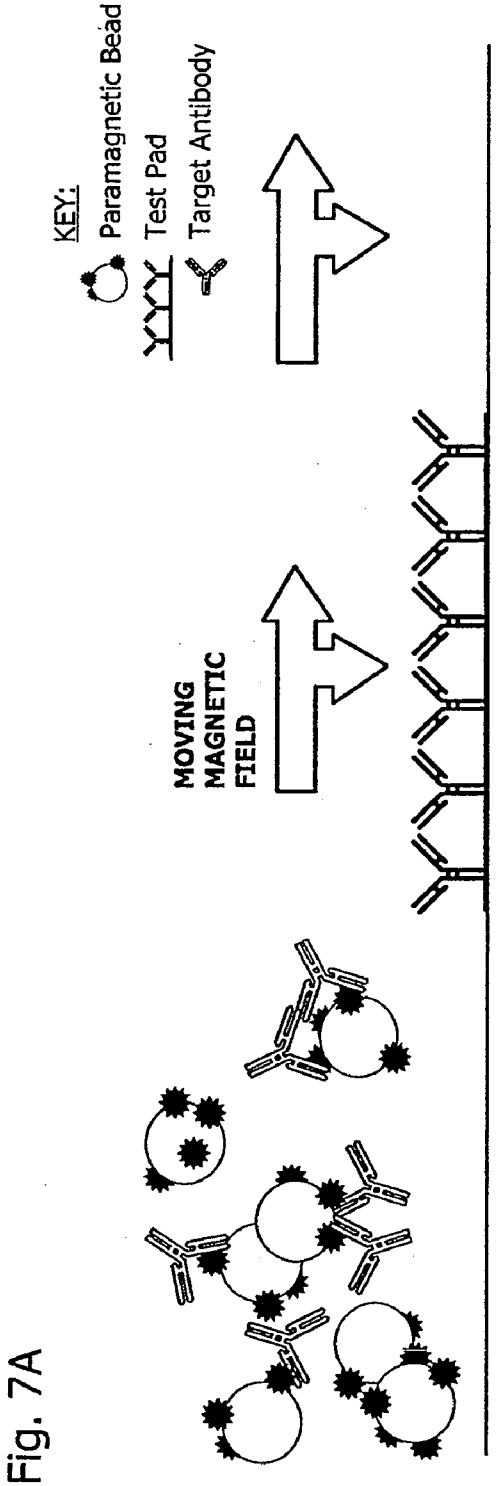

KEY:
Paramagnetic Bead
Test Pad
Target Antibody
MOVING MAGNETIC FIELD

Fig. 7A

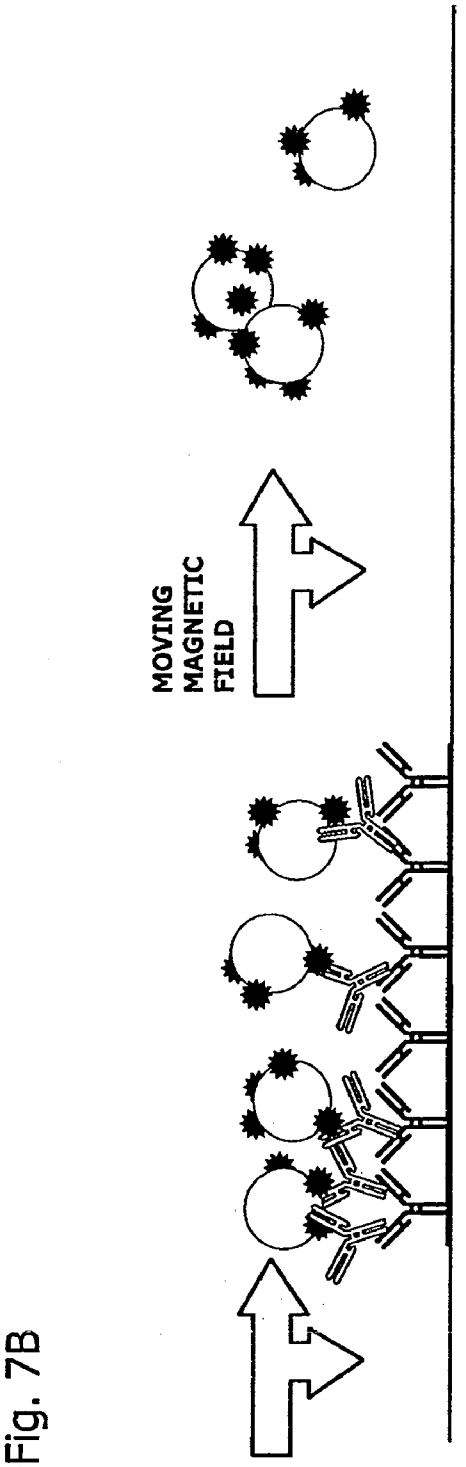

MOVING MAGNETIC FIELD

Fig. 7B

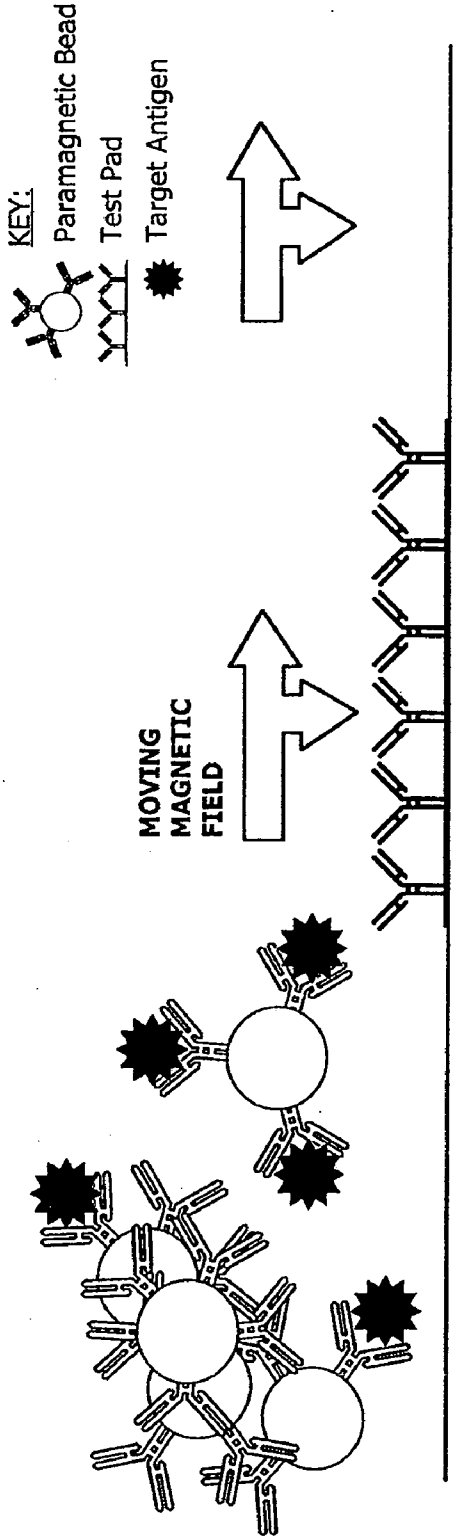

KEY:
Paramagnetic Bead
Test Pad
Target Antigen
MOVING MAGNETIC FIELD

Fig. 8A

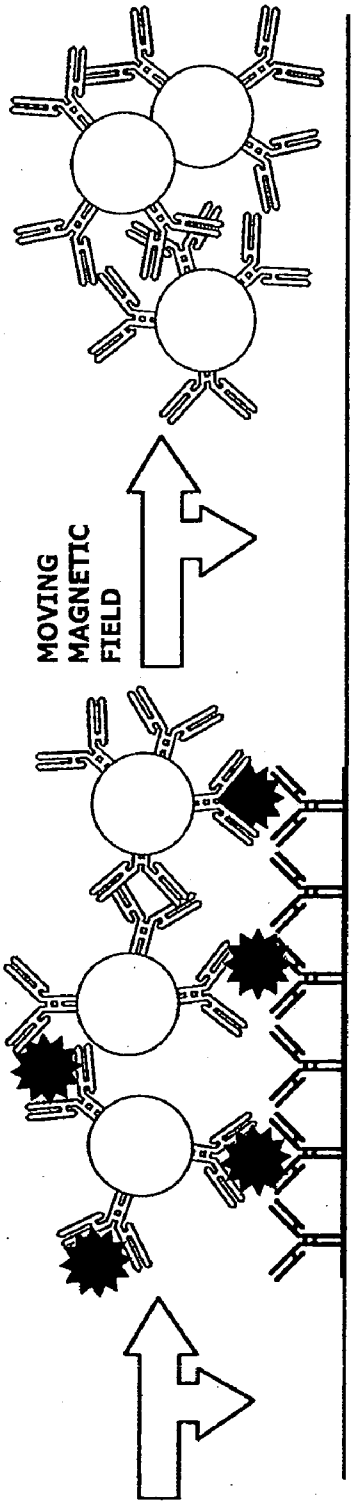

MOVING MAGNETIC FIELD

Fig. 8B

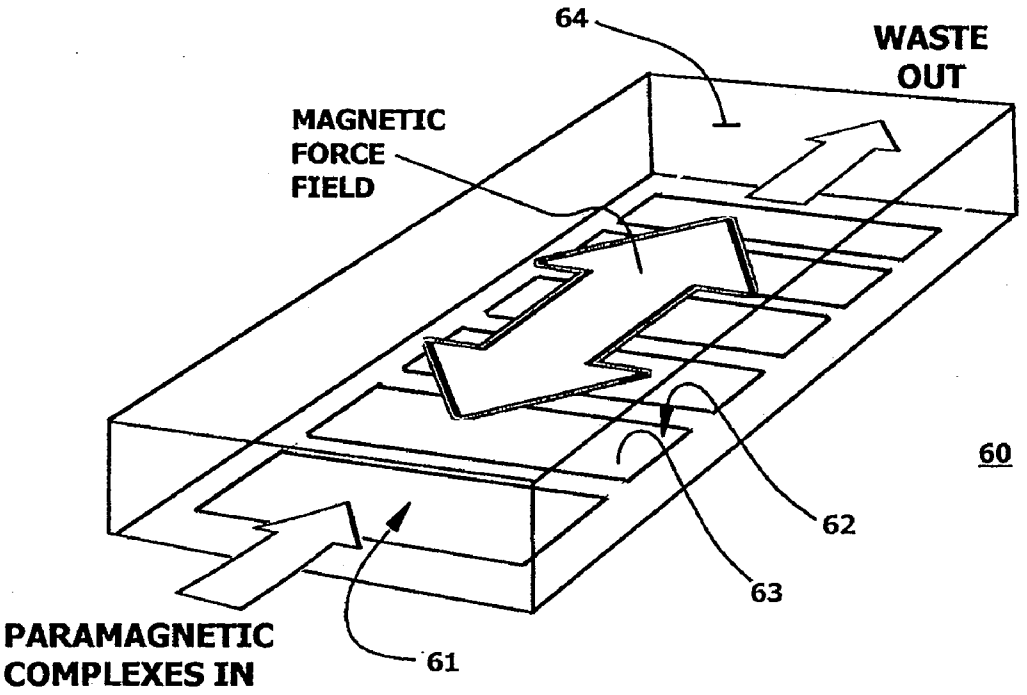

64
WASTE
OUT
MAGNETIC
FORCE
FIELD
60
62
63
PARAMAGNETIC
COMPLEXES IN
61

Fig. 9

Fig. 10

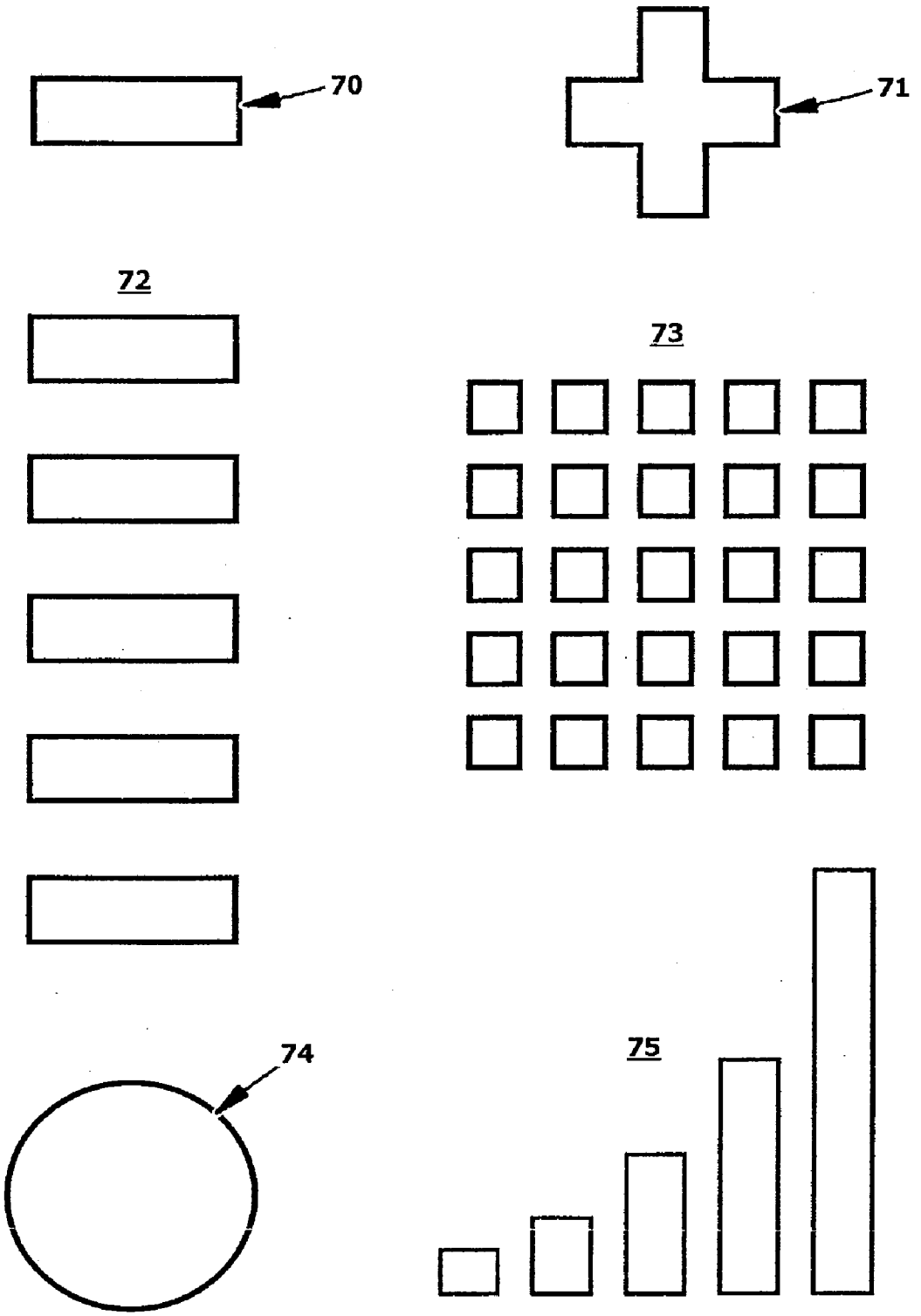


Fig. 11

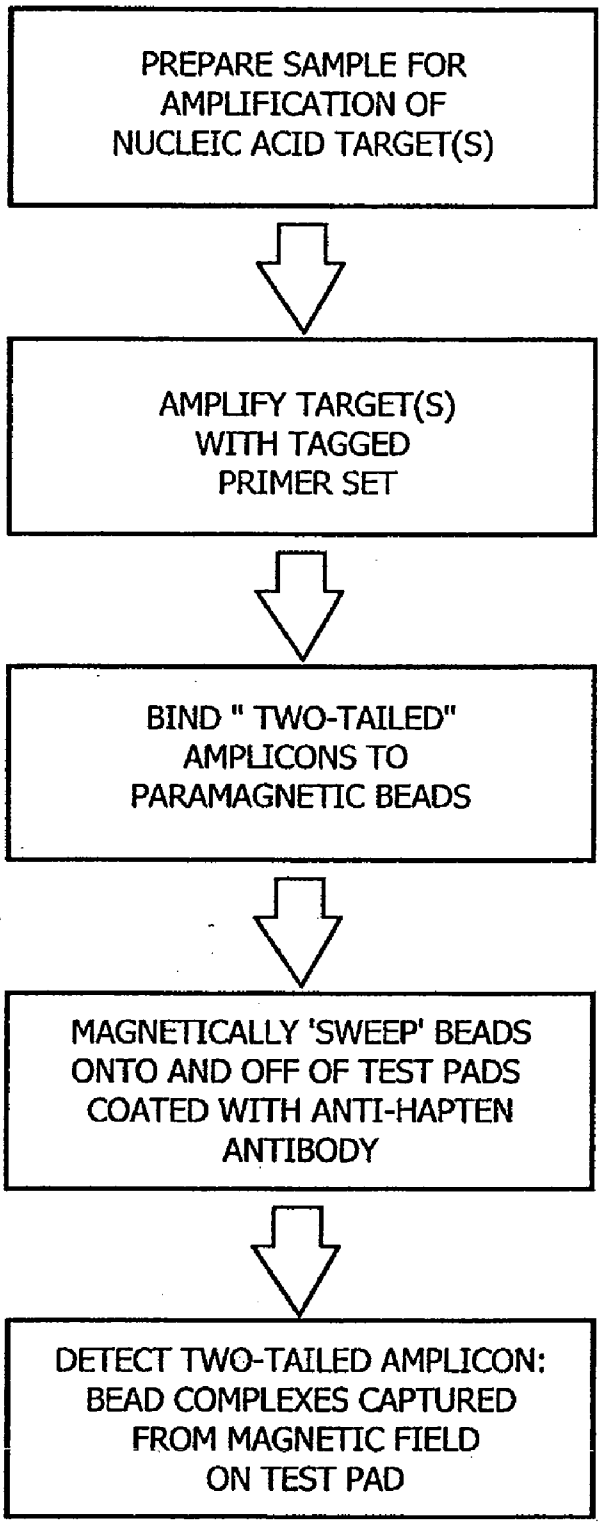

PREPARE SAMPLE FOR AMPLIFICATION OF NUCLEIC ACID TARGET(S)
AMPLIFY TARGET(S) WITH TAGGED PRIMER SET
BIND " TWO-TAILED" AMPLICONS TO PARAMAGNETIC BEADS
MAGNETICALLY 'SWEEP' BEADS ONTO AND OFF OF TEST PADS COATED WITH ANTI-HAPTEN ANTIBODY
DETECT TWO-TAILED AMPLICON: BEAD COMPLEXES CAPTURED FROM MAGNETIC FIELD ON TEST PAD

Fig. 12

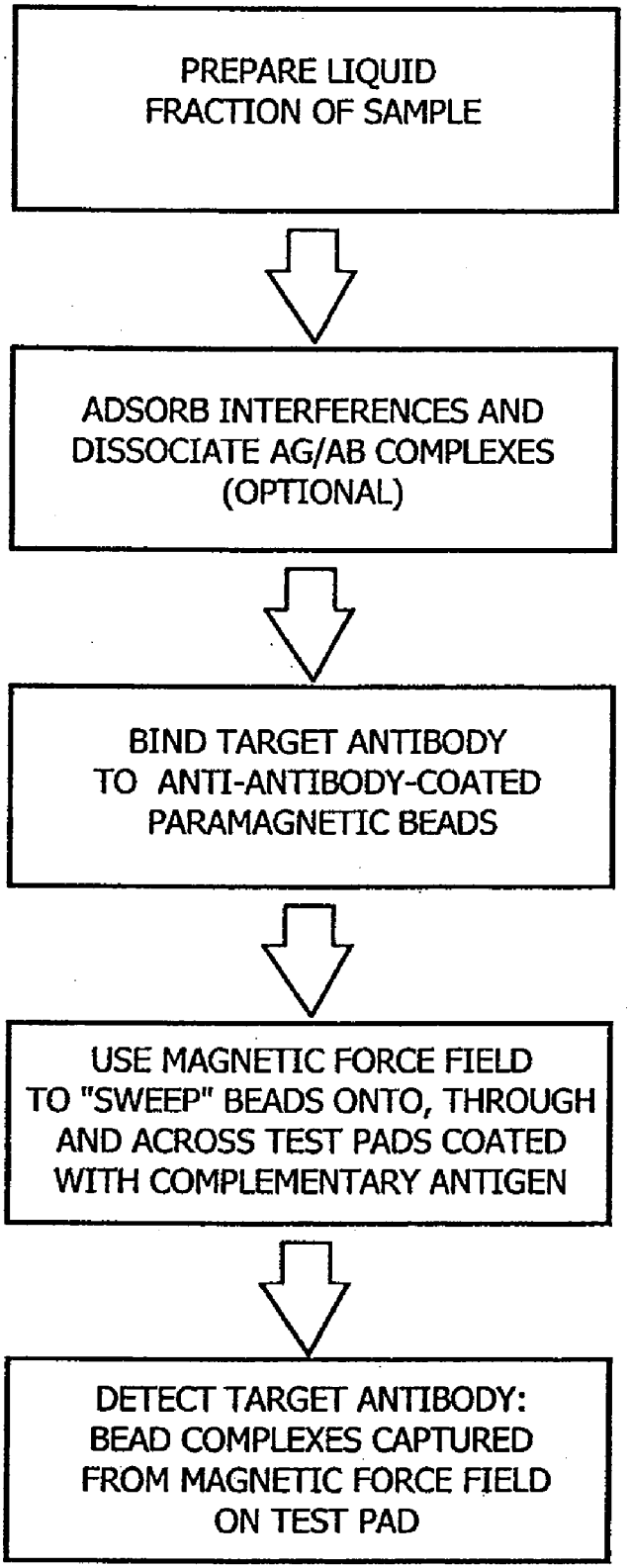

PREPARE LIQUID FRACTION OF SAMPLE
ADSORB INTERFERENCES AND DISSOCIATE AG/AB COMPLEXES (OPTIONAL)
BIND TARGET ANTIBODY TO ANTI-ANTIBODY-COATED PARAMAGNETIC BEADS
USE MAGNETIC FORCE FIELD TO "SWEEP" BEADS ONTO, THROUGH AND ACROSS TEST PADS COATED WITH COMPLEMENTARY ANTIGEN
DETECT TARGET ANTIBODY: BEAD COMPLEXES CAPTURED FROM MAGNETIC FORCE FIELD ON TEST PAD

Fig. 13

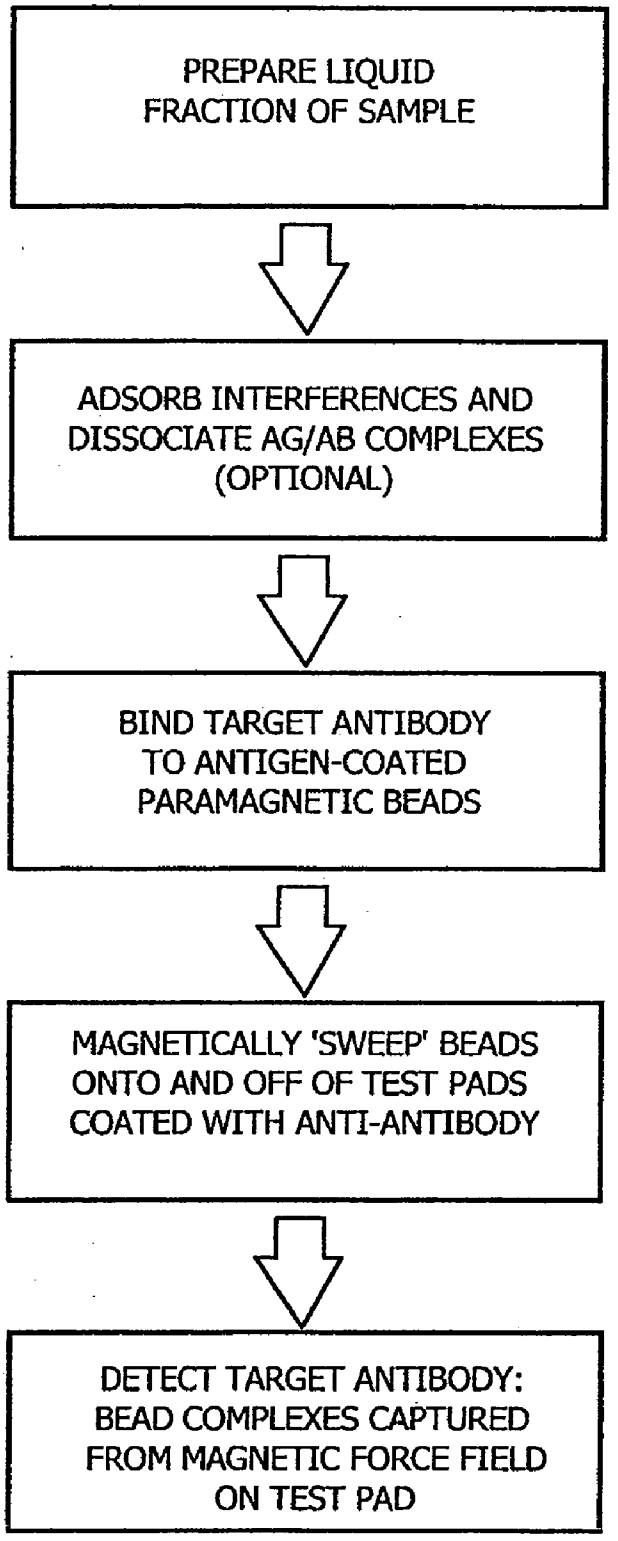

PREPARE LIQUID FRACTION OF SAMPLE
ADSORB INTERFERENCES AND DISSOCIATE AG/AB COMPLEXES (OPTIONAL)
BIND TARGET ANTIBODY TO ANTIGEN-COATED PARAMAGNETIC BEADS
MAGNETICALLY 'SWEEP' BEADS ONTO AND OFF OF TEST PADS COATED WITH ANTI-ANTIBODY
DETECT TARGET ANTIBODY: BEAD COMPLEXES CAPTURED FROM MAGNETIC FORCE FIELD ON TEST PAD

Fig. 14

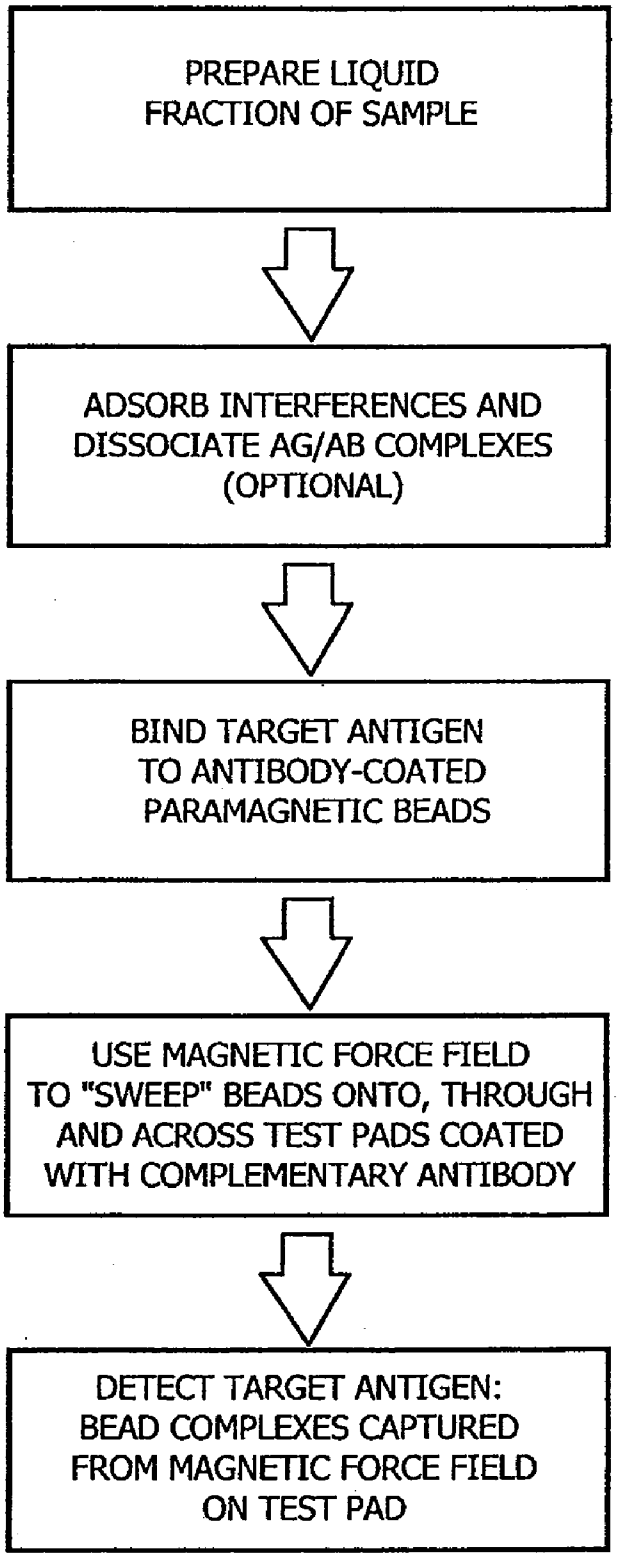

PREPARE LIQUID FRACTION OF SAMPLE
ADSORB INTERFERENCES AND DISSOCIATE AG/AB COMPLEXES (OPTIONAL)
BIND TARGET ANTIGEN TO ANTIBODY-COATED PARAMAGNETIC BEADS
USE MAGNETIC FORCE FIELD TO "SWEEP" BEADS ONTO, THROUGH AND ACROSS TEST PADS COATED WITH COMPLEMENTARY ANTIBODY
DETECT TARGET ANTIGEN: BEAD COMPLEXES CAPTURED FROM MAGNETIC FORCE FIELD ON TEST PAD

Fig. 15

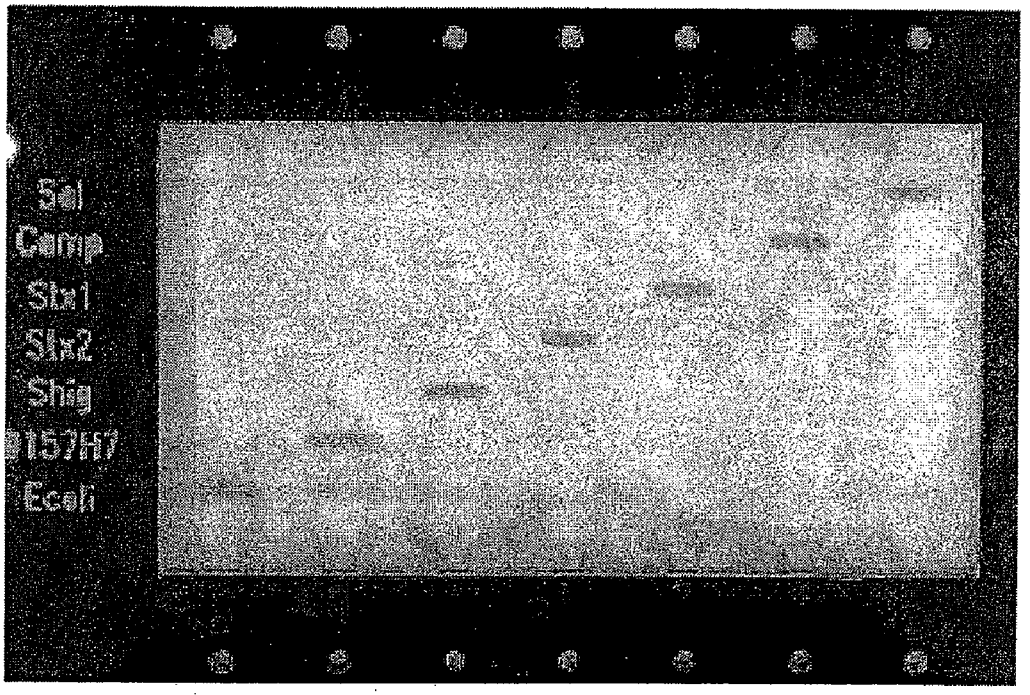
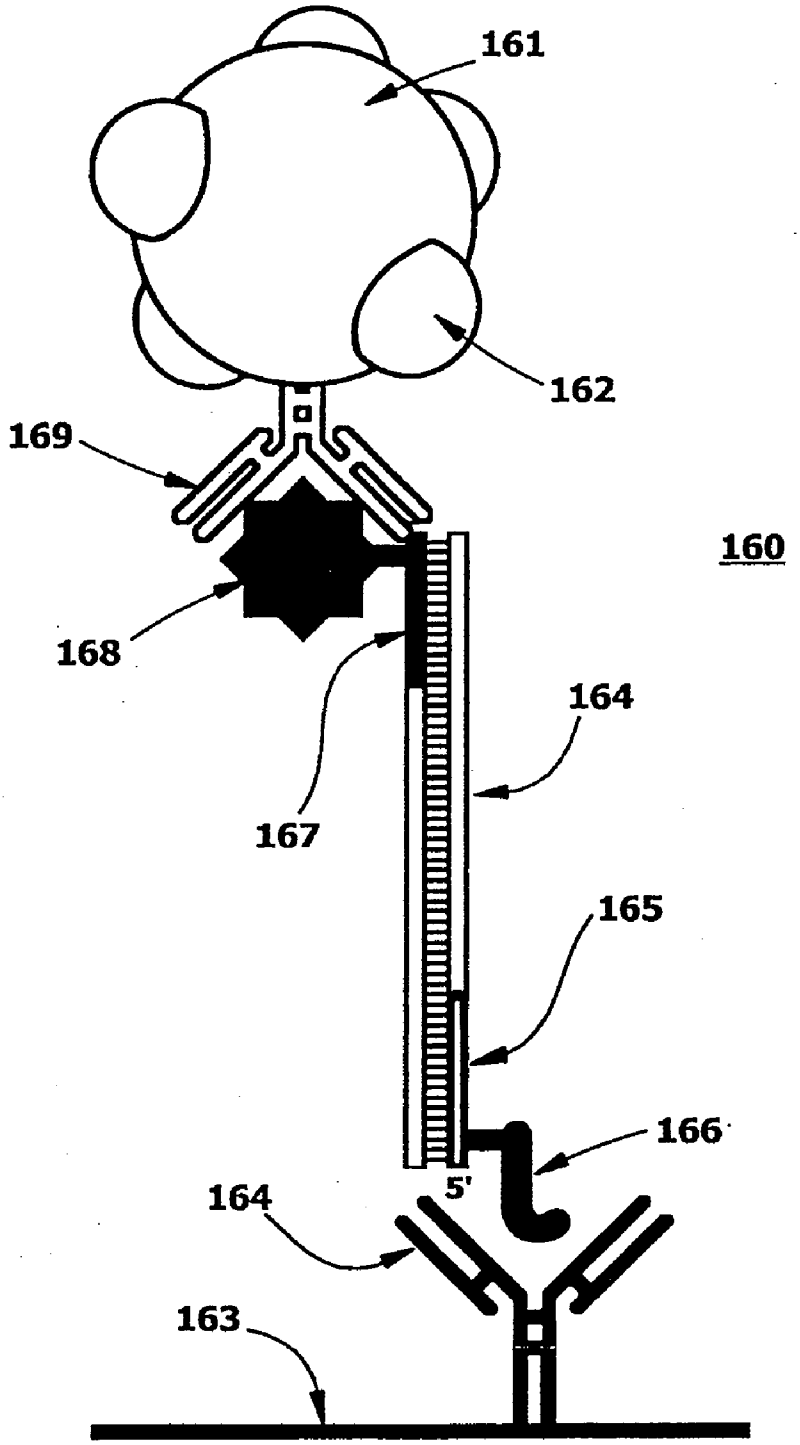


Fig. 16



RAPID MAGNETIC FLOW ASSAYS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation of International PCT Patent Application No. PCT/US2007/006585, filed Mar. 15, 2007, now pending, which claims the benefit under 35 U.S.C. 119(e) of U.S. Provisional Patent Application No. 60/782,649, filed Mar. 15, 2006, and U.S. Provisional Patent Application No. 60/844,811, filed Sep. 14, 2006. These applications are incorporated herein by reference in their entireties.

STATEMENT OF GOVERNMENT INTEREST

[0002] This invention was made with government support under Contract No. UO1 AI061187, awarded by the National Institutes of Health. The government has certain rights in this invention.

STATEMENT REGARDING SEQUENCE LISTING

[0003] The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is 660115_455C1_SEQUENCE_LISTING.txt. The text file is 6 KB, was created on Sep. 3, 2008, and is being submitted electronically via EFS-Web.

BACKGROUND OF THE INVENTION

[0004] 1. Field of the Invention

[0005] The present invention relates to the general fields of molecular biology and medical science, and more particularly to improved methods for nucleic acid and immunological bioassays.

[0006] 2. Description of the Related Art

[0007] There has been a dramatic transition in clinical laboratory diagnostic assays from the macroscale to the microscale, with specimen volume requirements decreasing from milliliters to microliters, and continuing reduction of assay times from hours to minutes.

[0008] These improvements are due in part to advances in materials and fabrication, to the rapidity of mass and heat transfer at the microscale, and to increases in detection sensitivity, but also represent a continuing effort at innovation.

[0009] Numerous heterogeneous binding assay systems are known in the prior art and need not be reviewed here. Particles, beads and microspheres, impregnated with color or having a higher diffraction index, are widely used to speed target isolation. The sensitivity of these assays can be improved with ELISA, with fluorescent dyes, by fluorescence quenching, with QDots as tags, for example, thereby achieving higher sensitivity, smaller test pads and larger arrays. Increasingly, nucleic acid assay targets have replaced serological testing. Conventional means for nucleic acid amplification have had a dramatic effect on assay sensitivity and robustness.

[0010] However, more can be accomplished to improve sensitivity and accelerate detection of the assay endpoint. Typically the problem is one of diffusion kinetics and mass transfer.

[0011] The zeta-potential at the shear boundary layer around particles in solution can slow the close approach needed for binding and immobilization of an assay target. In U.S. Pat. No. 6,720,411, particle colloids such as gold colloids coated with oligomers are aggregated in such a way that the color changes with the state of aggregation. As illustrated in Example 2B of U.S. Pat. No. 6,720,411, the color changes noted are reported to occur over several hours. The endpoint is temperature and salt sensitive and thus represents Brownian motion as path length, the counterion layer as a diffusion barrier, and reduction of interfacial tension as a driving potential. Antibodies can be detected by similar methods, as illustrated in U.S. Pat. No. 6,974,669. However, these detection methods are inherently slow.

[0012] Target migration and complexation rates in a solid chromatographic matrix also limit the sensitivity and velocity of endpoint detection in lateral flow assays. Immunochromatographic tests, commonly referred to as Lateral Flow Assays, have been widely used for qualitative and semi-quantitative assays relying on visual detection. One advantage is the wide variety of analytes that can be detected using this type of test. Consequently, a large industry exists for commercialization of this methodology. See, e.g., U.S. Pat. No. 5,120,643, U.S. Pat. No. 4,943,522, U.S. Pat. No. 5,770,460, U.S. Pat. No. 5,798,273, U.S. Pat. No. 5,504,013, U.S. Pat. No. 6,399,398, U.S. Pat. No. 5,275,785, U.S. Pat. No. 5,504,013, U.S. Pat. No. 5,602,040, U.S. Pat. No. 5,622,871, U.S. Pat. No. 5,656,503, U.S. Pat. No. 4,855,240, U.S. Pat. No. 5,591,645, U.S. Pat. No. 4,956,302, U.S. Pat. No. 5,075,078, and U.S. Pat. No. 6,368,876 and U.S. Pat. No. 648,982. Techniques for lateral flow assays are discussed in TechNotes #303 "Lateral Flow Tests" by Bang's Laboratories, Inc. (Fishers, Ind.), which is incorporated herein in full by reference.

[0013] As another example, in U.S. Pat. No. 5,989,813, amplicons are prepared by amplification of target nucleic acid sequences in the presence of forward and reverse primers conjugated with biotin and digoxigenin, respectively, for use in lateral flow assays. The amplicons are bound to particles with streptavidin and agglutinate in the presence of antibody to digoxigenin. By lateral flow in a sorbent, bifunctional amplicon complexes are detected as trapped aggregates excluded from the fibrous matrix. Other solids are interferences in the assay. In a second variant of the lateral flow format, the avidin conjugates are wicked into a membrane and migrate until encountering a detection strip coated with a capture agent. Accumulation of dyed particles at the detection strip is detected. The assays are generally dependent on flow rate in the materials, particle size and pore dimensions as well as laminar barriers to diffusion.

[0014] In Lateral Flow Assays, it is well known that capillary flow rate and adequate contact between the analyte and its corresponding capture antibody immobilized within the membrane are critical to the assay sensitivity. This demands careful membrane selection to optimize dwell time and flow rates. Contact between capture antibody and target analyte again involves convective and diffusional barriers to endpoint detection. These and other limitations of lateral flow assays are discussed in co-assigned US Patent Application 2007/0042427, "Microfluidic Laminar Flow Detection Strip", herein incorporated in full by reference.

[0015] It is not uncommon that magnetic beads are used to concentrate bioanalytes before or during assay (see for example US 2003/0032028). Beads have several advantages over arrays because beads have a higher specific surface area, move through the liquid sample matrix, and hence have more encounters per unit time with an assay target than the corre-

sponding array. Conceptually, use of magnetic microspheres is generally regarded as a concentration step, substituting for centrifugation or filtration.

[0016] Magnetic microbeads are also commonly used to position and contact analytes with reagents or solid substrates, as for example described in U.S. Pat. No. 5,660,990, U.S. Pat. No. 5,707,807, U.S. Pat. No. 6,815,160, 2002/0086443, 2002/0192676, 2003/0215825, 2004/0018611, 2004/01211364, 2005/0142582, and cumulative related citations representative of the prior art, all of which are incorporated here in full by reference. These examples show the breadth of the applications for microbeads. In US 2006/0292588, where magnetic control circuitry for bead washing is provided in an assay apparatus, time to assay endpoint is again the critical factor (FIG. 1 of US 2006/0292588, showing 5 hr to endpoint).

[0017] Magnetic beads have proven remarkably amenable to surface chemistry, and are routinely derivatized as assay reagents. Such chemistries include functional groups selected from carboxylate, amine, amide, hydrazide, anhydride, hydroxyl, sulfhydryl, chloromethyl, aldehyde, glycidyl (epoxy), and others. A broad range of applications exists.

[0018] In adapting microbeads to a microfluidics assay format, the problem of laminar convective and diffusional boundaries again must be overcome to optimize sensitivity and time to endpoint.

[0019] Accordingly, there remains a need for a generally applicable improvement in the sensitivity and speed of endpoint detection in nucleic acid and immunological bioassays.

[0020] Co-assigned patents and patent applications relevant to the development methods for nucleic acid and antibody bioassays in a microfluidic assay format include U.S. Pat. No. 6,743,399 ("Pumpless Microfluidics"), U.S. Pat. No. 6,488,896 ("Microfluidic Analysis Cartridge"), US Patent Applications 2005/0106066 ("Microfluidic Devices for Fluid Manipulation and Analysis"), 2002/0160518 ("Microfluidic Sedimentation"), 2003/0124619 ("Microscale Diffusion Immunoassay"), 2003/0175990 ("Microfluidic Channel Network Device"), 2005/0013732 ("Method and system for Microfluidic Manipulation, Amplification and Analysis of Fluids, For example, Bacteria Assays and Antiglobulin Testing"), US Patent Application 2007/0042427, "Microfluidic Laminar Flow Detection Strip", and unpublished documents "Microfluidic Cell Capture and Mixing Circuit", "Polymer Compositions and Hydrogels", "Microfluidic Mixing and Analytical Apparatus," "System and method for diagnosis of infectious diseases", and "Microscale Diffusion Immunoassay Utilizing Multivalent Reactants", all of which are hereby incorporated in full by reference.

BRIEF SUMMARY OF THE INVENTION

[0021] Surprisingly, ligand-tagged paramagnetic microbeads are readily extracted from a moving magnetic field by formation of molecular tethers with solid phase substrates coated with affinity ligand-binding molecules.

[0022] At odds with this finding, the prior art has taught that such molecular tethers are easily broken and that stationary magnetic fields are needed to keep paramagnetic beads immobilized during washing and separation of bound and unbound beads. Relevant to affinity capture, US 2004/0226348, hereby incorporated in full by reference, states for example, with respect to paramagnetic microbeads, "A major concern with the bead assay is the amount of force that a few covalent bonds has to hold a bead to the detection surface" (para 0007), indicating that the strength of a covalent bond is relatively weak. The disclosure continues, "Electromagnets can be controlled to exert a precise amount of force. This is critical in the stage of washing in an assay, where beads attached to a bottom testing surface are separated from beads that are unattached. During this stage, the precision in the amount of force applied to the beads is critical because the difference in force between moving an unattached bead and one that is tethered (i.e., attached) with a few covalent bonds (or biotin/avidin or DNA hybridization) may be extremely slight. Care must be taken to ensure that unattached beads are the only ones moved and the tethered beads remain attached to an intended surface" (para 0033). It was taught that, "The use of electromagnets eliminates the need to design precise flow mechanisms to keep beads in place" (para 0009). Similar teachings are reported in US 2004/0005718, where is stated, "Since magnetic beads to which probe DNA is attached are fixed to the substrate by being attracted by a magnetic force, the magnetic beads can be fixed to the substrate by a stronger force than the conventional bonding of probe DNA with the substrate" (para 0037), again teaching that a magnetic force is stronger than a molecular binding force.

[0023] It was thus unexpected that tagged paramagnetic beads can be affinity extracted from a moving magnetic field, not simply directed to or retained on a test pad by a stationary magnetic field. We found that a detectable endpoint for a bioassay can thus be achieved in one simple step wherein first a population of ligand-tagged paramagnetic microbeads is captured on an affinity-binding test pad as a magnetic field moves the bead complexes across the test pad, and second, as the magnetic field moves away, affinity tagged paramagnetic beads remain bound, but unbound paramagnetic beads are separated and pulled away to waste.

[0024] In the preferred method, the magnetic force field has both a perpendicular force vector and a lateral force vector. The paramagnetic beads are attracted to a surface or substrate by a magnetic force field emanating from the opposite side of the surface, and as the magnetic field moves laterally, the paramagnetic beads are dragged across the test pad while following the magnetic flux laterally. Tagged magnetic beads so readily adhere to the test pad in this way that visual detection endpoints may be used. Although a visual endpoint is preferable for its simplicity, the invention is not to be construed as limited to such.

[0025] We also show how this improvement in rapidity of the detection step can be integrated into various classes of assays for nucleic acids, direct and indirect assays for immunoactive targets, and other bioassays. Current detection time from sample introduction to detection is about 5 min, including filtration, extraction, and amplification.

While microfluidic devices are used in the embodiments of the examples reduced to practice herein, the invention again should not be construed as limited to such.

[0026] The method comprises the steps of:

[0027] a) Immobilizing an affinity capture agent within an area on a substrate within a fluid path, said fluid path with axis of flow, thereby forming a test pad area;

[0028] b) Binding a bioassay target molecule to a paramagnetic microbead reagent in a fluid and contacting the fluid with the substrate within said fluid path;

[0029] c) Sweeping the paramagnetic microbead reagent in the fluid into close contact with the affinity capture agent by moving a magnetic force field on a plane parallel to the axis of flow from outside to inside the test pad area, and thereby

affinity capturing any bioassay target molecule bound to the paramagnetic bead reagent from said magnetic force field in the form of a molecular detection complex; and upon forming said molecular detection complex, then sweeping from the test pad area any paramagnetic microbead reagent in the fluid not formed as molecular detection complex by moving the magnetic force field on a plane parallel to the axis of flow from inside to outside the test pad area; and,

[0030] d) Detecting said molecular detection complex in the test pad area.

[0031] In another aspect of the invention, we also show that peptidyl-conjugates to the 5' tail of amplification primer sets are generally applicable in polymerase-dependent amplification protocols and are further robust, surprisingly retaining full antigenicity and binding integrity following amplification. We show that an immobilized antibody, for example a monoclonal antibody, specific to a peptide-conjugated amplication primer will capture the products of amplification tagged with the primer. By using a second primer tagged with a second affinity ligand, rapid methods for forming target specific detection complexes are readily designed. Peptidyl-conjugated oligonucleotides have not previously been used as primers in PCR amplification, or in other amplification protocols, or used as means for tagging and discriminating mixed PCR products in multiplex target detection protocols. These detection complexes thus serve essentially as means for interrogating a peptidyl-primer amplicon library. Unexpectedly, this method has more breadth than prior art methods of tagging primers, which are limited to a few species of binding pairs, permitting simultaneous separation and detection of an essentially infinite number of amplicons by the step of tagging each amplicon with a unique peptide hapten (herein "peptidyl hapten") and employing the corresponding antibody to capture and immobilize it. The magnetic bead assay methods illustrated here are one embodiment of this discovery.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING(S)

[0032] FIGS. **1-4** depict affinity-immobilized paramagnetic target molecule binding complexes as detection complexes. Shown are four "sandwich" detection complexes involving a paramagnetic target molecule binding complex and a test pad.

[0033] FIG. **5** is a pictograph describing (in panel **5**A) the use of a vectored moving magnetic field to sweep paramagnetic two-tailed amplicon complexes across and over two test pads while magnetically contacting them with two species of capture antibodies immobilized on the test pads, and (in panel **5**B) the resultant immuno-immobilized complexes on the test pad bearing an antibody specific to the paramagnetic target molecule complexes.

[0034] FIG. **6** is a pictograph describing (in panel **6**A) the use of a vectored moving magnetic field to sweep paramagnetic antigen:antibody complexes across and over a test pad while magnetically contacting them with capture antigen immobilized on the test pad, and (in panel **6**B) the resultant immuno-immobilized complexes on the test pad.

[0035] FIG. **7** is a pictograph describing (in panel **7**A) the use of a vectored moving magnetic field to sweep paramagnetic target antibody:antigen complexes across and over a test pad while magnetically contacting them with capture anti-antibodies immobilized on the test pad, and (in panel **7**B) the resultant immuno-immobilized complexes on the test pad.

[0036] FIG. **8** is a pictograph describing (in panel **8**A) the use of a vectored moving magnetic field to sweep paramagnetic target antibody:target antigen complexes across and over a test pad while magnetically contacting them with capture antibodies immobilized on the test pad, and (in panel **8**B) the resultant immuno-immobilized complexes on the test pad.

[0037] FIG. **9** is a sketch showing the use of the method in a microfluidic detection chamber for a multiplex assay. The magnetic field can be used to sweep the paramagnetic target molecule complexes across and over multiple test pads, or to scrub the test pads back and forth with the complexes in order to form affinity-immobilized complexes.

[0038] FIG. **10** is a conceptual schematic of test pads and test pad arrays as may be useful in the method.

[0039] FIG. **11** is a flow chart depicting steps of a method for detection of affinity-immobilized amplicons.

[0040] FIG. **12** is a flow chart depicting steps of a method for detection of affinity-immobilized target antibody:antibody complexes with antigen.

[0041] FIG. **13** is a flow chart depicting steps of a method for detection of affinity-immobilized target antibody:antigen complexes with anti-antibody.

[0042] FIG. **14** is a flow chart depicting steps of a method for detection of affinity-immobilized target antigen:antibody complexes with complementary antibody.

[0043] FIG. **15** is a reproduction of a photograph of parallel detection chambers in a microfluidic card treated by the inventive method.

[0044] FIG. **16** pictographically depicts an affinity-immobilized molecular detection complex with complex paramagnetic microbead tethered to a solid phase by a two-tailed amplicon complex.

DETAILED DESCRIPTION OF THE INVENTION

[0045] The following definitions are provided as an aid in interpreting the claims and specification herein. Where works are cited by reference, and definitions contained therein are inconsistent in part or in whole with those supplied here, the definition used therein may supplement but shall not supersede or amend the definition provided herein.

1. DEFINITIONS

[0046] Test samples: Representative biosamples include, for example: blood, serum, plasma, buffy coat, saliva, wound exudates, pus, lung and other respiratory aspirates, nasal aspirates and washes, sinus drainage, bronchial lavage fluids, sputum, medial and inner ear aspirates, cyst aspirates, cerebral spinal fluid, stool, diarrhoeal fluid, urine, tears, mammary secretions, ovarian contents, ascites fluid, mucous, gastric fluid, gastrointestinal contents, urethral discharge, synovial fluid, peritoneal fluid, meconium, vaginal fluid or discharge, amniotic fluid, semen, penile discharge, or the like may be tested. Assay from swabs or lavages representative of mucosal secretions and epithelia are acceptable, for example mucosal swabs of the throat, tonsils, gingival, nasal passages, vagina, urethra, rectum, lower colon, and eyes, as are homogenates, lysates and digests of tissue specimens of all sorts. Mammalian cells are acceptable samples. Besides physiological fluids, samples of water, industrial discharges, food products, milk, air filtrates, and so forth are also test specimens. In some embodiments, test samples are placed directly in the device; in other embodiments, pre-analytical processing is contemplated.

[0047] Bioassay Target Molecule: or "analyte of interest", or "target molecule", may include a nucleic acid, a protein, an antigen, an antibody, a carbohydrate, a cell component, a lipid, a receptor ligand, a small molecule such as a drug, and so forth. Target nucleic acids include genes, portions of genes, regulatory sequences of genes, mRNAs, rRNAs, tRNAs, siRNAs, cDNA and may be single stranded, double stranded or triple stranded. Some nucleic acid targets have polymorphisms, deletions and alternate splice sequences. Multiple target domains may exist in a single molecule, for example an immunogen may include multiple antigenic determinants. An antibody includes variable regions, constant regions, and the Fc region, which is of value in immobilizing antibodies.

[0048] Pathogen: an organism associated with an infection or infectious disease.

[0049] Pathogenic condition: a condition of a mammalian host characterized by the absence of health, i.e., a disease, infirmity, morbidity, or a genetic trait associated with potential morbidity.

[0050] "Target nucleic acid sequence" or "template": As used herein, the term "target" refers to a nucleic acid sequence in a biosample that is to be amplified in the assay by a polymerase and detected. The "target" molecule can be present as a "spike" or as an uncharacterized analyte in a sample, and may consist of DNA, cDNA, gDNA, RNA, mRNA, rRNA, or miRNA, either synthetic or native to an organism. The "organism" is not limited to a mammal. The target nucleic acid sequence is a template for synthesis of a complementary sequence during amplification. Genomic target sequences are denoted by a listing of the order of the bases, listed by convention from 5' end to 3' end.

[0051] Reporter, "Label" or "Tag" refers to a biomolecule or modification of a biomolecule that can be detected by physical, chemical, electromagnetic and other related analytical techniques. Examples of detectable reporters include, but are not limited to, radioisotopes, fluorophores, chromophores, mass labels, electron dense particles, magnetic particles, dyed particles, spin labels, molecules that emit chemiluminescence, electrochemically active molecules, enzymes, cofactors, enzymes linked to nucleic acid probes, and enzyme substrates. Reporters are used in bioassays as reagents, and are often covalently attached to another molecule, adsorbed on a solid phase, or bound by specific affinity binding.

[0052] Ligand: any molecule for which there exists another molecule (i.e., an "antiligand" or ligand binding molecule) that binds with specific affinity to the ligand with stereochemical recognition or "fit" of some portion of the ligand by the ligand binding molecule. Forces between ligand and binding molecule are typically Van der Waals, hydrogen bond, hydrophobic bond, and electrostatic bond. Ligand binding is not typically covalent and is thus distinguished from "crosslinked" and "derivatized". As used herein, the term "ligand" is reserved for binding moieties that are not "Peptidyl haptens".

[0053] Peptidyl hapten: Refers to a subclass of haptens that is a peptide fragment. As used herein, peptidyl haptens, or "peptide haptens" are used with their complementary antibody to the peptide fragment as a means for capturing two-tailed amplicons on a solid phase.

[0054] Haptens are "molecular keys" in the Kekulean sense, that when bound to an immunogenic carrier and introduced into a vertebrate, will elicit formation of antibodies specific for the hapten or epitope. These molecular keys have stereochemical specificity, are generally exposed on the surface of the carrier, and are of lower molecular weight than the carrier. Illustrative examples include small-molecule derivatives of native proteins, RNA loop-stem structures, a drug or steroid such as digoxigenin, the carbohydrate side-chains that decorate a mucopeptide, and short chain peptides or helices of non-native proteins such as diphtheria toxin or toxoid. Even a dipeptide or a lipid, when conjugated on a suitable immunogenic carrier, can produce an antibody response, and affinity-captured antibody specific to the dipeptide or lipid itself, not the immunogen, can be produced by absorbing out the non-specific antibodies in an antiserum or by preparing a monoclonal antibody by lymphocyte selection. Although a hapten is not immunogenic of itself, it has very finely directed immunospecificity and is recognized by a very limited set of complementary antibodies.

[0055] As used herein, short chain peptides are a preferred hapten for tagging amplicons as used to create peptidyl-amplicon libraries because of their robust chemistry, compatibility with enzymes as primer labels, and essentially infinite immunospecificity.

[0056] Capture agent: or "affinity capture agent" is a generic term for a complementary partner in an affinity binding pair and is generally used to capture a ligand or hapten by binding it to a solid phase. Affinity binding pairs include streptavidin:biotin, antibody:antigen, hapten:antibody, peptidyl hapten:antibody, and antigen:antibody, for example, and either member of the affinity binding pair may be the capture agent.

[0057] Test pad area—or test strip, or test field, or simply "test pad", as used herein, is an area or zone occupied by an affinity capture agent. The area is 3-dimensional at a nanomolecular level and is generally formed on the surface of a substrate in a liquid flow path. The test pad is generally the site in the assay where the assay endpoint is observed or measured, and as such may be housed in a detection chamber with optical window.

[0058] Heterogeneous capture or immobilization: refers use of affinity binding pairs to concentrate an analyte or detection complex on a solid phase surface, particle, or porous adsorbent material, generally so that the analyte can be detected, concentrated or purified. Heterogeneous or solid phase capture may be achieved with capture agents such as immobilized antigen, antibody, avidin, nickel-NTA, lectin, or other ligand/receptor systems. As referred to herein, the molecular complex formed by heterogeneous capture is the "immobilized reporter complex" and may be the detection complex of a heterogeneous binding assay. Such complexes are stabilized by non-covalent and cooperative binding.

[0059] Amplification: As used here, the term "amplification" refers to a "template-dependent process" that results in an increase in the concentration of a nucleic acid sequence relative to its initial concentration. A "template-dependent process" is a process that involves "template-dependent extension" of a "primer" molecule. A "primer" molecule refers to a sequence of a nucleic acid that is complementary to a known portion of the target sequence. A "template dependent extension" refers to nucleic acid synthesis of RNA or DNA wherein the sequence of the newly synthesized strand of nucleic acid is dictated by the rules of complementary base pairing of the target nucleic acid and the primers.

[0060] Amplicon refers to a double stranded DNA product of a prior art amplification means, and includes double stranded DNA products formed from DNA and RNA templates.

[0061] Two-tailed Amplicon refers to a double stranded DNA product of a prior art amplification means in which tagged primer pairs are covalently incorporated, a first primer conjugated with one affinity tag, a second primer conjugated with a second affinity tag, the two tags being different. As used herein, the two-tailed amplicon functions as a "hetero-bifunctional" tether, and links a magnetic bead to a solid substrate.

[0062] Primer: as used herein, is a single-stranded polynucleotide or polynucleotide conjugate capable of acting as a point of initiation for template-directed DNA synthesis in the presence of a suitable polymerase and cofactors. Primers are generally at least 7 nucleotides long and, more typically range from 10 to 30 nucleotides in length, or longer. The term "primer pair" refers to a set of primers including a 5' "forward" or "upstream" primer that hybridizes with the complement of the 5' end of the DNA template to be amplified and a 3' "reverse" or "downstream" primer that hybridizes with the 3' end of the sequence to be amplified. Note that both primers have 5' and 3' ends and that primer extension always occurs in the direction of 5' to 3'. Therefore, chemical conjugation at or near the 5' end does not block primer extension by a suitable polymerase. Primers may be referred to as "first primer" and "second primer", indicating a primer pair in which the identity of the "forward" and "reverse" primers is interchangeable. Additional primers may be used in nested amplification.

[0063] In the preferred embodiment, the first primer is a monospecific or class-specific oligonucleotide conjugated to a peptide hapten or epitope recognized by a specific antibody. And the second "primer" is an oligonucleotide conjugated to a hapten, to a biotin, a digoxin, a steroid, a polysaccharide, an antigen or fragment thereof, a folic acid, a phycoerythrin dye, a fluorophore, to an Fc fragment of an antibody, to a nickel chelator such as NTA, or to a lectin, 2,4-dinitrophenyl, and so forth, at or near the 5' terminus.

[0064] Complementary (with respect to nucleic acids) refers to two single-stranded nucleic acid sequences that can hybridize to form a double helix. The matching of base pairs in the double helix of two complementary strands is not necessarily absolute. Selectivity of hybridization is a function of temperature of annealing, salt concentration, and solvent, and will generally occur under low stringency when there is as little as 55% identity over a stretch of at least 14-25 nucleotides. Stringency can be increased by methods well known in the art. See M. Kanehisa, *Nucleic Acids Res.* 12:203 (1984). Regarding hybridization of primers, a primer that is "perfectly complementary" has a sequence fully complementary across the entire length of the primer and has no mismatches. A "mismatch" refers to a site at which the base in the primer and the base in the target nucleic acid with which it is aligned are not complementary.

[0065] Complementary (with respect to immunobinding) refers to antibody:immunogen or antibody:hapten binding that is immunospecific.

[0066] Magnetic Microbead: refers to a "nanoparticle", "bead", or "microsphere", or by other terms as known in the art, having at least one dimension, such as apparent diameter or circumference, in the micron or nanometer range. An upper range of such dimensions is 600 um, but typically apparent diameter is under 200 nm, and may be 1-50 um or 5-20 nm, while not limited to such. Such particles may be composed of, contain cores of, or contain granular domains of, a paramagnetic or superparamagnetic material, such as the Fe_2O_3 and Fe_3O_4 (α-Fe crystal type),α'-FeCo, ϵ-Cobalt, CoPt, $CrPt_3$, $SmCo_5$, Nickel and nickel alloys, Cu_2MnAl, α-FeZr, $Nd_2Fe_{14}B$, NoTi, for example. Preferred are the Ferrites, defined as ferrimagnetic or ceramic compound materials consisting of various mixtures of iron oxides such as Hematite (Fe_2O_3) or Magnetite (Fe_3O_4) and iron oxides in alloys with other metals. These materials as used generally are particles having dimensions smaller than a magnetic domain, and may be formed into particles, beads or microspheres with binders such as latex polymers (generically), silica, as is generally well known and inclusive of such materials as are commercially available.

[0067] Particularly preferred are nanoparticles of Fe_3O_4 with diameters in the 50 nm-100 um range as are commercially available for magnetic bioseparations. These particles are "superparamagnetic", meaning that they are attracted to a magnetic field but retain no residual magnetism after the field is removed. Therefore, suspended superparamagnetic particles tagged to the biomaterial of interest can be removed from a matrix using a magnetic field, but they do not agglomerate (i.e., they stay suspended) after removal of the field. Also of interest are nickel and cobalt microbeads. These beads may be reactive with peptides containing histidine.

[0068] Paramagnetic beads have the property that they align themselves along magnetic flux lines and are attracted from areas of lower magnetic flux density to areas of higher magnetic flux density.

[0069] It should be recognized that magnetic microbeads may be composite materials. Such beads may further contain other micro- or nanoparticles agglomerated with a binder. Composites with RF-tags, QDots, up-converting fluorophores, colloid sols and clays, and the like are contemplated for use in the present invention. A magnetic bead need not be formed entirely of a magnetic material, but may instead comprise both magnetic and non-magnetic materials.

[0070] Microbeads may themselves be colloidal and have chromogenic properties, or may be combined with other colloidal metal particles with chromogenic properties. Mixed suspensions of differently modified microbeads may be used.

[0071] Microbeads are by no means simply commodities. They may be modified with surface active agents such as detergents to control their rheological properties, as in ferrofluids. The surface of microbeads may be modified by adsorption or covalent attachment of bioactive molecules, including immunoaffinity agents, antibodies, enzymes, dyes, fluorescent dyes, fluorescent quenchers, oligomers, peptide nucleomers, and the like, and more specifically by coating with streptavidin or single stranded DNA oligomers, for example. These and other cumulative prior art skills are incorporated herein in full without full recitation of their scope, as a full recitation is unnecessary to understand the principles of the current invention except insofar as to recognize that the microbeads of interest herein are comprised of at least one paramagnetic element therein, as would be readily recognized by those skilled in the prior arts.

[0072] Suitable matrices for microbeads include polystyrene, divinylbenzene, polyvinyltoluene, polyester, polyurethane, with optional functional groups selected from SO3, COOH, NH2, Glycidyl (COC), OH, Cl, Tosyl, aldehyde, and sulfhydryl. Particles often range from 0.3 to 5 um or larger. Latex particles of 100 nm, and 1, 5, 20, 50 or 100 um are

commercially available in bulk. Silica may be used as a matrix or as a capsule. Derivatized silane includes OH, NH2, COOH and more. Particles often range from 0.5 to 3 um. Dextran may also be used as a matrix. Particles often range from 20-50 nm. Polysaccharide may also be used with silane as silica fortified microbeads of particle size around 250 nm. Agarose and cellulose matrices include particles in the range of 1-10 um, and may be activated for introduction of functional groups. Protein particles, such as of gelatin and albumin, have long been used for magnetic microspheres. These are readily activated for amine, carboxyl, hydroxyl and sulfhydryl linkages with ligands or tags. Liposomes are somewhat more refractory to chemical derivatization, but have been used to make magnetic particles. Naked iron oxide, and other paramagnetic metal particles are also known, and may be derivatized by adding sulfhydryl groups or chelators. These particles often have sizes of 5 to 300 nm. Certain types of particle populations are known to be uniform in size; in others the heterogeneity may be controlled or selected.

[0073] Such microbeads may be readily prepared. For example, carboxyl-modified microbeads containing ~20-60% magnetite are made by dispersing a (magnetite)/styrene/divinylbenzene ferrofluid mixture in water, and emulsion-polymerizing the monomers to trap the magnetite in a polymer matrix of microbeads of ~1 μm diameter. The magnetite is thus dispersed throughout the solid beads. Other prior art means for synthesizing and modifying microbeads are commonly known.

[0074] Suitable microbeads for practicing the present invention may also be purchased from vendors such as Bang's Laboratories, Inc. (Fishers Ind.) and Polysciences, Inc (Warrington Pa.), as well as numerous suppliers of specialty modified microbeads such as Bioscience Beads (West Warwick R.I.). Tradenames of such beads, again not as a comprehensive recitation, include Estapor® SuperParaMagnetic Microspheres, COMPEL™ Uniform Magnetic Microspheres, Dynabeads® V MyOne™ Microspheres, and the like. Cobalt paramagnetic microbeads are sold as Dynabead's MyOne TALON. BioMag Plus microbeads from Polysciences have an irregular shape, and thus more surface area for affinity chemistry.

[0075] Populations—of microbeads are generally used to assay populations of assay targets. A population as used herein refers to a set of members sharing some common element or property. For example, a population of beads may be similar in that the beads share a common tag, such as an avidin coat, or a barcode. A population of nucleic acids comprising an assay target may simply share a target nucleic acid sequence, or may contain a common tag. A population of antibodies may share a common specificity. And so forth.

[0076] Paramagnetic and Superparamagnetic are taken as functionally synonymous for the present purposes. These materials when fabricated as microbeads, have the property of responding to an external magnetic field when present, but dissipating any residual magnetism immediately upon release of the external magnetic field, and are thus easily resuspended and remain monodisperse, but when placed in proximity to a magnetic field, clump tightly, the process being fully reversible by simply removing the magnetic field.

[0077] Magnetic Force Field: is the volume defined by the magnetic flux lines between two poles of a magnet or two faces of a coil. Electromagnets and driving circuitry can be used to generate magnetic fields and localized magnetic fields. Permanent magnets may also be used. Preferred permanent magnetic materials include NdFeB (Neodymium-Iron-Boron $Nd_2Fe_{14}B$), Ferrite (Strontium or Barium Ferrite), AlNiCo (Aluminum-Nickel-Cobalt), and SmCo (Samarium Cobalt). The magnetic forces within a magnetic force field follow the lines of magnetic flux. Magnetic forces are strongest where magnetic flux is most dense. Magnetic force fields penetrate most solids and liquids. A moving magnetic force field has two vectors: one in the direction of travel of the field and the other in the direction of the lines of magnetic flux.

[0078] Localized Magnetic Field: As used herein, a localized magnetic field is a magnetic field that substantially exists in the volume between the poles of two magnets, and may be attractive or repulsive.

[0079] Robustness: refers to the relative tolerance of an assay format to variability in execution, to materials substitutions, and to interferences, over a range of assay conditions. Robustness generally increases with the strength of the detection signal generated by a positive result. Robustness negatively correlates with the difficulty and complexity of the assay.

[0080] Specificity: Refers to the ability of an assay to reliably differentiate a true positive signal of the target biomarker from any background, erroneous or interfering signals.

[0081] Sensitivity: Refers to the lower limit of detection of an assay where a negative can no longer be reliably distinguished from a positive.

[0082] Assay endpoint: "Endpoint" or "datapoint" is used here as shorthand for a "result" from either qualitative or quantitative assays, and may refer to both stable endpoints where a constant plateau or level of reactant is attained, and to rate reactions, where the rate of appearance or disappearance of a reactant or product as a function of time (i.e., the slope) is the datapoint. Detection of a "molecular detection complex", also termed an "immobilized reporter complex", may constitute an assay endpoint.

[0083] Microfluidic cartridge: a "device", "card", or "chip" with fluidic structures and internal channels having microfluidic dimensions. These fluidic structures may include chambers, valves, vents, vias, pumps, inlets, nipples, and detection means, for example. Generally, microfluidic channels are fluid passages having at least one internal cross-sectional dimension that is less than about 500 μm and typically between about 0.1 μm and about 500 μm, but we extend the upper limit of the range to 600 um because the macroscopic character of the bead suspensions used here have a dramatic effect on the microfluidic flow regime, particularly as it relates to restrictions in the fluid path. Therefore, as defined herein, microfluidic channels are fluid passages having at least one internal cross-sectional dimension that is less than 600 um. The microfluidic flow regime is characterized by Poiseuille or "laminar" flow. The particle volume fraction (ϕ) and ratio of channel diameter to particle diameter (D/d) has a measurable effect on flow characteristics. (See for example, Staben M E et al. 2005. Particle transport in Poiseuille flow in narrow channels. Intl J Multiphase Flow 31:529-47, and references cited therein.)

[0084] Microfluidic cartridges may be fabricated from various materials using techniques such as laser stenciling, embossing, stamping, injection molding, masking, etching, and three-dimensional soft lithography. Laminated microfluidic cartridges are further fabricated with adhesive interlayers or by thermal adhesiveless bonding techniques, such by pres-

sure treatment of oriented polypropylene. The microarchitecture of laminated and molded microfluidic cartridges can differ.

[0085] Lateral flow Assay: refers to a class of conventional assays wherein particle aggregation, agglutination or binding is detected by applying a particle-containing fluid to a fibrous layer such as a permeable membrane and observing the chromatographic properties as the particles and particle aggregates move into and through the material. Penetration of clumps of particles is impeded, whereas free particles penetrate between the fibers. Similarly, free particles may accumulate as clumps in zones of the fibrous layer treated with affinity binding agents. The devices and methods described here are not lateral flow assays.

[0086] "Conventional" is a term designating that which is known in the prior art to which this invention relates.

[0087] "About" and "generally" are broadening expressions of inexactitude, describing a condition of being "more or less", "approximately", or "almost" in the sense of "just about", where variation would be insignificant, obvious, or of equivalent utility or function, and further indicating the existence of obvious minor exceptions to a norm, rule or limit.

[0088] Herein, where a "means for a function" is described, it should be understood that the scope of the invention is not limited to the mode or modes illustrated in the drawings alone, but also encompasses all means for performing the function that are described in this specification, and all other means commonly known in the art at the time of filing. A "prior art means" encompasses all means for performing the function as are known to one skilled in the art at the time of filing, including the cumulative knowledge in the art cited herein by reference to a few examples.

[0089] Means for extracting: refers to various cited elements of a device, such as a solid substrate, filter, filter plug, bead bed, frit, or column, for capturing target nucleic acids from a biological sample, and includes the cumulative knowledge in the art cited herein by reference to a few examples.

[0090] A means for polymerizing, for example, may refer to various species of molecular machinery described as polymerases and their cofactors and substrates, for example reverse transcriptases and TAQ polymerase, and includes the cumulative knowledge of enzymology cited herein by reference to a few examples.

[0091] Means for Amplifying: Include thermocycling and isothermal means. The first thermocycling technique was the polymerase chain reaction (referred to as PCR) which is described in detail in U.S. Pat. Nos. 4,683,195, 4,683,202 and 4,800,159, Ausubel et al. Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989), and in Innis et al., ("PCR Protocols", Academic Press, Inc., San Diego Calif., 1990). Polymerase chain reaction methodologies are well known in the art. Briefly, in PCR, two primer sequences are prepared that are complementary to regions on opposite complementary strands of a target sequence. An excess of deoxynucleoside triphosphates are added to a reaction mixture along with a DNA polymerase, e.g., Taq polymerase. If the target sequence is present in a sample, the primers will bind to the target and the polymerase will cause the primers to be extended along the marker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the template to form reaction products, excess primers will bind to the template and to the reaction products and the process is repeated. By adding fluorescent intercalating agents, PCR products can be detected in real time.

[0092] One isothermal technique is LAMP (loop-mediated isothermal amplification of DNA) and is described in Notomi, T. et al. *Nucl Acid Res* 2000 28:e63.

[0093] Strand Displacement Amplification (SDA) is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, i.e., nick translation (Walker et al. *Nucleic Acids Research,* 1992:1691-1696). A similar method, called Repair Chain Reaction (RCR), involves annealing several probes throughout a region targeted for amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA. Target specific sequences can also be detected using a cyclic probe reaction (CPR). In CPR, a probe having 3' and 5' sequences of non-specific DNA and a middle sequence of specific RNA is hybridised to DNA that is present in a sample. Upon hybridisation, the reaction is treated with RNase H, and the products of the probe identified as distinctive products that are released after digestion. The original template is annealed to another cycling probe and the reaction is repeated.

[0094] Another nucleic acid amplification technique is reverse transcription polymerase chain reaction (RT-PCR). First, complementary DNA (cDNA) is made from an RNA template, using a reverse transcriptase enzyme, and then PCR is performed on the resultant cDNA.

[0095] Another method for amplification is the ligase chain reaction ("LCR"), disclosed in EPO No. 320 308. In LCR, two complementary probe pairs are prepared, and in the presence of the target sequence, each pair will bind to opposite complementary strands of the target such that they abut. In the presence of a ligase, the two probe pairs will link to form a single unit. By temperature cycling, as in PCR, bound ligated units dissociate from the target and then serve as "target sequences" for ligation of excess probe pairs. U.S. Pat. No. 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence.

[0096] Qβ Replicase, may also be used as still another amplification method in the present invention. In this method, a replicative sequence of RNA that has a region complementary to that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence that can then be detected.

[0097] Still further amplification methods, described in GB Application No. 2 202 328, and in PCT Application No. PCT/US89/01025, may be used in accordance with the present invention. In the former application, "modified" primers are used in a PCR-like, template- and enzyme-dependent synthesis. The primers may be modified by labelling with a capture moiety (e.g., biotin) and/or a detector moiety (e.g., enzyme). In the latter application, an excess of labelled probes are added to a sample. In the presence of the target sequence, the probe binds and is cleaved catalytically. After cleavage, the target sequence is released intact to be bound by excess probe. Cleavage of the labelled probe signals the presence of the target sequence.

[0098] Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR (Kwoh et al., 1989, *Proc. Natl. Acad. Sci. U.S.A.,* 86: 1173; Gingeras et al., PCT Application WO 88/10315). In NASBA,

the nucleic acids can be prepared for amplification by standard phenol/chloroform extraction, heat denaturation of a clinical sample, treatment with lysis buffer and minispin columns for isolation of DNA and RNA or guanidinium chloride extraction of RNA. These amplification techniques involve annealing a primer which has target specific sequences. Following polymerisation, DNA/RNA hybrids are digested with RNase H while double stranded DNA molecules are heat denatured again. In either case the single stranded DNA is made fully double stranded by addition of second target specific primer, followed by polymerisation. The double-stranded DNA molecules are then multiply transcribed by an RNA polymerase such as T7 or SP6. In an isothermal cyclic reaction, the RNAs are reverse transcribed into single stranded DNA, which is then converted to double stranded DNA, and then transcribed once again with an RNA polymerase such as T7 or SP6. The resulting products, whether truncated or complete, indicate target specific sequences.

[0099] Davey et al., EPO No. 329 822 disclose a nucleic acid amplification process involving cyclically synthesising single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention. The ssRNA is a template for a first primer oligonucleotide, which is elongated by reverse transcriptase (RNA-dependent DNA polymerase). The RNA is then removed from the resulting DNA:RNA duplex by the action of ribonuclease H(RNase H, an RNase specific for RNA in duplex with either DNA or RNA). The resultant ssDNA is a template for a second primer, which also includes the sequences of an RNA polymerase promoter (exemplified by T7 RNA polymerase) 5' to its homology to the template. This primer is then extended by DNA polymerase (exemplified by the large "Klenow" fragment of *E. coli* DNA polymerase D, resulting in a double-stranded DNA ("dsDNA") molecule, having a sequence identical to that of the original RNA between the primers and having additionally, at one end, a promoter sequence. This promoter sequence can be used by the appropriate RNA polymerase to make many RNA copies of the DNA. These copies can then re-enter the cycle leading to very swift amplification. With proper choice of enzymes, this amplification can be done isothermally without addition of enzymes at each cycle. Because of the cyclical nature of this process, the starting sequence can be chosen to be in the form of either DNA or RNA.

[0100] Miller et al. in PCT Application WO 89/06700 disclose a nucleic acid sequence amplification scheme based on the hybridisation of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, i.e., new templates are not produced from the resultant RNA transcripts. Other amplification methods include "RACE" and "one-sided PCR" (Frohman, M. A., In: "PCR Protocols: A Guide to Methods and Applications", Academic Press, N.Y., 1990; Ohara et al., 1989, *Proc. Natl. Acad. Sci. U.S.A.*, 86: 5673-567).

[0101] Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide", thereby amplifying the di-oligonucleotide, may also be used in the amplification step of the present invention. Wu et al., (1989, *Genomics* 4: 560).

[0102] Means for detecting: as used herein, refers to an apparatus for displaying an endpoint, i.e., the result of an assay, and may include a detection channel and test pads, and a means for evaluation of a detection endpoint. Detection endpoints are evaluated by an observer visually in a test field, or by a machine equipped with a spectrophotometer, fluorometer, luminometer, photomultiplier tube, photodiode, nephlometer, photon counter, voltmeter, ammeter, pH meter, capacitative sensor, radio-frequency transmitter, magnetoresistometer, or Hall-effect device. Magnetic particles, beads and microspheres having impregnated color or having a higher diffraction index may be used to facilitate visual or machine-enhanced detection of an assay endpoint. Magnifying lenses in the cover plate, optical filters, colored fluids and labeling may be used to improve detection and interpretation of assay results. Means for detection of magnetic particles, beads and microspheres may also include embedded or coated "labels" or "tags" such as, but not limited to, dyes such as chromophores and fluorophores, for example Texas Red; radio frequency tags, plasmon resonance, spintronic, radiolabel, Raman scattering, chemoluminescence, or inductive moment as are known in the prior art. Colloidal particles with unique chromogenic signatures depending on their self-association are also anticipated to provide detectable endpoints. QDots, such as CdSe coated with ZnS, decorated on magnetic beads, or amalgamations of QDots and paramagnetic Fe_3O_4 microparticles, optionally in a sol gel microparticulate matrix or prepared in a reverse emulsion, are a convenient method of improving the sensitivity of an assay of the present invention, thereby permitting smaller test pads and larger arrays. Fluorescence quenching detection endpoints are also anticipated. A variety of substrate and product chromophores associated with enzyme-linked immunoassays are also well known in the art and provide a means for amplifying a detection signal so as to improve the sensitivity of the assay. Detection systems are optionally qualitative, quantitative or semi-quantitative. Visual detection is preferred for its simplicity, however detection means can involve visual detection, machine detection, manual detection or automated detection.

[0103] Unless the context requires otherwise, throughout the specification and claims which follow, the word "comprise" and variations thereof, such as, "comprises" and "comprising" are to be construed in an open, inclusive sense, that is as "including, but not limited to".

[0104] Reference throughout this specification to "one embodiment" or "an embodiment" means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, the appearances of the phrases "in one embodiment" or "in an embodiment" in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments.

2. DETAILED DESCRIPTION

[0105] Turning now to the figures, we will begin with the products of the process—detection complexes—and then describe their method of production. FIGS. **1** through **4** illustrate examples of amplicon capture on immobilized antibody, target antibody capture on immobilized antigen, target antibody capture on immobilized capture agent, and target antigen capture on immobilized antibody, respectively.

[0106] The detection complex of FIG. **1** depicts a paramagnetic bead **1** immobilized on test pad **2**. The tether between bead and antibody **3** is formed by a two-tailed amplicon **4**, in

this case with first primer **5** tagged with peptidyl hapten **6** and second primer **7** tagged with biotin **8**, for illustration. The paramagnetic beads are coated with bound avidin **9**.

[0107] As shown, the immobilized antibody on the test pad **1** has captured a "two-tailed amplicon" (**4**), i.e., an amplicon with peptidyl-oligomer-tagged primer at a first end and biotin-tagged primer at the opposite end. These two-tailed amplicons are synthesized during an amplification step by providing reagent primer sets in which biotin has been used to tag a second primer and peptide hapten the first primer by conventional chemistries. In this example, the biotin tagged amplicon is captured by the avidin-coated microbead, and the reporter bead complex in turn is then immobilized on the test pad. The two-tailed amplicon thus serves as a heterobifunctional tether. A sufficient number of immobilized beads, as present in a few microliters of reagent, result in a distinct visual coloration of the test pad. Biotin is only one such ligand useful in constructing these unique molecular detection complexes with magnetic beads.

[0108] Methods for preparation of affinity-modified microbeads are also commonly known. As would be obvious to one skilled in the art, composite magnetic beads can be prepared with materials such as QDots, fluorophores, dyes, enzymes, RFIDs, and so forth, so as to be readily detectable by alternative detection means when immobilized on the respective test pads. Detection can involve visual detection, machine detection, manual detection or automated detection. Methods for preparation of hapten-tagged primers are also readily extracted from the prior art.

[0109] Thus a "positive detection complex" results when an amplicon becomes tethered to a test pad as shown in FIG. **1**. Necessarily, only those amplicons bearing both tags are immobilized on any one test pad. Those test pads to which magnetic beads are tethered indicate a positive result for one specific two-tailed species of hapten-tagged amplicon. Those test pads to which no magnetic beads are tethered indicate a negative result for the respective hapten-tagged amplicon. Because the species of hapten are known and are assigned to a particular forward or reverse primer conjugate, the detection event can be interpreted as positive detection of the particular nucleic acid sequence corresponding to the target nucleic acid sequence under investigation.

[0110] In a preferred method, by using peptidyl-haptens (peptide epitopes) attached to the primer nucleotide sequence as a tag, large libraries of peptidyl-hapten-tagged amplicons can be prepared by amplification, and interrogated by the magnetic bead methods described here. Methods using the much more limited prior art toolbox of non-peptide ligands as haptens or binding agents are not so robust.

[0111] Clearly, the biotin:avidin affinity binding pair is one of many ligand binding pairs that might be chosen for affinity binding. Others include nickel:nickel binding complexes, as may be suitable to nickel-bearing microbeads. Or digoxin and digoxigenin and complementary antibodies, or the antibody Fc fragment and Protein A or Protein G. Antibody-coated microbeads may also be used to capture peptidyl hapten-tagged second primers (i.e., a unique peptidyl hapten on both primers), and so forth.

[0112] In FIG. **2**, we see a second detection complex, again taking advantage of a bifunctional tether. Paramagnetic bead **20** is immobilized on test pad **21** coated with antigen. The tether between bead and antigen **22** is formed by an antibody **23**. The paramagnetic beads are coated with bound anti-antibody **24**. Species specific anti-antibodies are useful for this method, as are also Protein A and Protein G.

[0113] In FIG. **3**, the roles are reversed. Paramagnetic bead **30** is immobilized on test pad **31** coated with an anti-antibody **32**. The tether between bead **30** and antibody **32** is again an antibody **33**, but the beads are now coated with antigen **34**. Species specific anti-antibodies are useful for this method.

[0114] In FIG. **4**, antigen is the target molecule of the assay. Paramagnetic bead **40** is coupled to test pad **41** coated with antibody **42** specific for the antigen. The tether between bead **40** and antibody **42** is now an antigen **43**. The beads are coated with an antibody **44** specific for the antigen. The antibodies are not necessarily identical, one antibody may be a hybridoma antibody, the other a polyvalent antibody, and the antibodies may bind to different recognition sites on a macromolecular antigen, or share a common binding site where multiple binding sites are present, for example when the antigen is a viral particle.

[0115] We now turn to the step in the method whereby the immobilized paramagnetic complex is produced. Examples of bead:amplicon capture on immobilized antibody, bead:target antibody capture on immobilized antigen, bead:target antibody capture on immobilized antibody capture agent, and bead:target antigen capture on immobilized antibody, respectively, will again be discussed.

[0116] In FIG. **5**, a key is used to describe the elements of the step. In the first panel, FIG. **5**A, paramagnetic bead:amplicon binding complexes on the left are in the process of being "swept" or "dragged" onto, through and across two patches of immobilized antibody illustrating the test pads. It can be appreciated that at a molecular level the test pads are not two-dimensional layers, but are in fact 3-dimensional surfaces coated with a layer of bound and unstirred water. The cone of the magnetic force field (long arrow) is moving parallel to the plane of the test pad, but the magnetic flux lines are experienced by paramagnetic particles as being directed downwardly (short arrow). The magnetic force is thus seen to have two vectors, one directed "downwardly" and the other "laterally" (relative to the plane of the test pad). Paramagnetic particles are attracted to the magnet from which the magnetic force field emanates, and the magnet is positioned under the plane of the test pad and is moving from left to right. The "J-hook" at the base of the amplicons (for illustration) represents a peptidyl hapten that is recognized by one of the test pads, which are coated with different antibodies (left vs. right test pad).

[0117] In the second panel, FIG. **5**B, the magnetic force field has moved past the test pads, "sweeping" or "dragging" with it unbound paramagnetic particles while—surprisingly—paramagnetic bead complexes bearing the amplicon have been captured and extracted from the magnetic field, and are seen in the panel to be immunoimmobilized on the test pad coated with capture antibody specific for the J-hook of the peptidyl hapten. This occurs much in the same way that a cable captures the tailhook of a landing airplane on an aircraft carrier. The bead complexes are dragged through the mat of antibody and liquid crystalline water bound to the test pad. The magnetic force pulls the complexes down into close approach and full contact with the antibody mat, contacting antibody and hapten. The magnetic force (which is a weak force) is not strong enough to rip the hapten from the primer, or even to rip the antibody from the test pad, but instead releases the bead complex, which remains immobilized on the test pad.

[0118] Note that the process of removing unbound paramagnetic material from the test pads after immunocapture could also be accomplished by repositioning the source of the magnetic field above the test pad. Paramagnetic beads will always move from a field of less dense magnetic flux lines to a field of more dense magnetic flux lines. Thus we can say that capture is accomplished by sweeping the beads from outside to inside the test pad area, and removal of unbound material is accomplished by sweeping the beads from inside to outside the test pad area, without reference to particular geometries. The magnetic field may also serve to remove the unbound material to waste.

[0119] Immobilization is specific. In this illustration, the peptide hapten is recognized only by the complementary antibody of the right test pad, not the left, and the bead complexes are therefore immobilized only on the right test pad. Detection of the immunoimmobilized bead complexes is thus a positive detection event and indicates here the presence of the target amplicon. Detection of the immobilized complexes can be as simple as a visual estimate of the color of the test pad before and after binding, or a comparison with positive and negative control test pads. Paramagnetic beads typically have a distinct color or can be suitably dyed. More complex detection means may also be used.

[0120] In FIG. **6**, a key is again used to describe the elements of the step. In the first panel, FIG. **6**A, paramagnetic bead:antibody binding complexes on the left are in the process of being "swept" or "dragged" onto, through and across a patch of immobilized antibody on the test pad. The bead antibody complexes consist of a mixture of target antibody (black body) and nonspecific antibodies (black tips), all of which have been bound to the beads by an anti-antibody, for example in the case of an assay for antigen-specific IgG immunoglobins in human serum, a mouse or hybridoma anti-human IgG antibody. The test pad is coated with adsorbed antigen specific for the antibody targeted in the assay.

[0121] It can again be seen that the magnetic force field (long arrow) is moving parallel to the plane of the test pad, but is experienced by paramagnetic particles as being directed downwardly (short arrow). There are thus two vectors to the magnetic force, the lateral vector corresponding to movement of the magnetic field from left to right and the perpendicular vector corresponding to the magnetic flux lines which are not shown. Paramagnetic particles are attracted to the magnet from which the magnetic flux lines emanate, and the magnet is positioned beneath the plane of the test pad and is moving from left to right. The paramagnetic particles will follow the motion of the magnetic force field, and are pulled against the test pad while being dragged from left to right.

[0122] In the second panel, FIG. **6**B, the magnetic force field has moved past the test pads, "sweeping" or "dragging" with it nonspecific antibody and unbound paramagnetic particles while—surprisingly—paramagnetic bead complexes bearing target antibody specific for the antigen have been captured and extracted from the magnetic field, and are seen in the panel to be immunoimmobilized on the test pad.

[0123] In FIG. **7**A, paramagnetic bead:antibody binding complexes on the left are in the process of being "swept" or "dragged" onto, through and across immobilized antibody on the test pad. The microbeads are coated with antigen complementary or specific for the target antibody of the assay. The test pad is coated with an anti-antibody, for example in the case of an assay for antigen-specific IgG immunoglobins in human serum, a mouse or hybridoma anti-human IgG antibody.

[0124] It can again be seen that the magnetic force field (long arrow) is moving parallel to the plane of the test pad, but is experienced by paramagnetic particles as having a downward vector (short arrow). Paramagnetic microbeads are attracted to the magnet from which the magnetic force field emanates, and the magnet is positioned beneath the plane of the test pad and is moving from left to right. The paramagnetic beads are thus pulled down on the test pad, in close contact with the capture agent, while simultaneously transversing the test pad from left to right.

[0125] In the second panel, FIG. **7**B, the magnetic force field has moved past the test pads, "sweeping" or "dragging" with it unbound paramagnetic particles while—surprisingly—paramagnetic bead complexes bearing target antibody have been captured and immunoextracted from the magnetic field.

[0126] In FIG. **8**A, paramagnetic bead:antigen complexes on the left are in the process of being "swept" or "dragged" onto, through and across immobilized antibody on the test pad. The beads are coated with antibody complementary for the target antigen of the assay. The test pad is coated with an anti-antigen antibody.

[0127] It can again be seen that the magnetic force field (long arrow) is moving parallel to the plane of the test pad, but is experienced by paramagnetic particles as being directed with a downward vector component (short arrow). Paramagnetic particles are attracted to the magnet from which the magnetic force field emanates, and the magnet is positioned beneath the plane of the test pad and is moving from left to right. The paramagnetic particles will follow the motion of the magnetic force field, and are pulled up against the test pad while being dragged from left to right.

[0128] In the second panel, FIG. **8**B, the magnetic force field has moved past the test pads, "sweeping" or "dragging" with it unbound paramagnetic particles while—surprisingly—paramagnetic bead complexes bearing target antigen have been captured and immunoextracted from the magnetic field.

[0129] Surprisingly, in the bound layer of water molecules on the test pad, the intermolecular forces of affinity binding are stronger than the magnetic forces on the particles. While not limited by theory, the invention is a way of solving a critical problem of bioassays, that of facilitating the close approach of target and target capture agent by dislodging the boundary or unstirred layer of water at the surface of the capture layer. At the nanoscale of microfluidics, this barrier is a critical barrier in affinity binding. Typically this problem has been overcome by extending incubation time or by convective close approach (for example as in the wicking effect of lateral flow) followed by diffusion and capture. Here we show that unbound paramagnetic complexes are first brought into contact with a capture surface or substrate under the direction of a magnetic force field and are then extracted from the magnetic field, while unbound paramagnetic substrates are dragged away from the capture surface or substrate by the continued lateral motion of the magnetic field.

[0130] The magnetic force field thus has two vectors, one directed "downwardly" (relative to the plane of the capture surface or test pad) and the other "laterally" (again relative to the plane of the capture surface or test pad). The downward vector penetrates the unstirred water layer around the capture

molecule, and draws the target molecule into the required close approach or "close encounter" where affinity binding can occur. The lateral vector is through the unstirred water layer, and again draws the target molecules into the required close approach to capture molecules, but further serves to differentiate bound and unbound material. Unbound paramagnetic molecular complexes remain with the moving magnetic field and continue their lateral path. Bound paramagnetic materials are immobilized at the site of capture and are not dislodged by the continuing lateral vector of the magnetic force field.

[0131] The magnetic force field is manipulated by moving its source (a permanent magnet or electromagnet) laterally across or through the plane of the test pad, and may be disengaged by withdrawing the magnet or turning off current to the electromagnet).

[0132] In FIG. **9**, illustrated is a simple device for conducting the method. It can be seen that the lateral motion of the magnetic force field is optionally bidirectional, here shown with a net motion in a fluid path of detection chamber **60** from upstream **61** (Paramagnetic Complexes In) to downstream (Waste Out). The source of the magnetic field is again "underneath" (or "behind") the test pad. This draws the target into close approach with the binding sites, and facilitates the detection step. In the capture step, the path of the magnetic field must necessarily contactingly traverse one or more test pads **62** coated with affinity capture agent **63**. In the detection step, a viewing window **64** permits detection of the bound complexes after the moving magnetic field has passed, thus simplifying detection. The magnetic field is further useful in directing unbound paramagnetic materials to waste. A multiplex detection device is shown, having a plurality of test pads.

[0133] Note that this approach to assays eliminates the "hook effect" characteristic of some lateral flow assays. The affinity-modified paramagnetic beads are reacted with the target molecule before seeing the capture agent, and when in excess, do not compete with the target molecule for binding on the test pad.

[0134] To assemble devices of the kind illustrated in FIG. **9**, the test pads are treated to immobilize a capture agent prior to assembly of the device, for example, on polystyrene, by plasma treatment of plastic areas delimited by a mask, followed by application of the capture agent and drying. Spotting of capture agent, for example with a laserjet printer, can eliminate the need for masking test pads. If necessary, test pads are "blocked" with blocking agents to prevent non-specific adsorption of target molecules prior to sealing the detection chamber or channel.

[0135] In FIG. **10** we show examples of possible test pad geometries. Test pads are a feature of the detection step of the method described herein. Test pads **70** and **71** constitute for example a negative and positive detection field for an assay and may be used as a pair. Test pad array **72** is a vertical stack of banded or striped test pads in the form of an array, not unlike that shown in FIG. **9**. Test pad array **73** is a rectangular array of individual test squares, each treated with a unique capture agent. Test pad **74** is circular and is adapted to inkjet printing. Test pad **75** is treated with a gradient of a capture agent so as to display a readily interpretable semi-quantitative endpoint.

[0136] Test pads have in common a test field bounded by an edge inside of which a bioactive capture agent is immobilized. While not a comprehensive list, the capture agent may be a protein such as an antibody, an anti-antibody, an anti peptidyl hapten antibody, Protein A, Protein G, or antigen, or a non-protein such as an aptimer, a carbohydrate antigen, a mucopolysaccharide, a binding protein such as folic acid binding protein or an avidin, or a nucleotide oligomer. Capture agents may also include denatured viral antigens and microbial antigens in general and cellular components or whole cells in general.

[0137] Note that test pads are not necessarily impermeable substrates, and may be porous or fibrous in character. The microbead fluid path in the magnetic field may be across or through the test pad area, as in from side-to-side or from front-to-back. The test pad architecture, at a molecular level, is inherently three-dimensional, although it may be represented as a two-dimensional plane.

[0138] Solid substrates for test pads include olefin or other thermoplastic materials such as polystyrene, polycarbonate, polypropylene, polyethylene terephthalate, polyether sulfone, polyvinyl chloride, polyvinyl acetate, copolymers of vinyl acetate and vinyl chloride, and polyamides and also inorganic materials such as glass. Certain fibrous or porous supports such as nitrocellulose, nylon, hydrogel, and polyethylene may also be applied as test pads, and may be pretreated with capture agent for ease of assembly. To enhance binding of capture agents, crosslinked proteins are sometimes employed. Drying also promotes irreversible binding of the capture agent.

[0139] A preferred method for pretreating plastic prior to adsorbing the capture agent is low pressure gas plasma treatment. Exposure of the surface to pure oxygen or nitrogen produces an activated hydroxylated and carboxylated substrate layer or an activated aminated and nitroxidated layer, respectively. Argon may also be used. In one embodiment, polystyrene plastic is used as the substrate for immobilizing capture agent. Masking, followed by gas plasma treatment is used to activate designated areas as test pads. The capture agent is applied, dried in place, and the mask is removed. When antibody is used as the capture agent, application by hand or with an automated printer is followed by drying and blocking. Other capture agents may require modified protocols as are known in the art.

[0140] Techniques for surface activation are reviewed in Chan et al. (1996) *Surface Science Reports* 24:1-54 and in Garbassi et al. (1998) Polymer Surfaces-From Physics to Technology (John Wiley pp 238-241), and in U.S. Pat. No. 6,955,738, which describes hydrophilization and functionalization of polymer surfaces and is incorporated herein in its entirety by reference.

[0141] We now disclose integrated assay methods relying on a step for laterally moving magnetic fields to contactingly capture and extract target analytes from biological samples.

[0142] Turning to FIG. **11**, an assay for a target nucleic acid sequence becomes the steps of first preparing the sample for amplification of the target sequence, amplifying the target by a PCR or related isothermal protocol whereby tagged primers are incorporated into the product amplicon, then binding those tagged "two-tailed" amplicons on paramagnetic beads coated with an affinity binding agent, and magnetically sweeping or dragging the beads into close contact with a test pad area with immobilized capture antibody so as to form immunoimmobilized paramagnetic complexes of test pad: capture antibody:amplicon with hapten tag of first primer and ligand tag of second primer:binding agent and paramagnetic bead (i.e., the detection complex), before sweeping from the

test pad any un-immobilized paramagnetic material. And finally a step for detecting any molecular detection complexes on the test pad.

[0143] Preparation of a sample may involve lysing cells to release the target nucleic acids, removing interferences such as hemoglobin from a blood lysate by selective adsorption and elution of the nucleic acids from a glass solid phase, and dissolution of the nucleic acids with a suitable buffer for a polymerase. Also required in some applications are preliminary steps for reverse transcription, as when mRNA contains the target sequences and must be converted to duplex DNA before amplification.

[0144] In the step for amplification, multiplex or nested primer sets may be used. The method uses a second primer with tag suitable for complexation with an affinity binding agent on the paramagnetic beads, and often this a biotin tag as illustrated in FIG. **1**. The method uses a first primer with tag suitable for immunoaffinity immobilization of the formed amplicon:bead complex on the surface of the test pad.

[0145] So two levels of affinity capture are involved, the first being the binding of a ligand-tagged amplicon on the paramagnetic bead, and the second the immobilization or capture of an amplicon:bead binding complexes on the test pad (forming the detection complex or immobilized reporter complex). Various affinity binding agents may be used in each phase of formation of the detection complex. However, the advantage of using capture antibodies for second phase immobilization is the specificity of antibody:peptidyl hapten binding, which permits design of protocols for simultaneous assay of multiple target nucleic acid sequences. Immunoimmobilization of target analyte with antibody capture agent is a preferred embodiment, but the invention is not limited to such.

[0146] Having formed the paramagnetic bead:amplicon binding complexes in free solution, the next step is to use a magnetic field to localize and contact the analyte complexes with the test pad so that the immunoimmobilized detection complexes can be formed. The magnetic field is moved and optionally modulated to perform this. Lateral motion of the magnetic field sweeps or drags the bead complexes onto the test pad, through the unstirred layer and the 3-dimensional network of bound capture antibody, and finally across the test pad, where unbound paramagnetic material is carried off the test pad and away with the lateral motion of the magnetic force. This step promotes binding interactions without the need for multi-minute incubations.

[0147] In the detection step, the double-stranded, two-tailed amplicon, bound by avidin:biotin on one end (for example) and antibody:peptidyl hapten on the other (for example), is sufficiently strong to selectively tether the paramagnetic bead to the test pad and resist delocalization by the moving magnetic force field. It can be said that the capture antibody "extracts" the amplicon:bead complexes from the moving magnetic field. Sufficient numbers of bound bead complexes are readily identified and form a positive result by visual endpoint. A visual detection step is illustrated.

[0148] It should be noted that the primer set is essentially a first assay reagent, and may be prepared and placed in an assay device or kit, optionally in dried form, at any time prior to performing the assay. Similarly, the beads are essentially a second assay reagent, and may be sensitized with the desired binding agent, and optionally dried in place prior to the assay. Test pads are prepared in advance of the assay itself and may be rehydrated prior to use or rehydrated by the test sample in performance of the assay. Drying promotes irreversible binding of the capture agent to the test pad substrate. Reagents for sample preparation and amplification may also be prepared separately.

[0149] In FIG. **12** we see the same principles illustrated in a target antibody assay. The sample is first processed to prepare a liquid fraction, which might be serum or plasma, a paracellular fluid, saliva, or other biological sample. Generally any solid fraction of the sample is separated from the aqueous liquid fraction.

[0150] Optionally, interferences are then adsorbed and any antibody:target antigen complexes in the biological sample are disrupted so as to release the analytical target.

[0151] The target antibody in free solution is then bound by paramagnetic beads coated with an anti-antibody. This method is of use, for example, when a particular class of target antibody is of interest, as in distinguishing acute, convalescent, and chronic stages of infection, or when all antibody in the sample is to be interrogated for specificity to a plurality of antigens.

[0152] In the detection step, the bead:antibody:antibody:antigen tether, is sufficiently strong to selectively anchor the paramagnetic bead to the test pad and resist disruption by the magnetic field. Sufficient numbers of bound bead complexes are readily identified and form a visually positive detection endpoint. The detection complex is formed of test pad:antigen:target antibody:affinity bound paramagnetic bead. Alternatively, the detection complex may contain an enzyme, for example, and may be further developed for detection by enzymatic assay.

[0153] The common step in all these assays is to simultaneously use a magnetic field a) to localize and contact the analyte:bead complexes with the test pad so that the immobilized detection complex can be formed and further b) to separate bound and unbound paramagnetic bead complexes. This speeds the analytical process. The magnetic field is moved and optionally modulated to perform this. Lateral motion of the magnetic field sweeps or drags the bead complexes onto the test pad, through the unstirred layer and the 3-dimensional network of bound capture antigen, and finally across the test pad, where unbound paramagnetic material is carried off the test pad and away with the lateral motion of the magnetic force. Paramagnetic bead complexes bearing target antibody remain behind, immuno-immobilized on complementary, irreversibly adsorbed antigen on the test pad.

[0154] Clearly the bifunctional or "two-tailed" tether confers assay specificity. Using FIG. **12** for illustration, which is copacetic with FIGS. **2** and **6**, nonspecific antibody may be bound to the paramagnetic beads, but would not be captured by the antigen on the test pads, so no false-positive detection complex will form. Anti-antibodies directed at the Fc fragment of the target antibody are preferable for this assay so that the variable regions of the target antibody arms are free to recognize and bind to the bound antigen.

[0155] It should be noted that the beads are essentially a first assay reagent, and may be sensitized with the desired binding agent, and optionally dried in place prior to the assay. Test pads are prepared in advance of the assay itself and may be rehydrated prior to use or rehydrated by the test sample in performance of the assay. Reagents for sample preparation may also be prepared separately.

[0156] In FIG. **13**, the method differs from that of FIG. **12** essentially by the polarity of the tether. In the sample preparation step, generally, the solid fraction of the sample is sepa-

rated from the aqueous liquid fraction. If needed, interferences are then adsorbed and any antibody:target antigen complexes in the biological sample are disrupted so as to release the analytical target.

[0157] The target antibody in free solution is then bound by paramagnetic beads coated with complementary antigen, forming immunospecific antibody:antigen complexes on the bead (also termed a "reporter:analyte complex".

[0158] The next step is common to all these assays and involves the simultaneous use a magnetic field to a) localize and contact the analyte:bead complexes with the test pad so that the immobilized detection complex can be formed and b) to separate bound and unbound paramagnetic bead complexes. Lateral motion of the magnetic field sweeps or drags the bead complexes onto the test pad, through the unstirred layer with a downward vector on the paramagnetic beads, penetrating the 3-dimensional network of bound capture anti-antibody on the test pad, and finally across the test pad, whereupon unbound paramagnetic material is carried away with the lateral motion of the magnetic force. Paramagnetic bead complexes bearing target antibody remain trapped by immunoimmobilization on adsorbed anti-antibody on the test pad.

[0159] In the detection step, the bead:antigen:antibody:antibody tether, is sufficiently strong to selectively anchor the paramagnetic bead to the test pad and resist disruption by the magnetic field. Sufficient numbers of bound bead complexes are readily identified and form a positive visual detection endpoint. The detection complex is formed of test pad:antibody:target antibody:affinity bound paramagnetic bead. The detection endpoint may be further developed to amplify the detection sensitivity, for example by excitation of a fluorophore.

[0160] Clearly the bifunctional tether confers assay specificity. Using FIG. **13** for illustration, which corresponds to FIGS. **3** and **7**, the capture anti-antibody on the test pad in panel **13**B will likely capture a broad spectrum of antibodies in the sample, but only those immunocomplexed by paramagnetic beads will result in a positive assay. Thus the bifunctional specificity of the tether ensures assay specificity. Note that, however, the paramagnetic bead reagent is a mixture of beads coated with either a first antigen and beads coated with a second antigen, an immunoimmobilized positive assay endpoint will form if antibodies to either antigen are present in the sample. The identity of individual antibodies to particular antigens is, however, obtained with the method of FIG. **12**, even in a multiplexed bead format.

[0161] It should be noted that the beads are essentially a first assay reagent, and may be sensitized with the desired binding agent, and optionally dried in place prior to the assay. Test pads are prepared in advance of the assay itself and may be rehydrated prior to use or rehydrated by the test sample in performance of the assay. Reagents for sample preparation may also be prepared separately.

[0162] Similarly, individual antigens in a biological test sample may be identified by the method of FIG. **14**. In FIG. **14** we see the same principles illustrated to assay for a target antigen. The sample is first processed to prepare a liquid fraction containing a solution or suspension of the target antigen.

[0163] Optionally, interferences are adsorbed and any antibody:target antigen complexes in the biological sample are disrupted so as to release the analytical target.

[0164] The target antigen in free solution is then bound by paramagnetic beads coated with an antibody complementary for the antigen. Multiple antigens may be targeted simultaneously. This method is of use, for example, when a sample is suspected of carrying an enteric pathogen, a virus, or a marker released from malignant cells.

[0165] The common step in all these assays is use a magnetic field to a) localize and contact the analyte:bead complexes with the test pad so that the immobilized detection complex can be formed and to b) separate bound and unbound paramagnetic bead complexes. Essentially this is done simultaneously, thus speeding the assay and eliminating multi-minute incubations for the binding interaction.

[0166] The step for magnetic sweeping is comprised of applying a magnetic force to said paramagnetic bead reagent, wherein said magnetic force comprises generally lateral and generally perpendicular force vectors generated by a moving magnetic force field comprising flux lines extending from less dense to more dense. Because paramagnetic beads move from areas of less dense magnetic flux to areas of more dense magnetic flux, the magnetic force pulls the beads onto and into the arms of the capture agent. Because the magnetic field is moving laterally, the magnetic force sweeps or pulls the beads laterally over and across the test pad, separating bound and unbound materials as it goes. Rates of motion (linear velocity) for the magnetic sweep have been in the range of 25 to 100 mm/min (up to about 0.2 cm/sec). This step can be performed manually, or can be performed with an automated or semi-automated apparatus.

[0167] FIG. **15** illustrates a result of the assay. Seven vertically elongate detection chambers are placed side by side on a microfluidic cartridge under an optical window. Within each detection chamber are seven test pads stacked vertically. Each test pad is about 0.5×2 mm in size. Paramagnetic microbead reporter complexes are added to the detection chamber via a sample port and the beads are drawn up the detection chamber by a magnetic field originating from a magnet behind the cartridge. This magnetic field serves to a) draw the reporter complexes to the site of immobilization and b) remove unbound material. The result is a striking rust colored band where reporter complexes are bound to the corresponding antibody on a test pad. Seven amplicons were used in preparation of this test cartridge, and seven corresponding antibody test pads were prepared in each detection chamber. The result thus appears as a "stairstep" from left to right.

[0168] We can thus, in general, characterize the method as a rapid bioassay protocol comprising a step of moving a magnetic force field from outside to inside a test pad area so as to sweep a paramagnetic bead reagent in a fluid into close contact with an affinity capture agent in said test pad area, and thereby affinity capturing or extracting any bioassay target molecule bound to the paramagnetic bead reagent from the magnetic force field in the form of an immobilized paramagnetic microbead complex; and upon forming the immobilized paramagnetic bead complex (i.e., the detection complex), then moving the magnetic force field from inside to outside the test pad area so as to sweep from the test pad area any paramagnetic bead reagent not formed as immobilized paramagnetic complex, before detecting the detection complex, although it should be clear that, simplicity of description aside, the sweeping step in fact simultaneously integrates multiple simultaneous acts of formation of immobilized bead complexes and parallel acts of separation of not immobilized materials.

[0169] Surprisingly, the tether is sufficiently strong to selectively anchor the paramagnetic bead to the test pad while resisting the separating force of the magnetic field. In the detection step, sufficient numbers of bound bead complexes are readily identified and form a visual detection endpoint. The detection complex comprises bead:antibody:antigen:antibody:test pad, and may be further developed to increase assay sensitivity, for example by exciting an RFID tag or a fluorophore embedded in the bead matrix. The bead thus acts as a reporter group itself, or as a complex with accessory reporter groups.

[0170] Clearly the bifunctional or "two-tailed" tether confers assay specificity. Using FIG. **14** for illustration, which is copacetic with FIGS. **4** and **8**, specific antibody binds the target antigen, such as a drug or other small molecule, to the bead, and the target antigen:bead binding complex is bound to the test pad again by another antibody specific to the target analyte. Specificity and robustness is also demonstrated in FIG. **15**.

[0171] It should be noted that the beads are essentially a first assay reagent, and may be sensitized with the desired binding agent, and optionally dried in place prior to the assay. Test pads are prepared in advance of the assay itself, are advantageously dried in place, and may be rehydrated prior to use or rehydrated by the test sample in performance of the assay. Reagents for sample preparation may also be prepared separately before use.

[0172] In the various applications noted above, we have developed a preference for monosized bead reagents with high density relative to typical aqueous solutions. Metallic microbeads settle quickly in micron-sized flow paths and the beads are not readily resuspended during washing. Interestingly, in certain microfluidic applications, a magnet is no longer used for routine washing and rinsing of magnetic beads. These preferred beads are also readily detected visually. Labelled test pads appear as brightly rust colored spots or bands on a white or clear background.

[0173] The size of magnetic beads preferred in the assay are about 0.01 to 50 microns, more preferably 0.5 to 10 microns, and most preferentially 0.8 to 2.8 microns, mean diameter. Homogeneously sized beads are preferred. Suitable beads may be obtained from Dynal Invitrogen (Carlsbad Calif.), Agencourt Bioscience Corp (Beverly Mass.), Bang's Laboratories, Inc. (Fishers Ind.), Polysciences, Inc (Warrington Pa.), Bioscience Beads (West Warwick R.I.), Bruker Daltonics (Nashville Tenn.) and AGOWA (Berlin Del.), for example.

[0174] The magnetic beads may be in the form of a ferrofluid, taken broadly. In operation, in traversing the test pad, the method serves as a sort of magnetic fluidized bed reactor for extraction of affinity captured beads and separation out of nonspecifically labeled beads, reagents and assay materials.

[0175] To effect motion of the magnetic force field relative to the test pad, several alternative embodiments are possible: a) The magnet itself can be moved. Movement can be manual or powered with a stepper motor, servo motor, voice coil or with a spring-loaded mechanism and an x-z or y-z carriage can be constructed and automated. Alternatively, b) the test pad may be moved relative to the magnetic field by similar means. And if electromagnets are used in place of permanent magnets, c) an array of electromagnets can be actuated in sequence to redirect the magnetic field. It is possible to build a solid state system where a series of electromagnets are used to move the beads in a chamber. However, the methods of the inventions should not be construed as being limited to a microfluidic device. Adaption to laminar flow, lateral flow, capillary, dipstick, multiwell plate, and test tube formats is also contemplated.

[0176] In a preferred apparatus, as built, a stepper motor is used to move a rare earth magnet (neodymium) in an undercarriage mounted in close proximity to the detection chamber of a microfluidic device. Simple software commands are used to move the undercarriage along y-axis of the detection chamber (see FIG. **9**). The speed of translation is adjustable and the carriage may be lowered in the z-axis to weaken the magnetic field in the chamber. Because paramagnetic beads line up on magnetic flux lines and are attracted to areas of higher magnetic flux, they cannot be repelled by the magnet and the orientation of the poles of the magnet is reversible.

[0177] The preferred apparatus accepts a microfluidic cartridge with detection chamber or "microchannel" configured in the body, the microchannel comprising a fluid path with axis of flow and with upper and lower aspects.

[0178] Within the microchannel is a test pad or solid phase element, which comprises an affinity capture agent for the analyte or for an analyte binding complex. A means is provided for introducing a population of paramagnetic microbeads in a fluid into the microchannel, generally by assembling the cartridge with dehydrated beads inside and then rehydrating the beads in test sample fluid so that the beads complex target analyte. Also provided is a means for moving a magnetic force field along a plane parallel to the axis of flow of said fluid path, so as to sweep the population of paramagnetic microbeads in said fluid into close contact with said affinity capture agent, thereby affinity capturing any bioassay target molecule bound to said population of paramagnetic beads from the magnetic force field in the form of an molecular detection complex, and sweeping from the solid phase element any paramagnetic bead reagent not formed as molecular detection complex.

[0179] The means for moving a magnetic force field comprises a subassembly external to said microfluidic cartridge, said subassembly with moveable carriage with track upon which said carriage is mounted, said track mounted in a plane parallel to said axis of flow, said carriage further comprising a first magnet, the subassembly further configured to move the magnet along said track, first bringing the magnetic force field into proximity to said test pad and then distancing the magnetic force field from said test pad element.

[0180] Neodynium (NdFeB) magnets obtained from K&J Magnetics (Jamison Pa.) were found to be suitable. Magnets designated D38, D40, and D44 were used. These magnets are cylindrical with poles on the long axis and have a Curie temperature of about 300° C. (maximum operating temperature of 80° C.). The magnets are Grade N52 neodynium and have a surface field strength of 4600 to 5000 Gauss. It should be recalled that magnetic field force is inversely proportionate to the 4th power of the distance. Proximity to the test pad is in the range of 0.2 to 1.2 mm for these particular magnets. The diameter of the magnets range from to about 5 to 10 mm at the poles. For reference, the test pads themselves are about 0.5 mm×2 mm, with the long axis perpendicular to the traverse of the magnet.

[0181] Magnets with a triangular cross-section (prism magnets) and poles on two facets may also be used. These magnets have a sharply focused flux density above the apex of the facets.

[0182] Another aspect of the invention is use of peptidyl primer tagged amplicons in assays for nucleic acids. A num-

ber of methods are now available for manufacture of specific peptide epitopes attached to oligonucleotide probes or primers (see C.-H. Tung and S. Stein, *Bioconjugate Chem.*, 2000, 11, 605-618; E. Vives and B. Lebleu, *Tetrahedron Lett.*, 1997, 38, 1183-1186; R. Eritja, A. Pons, M. Escarcellar, E. Giralt, and F. Albericio, *Tetrahedron Lett.*, 1991, 47, 4113-4120; J. P. Bongartz, A. M. Aubertin, P. G. Milhaud, and B. Lebleu, *Nucleic. Acids Res.*, 1994, 22, 4681-4688; C.-H. Tung, M. J. Rudolph, and S. Stein, *Bioconjugate Chem.*, 1991, 2, 461-465; J. G. Harrison and S. Balasubramanian, *Nucleic. Acids Res.*, 1998, 26, 3136-3145; S. Soukchareun, J. Haralambidis, and G. Tregear, *Bioconjugate Chem.*, 1998, 9, 466-475; K. Arar, A.-M. Aubertin, A.-C. Roche, M. Monsigny, and M. Mayer, *Bioconjugate Chem.*, 1995, 6, 573-577; and, for an example of the use of the native ligation technique see: D. A. Stetsenko and M. J. Gait, *J Organic Chem.*, 2000, 65, 4900-4908). See also US 20006/0263816, incorporated herein in full by reference.

[0183] The peptidyl hapten conjugated primers of this method are satisfactorily synthesized by the above chemistries and others. We disclose here that primers of this class are compatible with PCR methods and with molecular biological nucleic acid amplifications in general. For use in assays, the amplification product with peptide-tagged primer-labelled amplicons is first captured by an affinity capture agent specific for a ligand on the second primer of the amplification primer set and bound to a magnetic microbead. The amplicon-bead complex is then interacted with peptidyl hapten-specific antibodies on the testpad and only those bead complexes with the peptide :amplicon molecular complex are captured by the testpad. This method permits screening of peptidyl-amplicon libraries by heterogeneous binding assays using magnetic bead technology.

[0184] The method results in an inventive composition as a product: a molecular detection complex comprising a two-tailed amplicon with first end and second end, said first end comprising a first primer covalently conjugated with a peptidyl hapten, and said second end comprising a second primer covalently conjugated with a ligand, said first end further comprising a ligand-bound ligand binding agent-coated reporter group, and said second end further comprising a peptidyl hapten bound anti-peptidyl hapten antibody immobilized on a solid phase.

[0185] This aspect of the invention is illustrated in FIG. **16**, which shows a molecular detection complex with two-tailed amplicon as tether, as in FIG. **1**, but here not involving biotin, and utilizing a more complex magnetic microbead than that described in FIG. **1**.

[0186] In FIG. **16**, the magnetic microbead **161** contains inclusion bodies or patches **162** of QDot, Texas Red, phycoerythrin, or other fluor, covalently attached or immobilized in the magnetic microbead matrix, here a latex binder with embedded particles of a ferrofluid. The microbead further comprises adsorbed antibody **169**. This is the reporter group. The tether consists of amplicon **164** with first primer **165** and peptidyl hapten tag **166**. Primer conjugates are incorporated into the amplicon during amplification. Anti-peptidyl hapten antibody **166** immobilizes the tether to solid substrate **163**, which may be another bead or a fiber or a test pad. The second primer **167** is conjugated with digoxigenin (for example). And the antibody in the reporter group is specific for digoxigenin. The reporter group is thus immobilized in a complex comprising at least 5 non-covalent bonds—bead:antibody; antibody:ligand; DNA:DNA; peptide:antibody; and antibody:solid phase. Yet the assay method described here permits the tether and reporter group to be extracted with high specificity and robustness from a moving magnetic field in which the paramagnetic beads are carried.

[0187] It should be noted that soluble reporter groups and fluorophore dyed latex beads may be used with the two-tailed amplicons of the present invention. By barcoding the fluorophore beads and coating uniformly labeled bead populations with peptidyl hapten specific antibodies, bead libraries can be synthesized for analysis of mixed populations of two-tailed amplicons or of two-tailed amplicon libraries, and the resulting affinity binding complexes with pairs of beads tethered by the two-tailed amplicons can then be sorted or assayed using dual excitation fluorometry, a sort of liquid microarray. These assays may be performed, for example, in a microfluidic cartridge configured as a fluorescent particle sorter, or in a flow luminometer. In a preferred assay method, the reporter group is a fluorophore of one emission frequency and the barcoded latex bead is selected from those of the prior art.

[0188] Assays of the method described herein are generally amenable to the preparation of devices, apparatuses, and kits for their performance.

EXAMPLE 1

A) Preparation of Primer Sets

[0189] Reverse primers were first prepared and HPLC purified. Peptides were derivatized with n-terminal hydrazine before use. Oligonucleotides were treated with succinimidyl 4-formylbenzoate in formamide and then reacted with the hydrazine derivatized peptides to form hapten-tagged primers.

[0190] The following peptidyl hapten-tagged primers were used.

5'-Peptidyl Oligomers

Primer	Primer Sequence*	Peptide Sequence**
A	CGCCAGTACGATATTCAG (SEQ ID NO:1)	(HNA) EQKLISEEDL (NH2) (SEQ ID NO:8)
B	ACCTGGACATCACGGCTTTCAAC (SEQ ID NO:2)	(HNA) YPYDVPDYA (NH2) (SEQ ID NO:9)
C	CCTATTGCAGAGCGAATGAC (SEQ ID NO:3)	(HNA) YTDIEMNRLGK (NH2) (SEQ ID NO:10)
D	TGAACTCCATTAACGCCAGA (SEQ ID NO:4)	(HNA) CEEEEYMPME (NH2) (SEQ ID NO:11)
E	CGACCTGACCAAATGCCAG (SEQ ID NO:5)	(HNA) TDFYLK (NH2) (SEQ ID NO:12)
F	CCTATAACAGCACCCACTATACGG (SEQ ID NO:6)	(HNA) DTYRYI (NH2) (SEQ ID NO:13)
G	CTCTGCGAGCATGGTCTGG (SEQ ID NO:7)	(HNA) QPELAPEDPED (NH2) (SEQ ID NO:14)

[0191] These peptide epitopes were selected based on the availability of complementary antibodies. Alternate peptide conjugation chemistries may also be used. Forward primers were all conjugated with biotin.

B) Preparation of Paramagnetic Microbeads

[0192] Monodisperse streptavidin-coated magnetic beads (MyOne Streptavidin Cl Dynabeads) were purchased from

Dynal, Carlsbad Calif. and washed and resuspended in 0.9× PBS, 30 mg/mL BSA and 1% TritonX100 with 5% (v/v) of a solution of 80 mM $MgCl_2$, 0.24% TritonX100, 1% BSA, in 0.5M TRIS pH 8 before use.

C) Preparation of Test Pads

[0193] A microfluidic device was built from stencil-cut laminates and contained multiple detection chambers of the form illustrated in FIG. **9**. Each detection chamber was formed with an inlet port and an outlet port fluidically connected to the detection chamber by microfluidic channels. Sufficient detection chambers were built for the experiment.

[0194] Before final assembly, test pads in the detection chamber were masked and plasma treated with oxygen gas. Peptidyl hapten-specific antibodies (Research Diagnostics, Flanders N.J.) and negative control solution were spotted on the test pads, 1 uL per pad, and dried in place under vacuum. Each detection chamber contained one test pad corresponding to each primer set and a negative control. The fully assembled device was treated with blocking/wash solution consisting of 0.9×PBS, 30 mg/mL BSA and 1% TritonX100 to passivate untreated plastic surfaces. The blocking solution was removed before use and the chambers were dried.

D) Assay Protocol

[0195] Using known DNA samples from enteric pathogens, PCR was performed with the prepared primer sets (above) for 35 cycles. Platinum Quantitative RT-PCR Thermoscript One-Step System reagents were used for the amplification. Successful amplification was confirmed by 5% agarose gel electrophoresis. Amplicon 10 uL was then resuspended with 5 uL of beads (above) in about 20 uL of buffer containing 10 mM $MgCl_2$, 0.5% BSA, 0.1% TritonX100 and 5 mM TRIS Buffer pH 8 and the bulk of this solution was loaded into a detection chamber. Each amplicon product corresponded to a single primer set and was loaded into a separate detection chamber.

[0196] The beads were first captured with a magnet positioned on the bottom of the detection chamber and the excess solution was removed. The magnet was then used to smear the bead paste onto, through and across the test pads, and the mixture was then allowed to incubate 1 min. With the magnet positioned on the bottom of the well, the well was gradually filled with blocking solution. The magnet was moved along the flow of the buffer, creating a bead front on the bottom layer of the detection chamber. The magnet was then shifted to the top of the detection chamber, lifting unbound beads out of the test pad areas. The unbound material could be resuspended in flowing buffer and rinsed to waste. The test pads were then rinsed with 1 volume of fresh buffer. Bright orange test pad "stripes" were immediately visible and were determined to correctly reflect specificity of binding of the hapten-tagged amplicon to the test pad containing the complementary antibody. Because the detection chambers were aligned in parallel when constructed, a stairstep pattern was evident after all the amplicon bead mixtures were processed because each tagged amplicon was bound by only one test pad in each detection chamber.

[0197] Upon clearing, positive tests were immediately visible as bright orange bands corresponding to the location of particular test strips. Negative test strips and negative controls remained translucent and uncolored. The results could be easily decoded by matching the location of the stained test pad with a key of the antibodies used in spotting.

EXAMPLE 2

[0198] PCR amplification was performed in a microfluidic device as follows:

[0199] A microfluidic device was built from stencil-cut laminates. Before final assembly, biotin- and hapten-tagged primer pairs, dATP, dCTP, dGTP and dTTP, TAQ polymerase, and a matrix consisting of TritonX100, BSA, PEG and Trehalose plus magnesium chloride were deposited in the amplification channel or chamber and dried in place under vacuum. Streptavidin-coated magnetic beads (Dynal MyOne Streptavidin Cl, Carlsbad Calif.) were spotted and dried in a chamber adjoining the amplification channels or chambers. Test pad areas in the detection chamber were stenciled (see FIG. **21** for general approach) and gas plasma treated, before antibody solutions were applied and dried in place. Antibody spots were blocked with StabilCoat (SurModics, Eden Prairie Minn.). The device was then treated with a TritonX100:BSA buffer to passivate untreated plastic surfaces.

The following reagents were also prepared:

Lysis Buffer

[0200] 4.5M Guanidinium thiocyanate
[0201] 5% TritonX100
[0202] 1% Sarcosine
[0203] 50 mM MES, pH 5.5
[0204] 20 mM EDTA

Wash Reagent

[0205] Anhydrous ethanol

Elution Buffer E11

[0206] 1% TritonX100
[0207] 0.1 mM EDTA
[0208] 20 mM TRIS pH8.0
[0209] 50 U RNAsin (Promega)

Rehydration Buffer

[0210] 1% TritonX100
[0211] 0.5% NaCl
[0212] 10 mg/mL Bovine Serum Albumin
[0213] 50 mM TRIS pH 8.0

[0214] Lysis Buffer, Wash Reagent, Elution Buffer, and Rehydration Buffer were aliquoted into sealed blister packs in designated chambers of the device. The device was then fully assembled and placed in a pneumatic controller with variable temperature TEC heating blocks positioned under the PCR fluidics and thermal interface assembly.

[0215] Clinical swab samples from diarrhoeal patients known to contain pathogenic microorganisms were handled with gloves in a biosafety cabinet. Each rectal swab was mixed vigorously with 400 uL of TE to solubilize the contents. Using filter-plugged pipet tips, about 400 uL of homogenate was then transferred to the sample port of the microfluidic device and the sample port was closed. All other steps were performed in the single-entry device, with no other operator exposure.

[0216] The remaining assay steps were automated.

[0217] An on-board sanitary bellows pump was used to pull sample through a pre-filter consisting of a depth filter ele-

ment, made of polypropylene for example, supported on a laser-cut plastic ribs. A valve was then used to close the sample port. The crude filtrate was then mixed with lysis buffer and drawn through a glass fiber filter to trap nucleic acids, and the filter retentate was rinsed thoroughly with ethanol. All rinses were sequestered in an onboard waste receptacle which vents through a 0.45 micron hydrophobic membrane filter. The nucleic acids on the glass fiber membrane were then eluted with elution buffer and ported into the reaction channel containing primers, dNTPs, polymerase, magnesium, buffer and surface active agents in dehydrated form. The reaction mixture, in a volume of about 50 uL, was then heated to 95° C. in the PCR fluidics and thermal interface assembly for about 10 sec to effect denaturation of double stranded sequences and secondary structure in the sample. Heating and cooling was supplied by external Peltier chips mounted on suitable heat sinks and PID controlled within a 1° C. range from setpoint. Immediately thereafter, the temperature was returned to about 60° C. for a first round of annealing and extension, which was continued for about 20 sec. Thermocycling was repeated for 40 cycles over an 18 min period.

[0218] Following extraction and amplification, the amplicon products were moved to a mag mix chamber for mixing streptavidin-labelled magnetic beads (Dynal, MyOne Streptavidin C1) which had been rehydrated in Rehydration Buffer. This mixture was incubated with gentle mixing and then transferred to a MagnaFlow chamber. Optionally the reaction mix can be rinsed to remove unreacted hapten-conjugated primer while holding the magnetic beads in place. Using permanent magnets mounted on an X-Y stage, the coated beads with putative target amplicon were brought into contact with the capture antibody test pads or array in the detection chamber, and unbound beads were moved away from the test pads with a moving magnetic field and sent to waste. Primers and non-specific amplicons were rinsed from the chamber with an excess of rehydration buffer, which again was discarded into on-board waste.

[0219] Upon clearing, positive tests were readily visible as orange bands corresponding to the location of particular test strips. Negative test strips remained translucent and uncolored. Time following transfer of amplification mixture to detection event was about 4 min. Knowing the identity of each immobilized antibody, the results could be easily decoded. In best practice to this date, the time from amplification to data presentation is less than 4 minutes.

[0220] In a test run with clinical samples, pathogens in 46 out of 47 stools were scored correctly in screening with the Magnaflow device. One sample previously identified as containing *Salmonella* by culture was identified as also containing enterotoxigenic *E. Coli O*157H1 by Magnaflow, (i.e., a double infection). For this example, the following primer pairs were obtained by custom synthesis and chemically conjugated by methods known in the art.

5'-Peptidyl Oligomers

Target	3' Target Gene Primer Sequence	Hapten conjugate
InvA	CAATGTAGAACGACCCCATAAACA (SEQ ID NO:15)	EQKLISEEDL' (SEQ ID NO:8)
Gyrase A	GCCATTCTAACCAAAGCATCATA (SEQ ID NO:16)	DTYRYI' (SEQ ID NO:13)
ipaH	ACTCCCGACACGCCATAGAA (SEQ ID NO:17)	QPELAPEDPED' (SEQ ID NO:14)
Eae	CTATCCAACAAGTTCAATTCATCC (SEQ ID NO:18)	TDFYLK' (SEQ ID NO:12)
Stx1A	AGACGTATGTAGATTCGCTGAA (SEQ ID NO:19)	YTDIEMNRLGK' (SEQ ID NO:10)
Stx2A	CTGGATGCATCTCTGGTCAT (SEQ ID NO:20)	CEEEEYMPME' (SEQ ID NO:11)
MalB	GGCGAATACCCAGCGACAT (SEQ ID NO:21)	YPYDVPDYA' (SEQ ID NO:9)

-continued

5'-Biotinylated Oligomers

Target	5'-Target Gene Primer Sequence	Primer Conjugate
InvA	TATCTGGTTGATTTCCTGATCGC (SEQ ID NO:22)	Biotin
Gyrase A	AAAATGATGAGGCAAAAAGTAGAACA (SEQ ID NO:23)	Biotin
ipaH	GGACATTGCCCGGGATAAA (SEQ ID NO:24)	Biotin
Eae	TTACCCGACGCCTCAAAC (SEQ ID NO:25)	Biotin
Stx1A	AGACGTATGTAGATTCGCTGAA (SEQ ID NO:26)	Biotin
Stx2A	GGAATGCAAATCAGTCGTCA (SEQ ID NO:27)	Biotin
MalB	GCCGATGCCAAATCGTCAG (SEQ ID NO:28)	Biotin

[0221] Forward primers for this example were conjugated with biotin. Reverse primers were conjugated with peptide haptens for which antibodies were available (Research Diagnostics, Flanders N.J.). Covalent attachment of the haptens was at the 5' terminus of the oligomer. Peptides were activated at the amino terminus for coupling.

EXAMPLE 3

[0222] A result of an assay in which the targets of Example 2 were extracted, amplified and detected is shown in FIG. **6**.

EXAMPLE 4

[0223] A respiratory panel containing biotinylated and peptidyl hapten-tagged primer pairs is designed. The primers are synthesized and then deposited in separate amplification channels or chambers of a device. Following the procedure of Example 2, throat swab washings are analyzed. A mini-bead impact mill is used to prepare the sample prior to analysis. A result is displayed in the detection chamber. The product is packaged as a kit.

EXAMPLE 5

[0224] A sexually transmitted disease panel containing biotinylated and peptidyl hapten-tagged primer pairs is designed and the primers are synthesized. The primers are

then deposited in separate amplification channels or chambers of a device. Following the procedure of Example 2, vaginal swab washings are analyzed. A detection endpoint is displayed in the detection chamber. The product is packaged as a kit.

EXAMPLE 6

[0225] An oncogene panel containing biotinylated and peptidyl hapten-tagged primer pairs is designed and the primers are synthesized. The primers are then deposited in a common amplification channel or chamber. Following PCR amplification, the amplification products are detected in a detection station. The product is packaged as a kit.

[0226] While the above description contains specificities, these specificities should not be construed as limitations on the scope of the invention, but rather as exemplifications of embodiments of the invention. That is to say, the foregoing description of the invention is exemplary for purposes of illustration and explanation. Without departing from the spirit and scope of this invention, one skilled in the art can make various changes and modifications to the invention to adapt it to various usages and conditions without inventive step. As such, these changes and modifications are properly, equitably, and intended to be within the full range of equivalence of the following claims. Thus the scope of the invention should be determined by the appended claims and their legal equivalents, rather than by the examples given.

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1-4. (canceled)

5. A method for multiplex target nucleic acid detection by heterogeneous binding assay, comprising:

a) preparing an amplification reagent comprising an amplification primer set having a first primer tagged with a peptidyl hapten tag and a second primer tagged with a ligand tag, wherein said first primer and said second primer are complementary to flanking sequences of a nucleic acid target sequence to be assayed;

b) preparing a paramagnetic bead reagent comprising a paramagnetic microbead with a binding agent, wherein said binding agent has a binding affinity for said ligand tag of said second primer;

c) preparing a test pad area with immobilized capture antibody, wherein said capture antibody has a binding affinity for said peptidyl hapten tag of said first primer;

d) processing a biological sample to release a nucleic acid fraction;

e) contacting said nucleic acid fraction with said amplification reagent;

f) performing an amplification to yield amplification products; and

g) assaying said amplification products for a two-tailed amplicon labeled with said peptidyl hapten tag of said first primer on a first end of said two-tailed amplicon and said ligand tag of said second primer on a second end of said two-tailed amplicon by:

i) contacting said amplification products with said paramagnetic bead reagent, thereby binding said ligand tag of said second end of said two-tailed amplicon to said binding agent of said paramagnetic bead reagent to yield a two-tailed amplicon paramagnetic bead complex;

ii) sweeping said two-tailed amplicon paramagnetic bead complex into close contact with said test pad area by moving a magnetic force field from outside to inside said test pad area, thereby binding said peptidyl hapten tag of said first end of said two-tailed amplicon of said two-tailed amplicon paramagnetic bead complex to said capture antibody of said test pad area to yield an immunoimmobilized paramagnetic reporter complex;

iii) sweeping from said test pad area any unbound two-tailed amplicon paramagnetic bead complex by moving said magnetic force field from inside to outside said test pad area; and

iv) detecting the presence of said immunoimmobilized paramagnetic reporter complex on said test pad area.

6. The method of claim **5** wherein said ligand tag of said amplification reagent is biotin and said binding agent of said paramagnetic bead reagent is an avidin.

7. The method of claim **5** wherein said peptidyl hapten tag is a peptide with 2 to 100 amino acid residues.

8. The method of claim **5** wherein said immunoimmobilized paramagnetic complex on said test pad is detected visually.

9. The method of claim **5** wherein said amplification step comprises a thermocycling protocol or isothermal protocol.

10. The method of claim **5** wherein said amplification step further comprises reverse transcription.

11. The method of claim **5** wherein said amplification step comprises nested amplification.

12. The method of claim **5** further comprising a multiplex target detection protocol.

13. A kit for performing the method of claim **5**, said kit comprising said amplification reagent and said test pad area.

14-33. (canceled)

34. A molecular detection complex comprising a two-tailed amplicon having a first end and a second end, said first end comprising a first primer covalently conjugated with a peptidyl hapten tag, and said second end comprising a second primer covalently conjugated with a ligand tag, wherein said first end further comprises a peptidyl hapten-bound anti-peptidyl hapten antibody immobilized on a solid phase, and wherein said second end further comprises a ligand-bound ligand binding agent-coated reporter group.

35. The molecular detection complex of claim **34**, wherein said ligand-bound ligand binding agent-coated reporter group is a magnetic microbead coated with said ligand binding agent, and wherein said solid phase is a test pad area.

36. An apparatus for forming and purifying the molecular detection complex of claim **35**, comprising:

a) a microfluidic cartridge comprising a substrate and a microchannel in said substrate, said microchannel comprising a fluid path with axis of flow and with upper and lower aspects;

b) a test pad in said microchannel, said test pad comprising an affinity capture agent comprising an anti-peptidyl hapten antibody immobilized on a solid phase;

c) a means for introducing a fluid comprising (i) a paramagnetic microbead comprising said reporter group and (ii) a two-tailed amplicon into said microchannel; and

d) a means for moving a magnetic force field along a plane parallel to said axis of flow of said microfluidic channel to (i) sweep said paramagnetic microbead in said fluid into close contact with said affinity capture agent, thereby binding said paramagnetic microbead to said affinity capture agent and binding said two-tailed amplicon to said paramagnetic microbead to yield said molecular detection complex, and (ii) sweep from said test pad any unbound paramagnetic microbead and any unbound two-tailed amplicon.

37-43. (canceled)

44. A method for rapid bioassay comprising:

a) preparing an amplification primer set comprising a first primer comprising a peptidyl hapten tag and a second primer comprising a ligand tag;

b) forming a two-tailed amplicon product comprising said peptidyl hapten tag of said first primer on a first end and said ligand tag of said second primer on a second end by amplifying a nucleic acid target with said amplification primer set;

c) complexing said two-tailed amplicon product to a reporter group having a binding affinity for said ligand tag to yield a two-tailed amplicon reporter group complex;

d) capturing said two-tailed amplicon reporter group complex on a solid phase having an immobilized capture antibody, wherein said capture antibody has a binding affinity for said peptidyl hapten tag, to yield an immobilized reporter group complex; and,

e) detecting said immobilized reporter group complex.

45. The method of claim **44** wherein said reporter group is a magnetic microbead and said solid phase is a test pad.

46. The method of claim **44** wherein said reporter group is a fluorophore and said solid phase is a barcoded latex bead.

47. The method of claim **44**, wherein said steps a) through c) are performed for a plurality of first primers conjugated

with different peptidyl hapten tags and a plurality of two-tailed amplicon products are captured and detected on a plurality of solid phases, each with an antibody specific for one of said different peptidyl hapten tags.

48. The method of claim **45**, wherein said steps a) through c) are performed for a plurality of first primers conjugated with different peptidyl hapten tags and a plurality of two-tailed amplicon products are magnetically captured and detected on a plurality of solid test pads, each with an antibody specific for one of said different peptidyl hapten tags.

49. The method for multiplex target nucleic acid detection by heterogeneous binding assay of claim **12**, wherein said amplification reagent comprises a plurality of first primers, each tagged with a different peptidyl hapten tag, and wherein said test pad area comprises a plurality of test pads, each with an immobilized monoclonal capture antibody having a binding affinity for one of the peptidyl hapten tags of the plurality of first primers.

50. The method for multiplex target nucleic acid detection by heterogeneous binding assay of claim **12**, wherein said test pad area comprises a plurality of test pads, each with an immobilized monoclonal capture antibody having a binding affinity for a different peptidyl hapten tagged amplicon.

51. A method for multiplex target nucleic acid detection by heterogeneous binding assay, comprising:

a) preparing a plurality of amplification reagents, each comprising an amplification primer set having a first primer tagged with a peptidyl hapten tag and a second primer tagged with an affinity tag, wherein each pair of said first primers and said second primers are complementary to flanking sequences of a plurality of nucleic acid target sequences to be assayed;

b) preparing a plurality of paramagnetic bead reagents, each comprising a paramagnetic microbead with a binding agent, wherein each of said binding agents has a binding affinity for one of said affinity tags of said second primers;

c) preparing a plurality of test pads in a test pad area, each test pad having a different immobilized capture antibody having a binding affinity for one of said peptidyl hapten tags of said first primers;

d) processing a biological sample to release a nucleic acid fraction;

e) contacting said nucleic acid fraction with said amplification reagents;

f) performing an amplification to yield amplification products; and

g) assaying said amplification products for two-tailed amplicons labeled with one of said peptidyl hapten tags of said first primers on a first end and one of said affinity tags of said second primers on a second end by:

i) contacting said amplification products with said paramagnetic bead reagents, thereby binding said affinity tags of said second ends of said two-tailed amplicons to said binding agents of said paramagnetic bead reagents to yield two-tailed amplicon paramagnetic bead complexes;

ii) sweeping said two-tailed amplicon paramagnetic bead complexes into close contact with said plurality of test pads by moving a magnetic force field from outside to inside said test pad area, thereby binding said peptidyl hapten tags of said first ends of said two-tailed amplicon of said two-tailed amplicon paramagnetic bead complex to said capture antibodies of said test pads to yield immobilized paramagnetic reporter complexes;

iii) sweeping from said test pad area any unbound two-tailed amplicon paramagnetic bead complexes by moving said magnetic force field from inside to outside said test pad area; and

iv) detecting the presence of said immunoimmobilized paramagnetic reporter complexes on each of said test pads.

52. The method for multiplex target nucleic acid detection by heterogeneous binding assay of claim **51**, wherein each of said affinity tags is a peptidyl hapten tag and each of said binding agents is an anti-peptidyl hapten antibody.

53. A molecular detection complex comprising a two-tailed amplicon having a first end and a second end, said first end comprising a first primer covalently conjugated with a first peptidyl hapten tag, and said second end comprising a second primer covalently conjugated with a second peptidyl hapten tag, wherein said first end further comprises a first peptidyl hapten-bound anti-first peptidyl hapten antibody immobilized on a solid phase, and wherein said second end further comprises a second peptidyl hapten-bound anti-second peptidyl hapten antibody-coated reporter group.

54. A method for rapid bioassay comprising:

a) preparing an amplification primer set comprising a first primer comprising a first peptidyl hapten tag and a second primer comprising a second peptidyl hapten tag;

b) forming a two-tailed amplicon product comprising said first peptidyl hapten tag of said first primer on a first end and said second peptidyl hapten tag of said second primer on a second end by amplifying a nucleic acid target with said amplification primer set;

c) complexing said two-tailed amplicon product to a reporter group having a binding affinity for said second peptidyl hapten tag of said second primer to yield a two-tailed amplicon reporter group complex;

d) capturing said two-tailed amplicon reporter group complex on a solid phase having an immobilized capture antibody, wherein said capture antibody has a binding affinity for said first peptidyl hapten tag, to yield an immobilized reporter group complex; and

e) detecting said immobilized reporter group complex.

55. A kit for performing a multiplex nucleic acid bioassay with multiplex detection of two-tailed amplicons, said kit comprising:

a first assay reagent having a peptidyl-hapten conjugated first amplification primer;

a plurality of test pads, each of said test pad comprising a dehydrated first affinity binding agent with affinity for said peptidyl-hapten of said first primer; and

a second assay reagent comprising a bead reagent as a reporter group, wherein said bead reagent is coated with a second affinity binding agent.

56. The kit of claim **55** for performing a panel assay for multiplex detection of multiple nucleic acid targets.

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