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(54) Title: BIO-BARCODE BASED DETECTION OF TARGET ANALYTES

(57) Abstract: The present invention relates to screening methods, compositions, and kits for detecting for the presence or absence of one or more target analytes, e.g. biomolecules, in a sample. In particular, the present invention relates to a method that utilizes reporter oligonucleotides as biochemical barcodes for detecting multiple protein structures or other target analytes in a solution.

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BIO-BARCODE BASED DETECTION OF TARGET ANALYTES

FIELD OF THE INVENTION:

The present invention relates to a screening method for detecting for the presence or absence of one or more target analytes, e.g., proteins, nucleic acids, or other compounds in a sample. In particular, the present invention relates to a method that utilizes reporter oligonucleotides as biochemical barcodes for detecting one or more analytes in a solution.

CROSS-REFERENCE:

This application claims the benefit of provisional application Nos. 60/506,708, filed Sept. 25, 2003; 60/482,979, filed June 27, 2003; 60/496,893, filed August 21, 2003; 60/515,243, filed Oct. 28, 2003; 60/530,797, filed Dec. 18, 2003 and is a continuation-in-part of U.S. patent application serial no. 10/108,211, filed March 27, 2002, which in turn claims the benefit of U.S. Provisional application nos. 60/192,699, filed March 28, 2000; and 60/350,560, filed November 13, 2001, which are incorporated by reference in their entirety, and which is a continuation-in-part of U.S. patent application serial no. 09/820,279, filed March 28, 2001. The work reported in this application is funded, in part, by NSF, ARO, AFOSR, DARPA, and NIH grants. Accordingly, the U.S. government has certain rights to the invention described in this application.

BACKGROUND OF THE INVENTION:

The detection of analytes is important for both molecular biology research and medical applications. Diagnostic methods based on fluorescence, mass spectroscopy, gel electrophoresis, laser scanning and electrochemistry are now available for identifying a variety of protein structures.[1-4] Antibody-based reactions are widely used to identify the genetic protein variants of blood cells, diagnose diseases, localize molecular probes in tissue, and purify molecules or effect separation processes.[5] For medical diagnostic applications (e.g. malaria and HIV), antibody tests such as the

enzyme-linked immunosorbent assay, Western blotting, and indirect fluorescent antibody tests are extremely useful for identifying single target protein structures. [6,7] Rapid and simultaneous sample screening for the presence of multiple antibodies would be beneficial in both research and clinical applications. However, it is difficult, expensive, and time-consuming to simultaneously detect several protein structures under assay conditions using the aforementioned related protocols.

Polymerase chain reaction (PCR) and other forms of target amplification have enabled rapid advances in the development of powerful tools for detecting and quantifying DNA targets of interest for research, forensic, and clinical applications [26-32]. The development of comparable target amplification methods for proteins could dramatically improve medical diagnostics and the developing field of proteomics [33-36]. Although one cannot yet chemically duplicate protein targets, it is possible to tag such targets with oligonucleotide markers that can be subsequently amplified with PCR and then use DNA detection to identify the target of interest [37-45]. This approach, often referred to as immuno-PCR, allows one to detect proteins with DNA labels in a variety of different formats (Figure 5). To date, all immuno-PCR approaches involve heterogeneous assays, which involve initial immobilization of a target analyte to a surface with subsequent detection using an antibody with a DNA label (for example, see U.S. Patent nos. 5,635,602, and 5,665,539). The DNA label is typically strongly bound to the antibody (either through covalent interactions or strepavidin-biotin binding). Although theses approaches are notable advances in protein detection, they have several drawbacks: 1) limited sensitivity because of a low ratio of DNA identification sequence to detection antibody; 2) slow target binding kinetics due to the heterogeneous nature of the target capture procedure, which increases assay time and decreases assay sensitivity (Step 3 in Figure 5); 3) complex conjugation chemistries that are required to chemically link the antibody and DNA-markers (Step 4 in Figure 5); and 4) require a PCR amplification step[45]. Therefore, a sensitive, and rapid method for detecting target analytes in a sample that is amenable to multiplexing and easy to implement is needed.

For DNA detection methods, many assays have been developed using radioactive labels, molecular fluorophores, chemiluminescence schemes,

electrochemical tags, and most recently, nanostructure-based labels.[61-70] Although some nanostructure-based methods are approaching PCR in terms of sensitivity, none thus far have achieved the 1-10 copy sensitivity level offered by PCR. A methodology that allows for PCR-like signal amplification without the complexity, expense, and time and labor intensive aspects associated with PCR would provide significant advantages over such PCR-based methods.

SUMMARY OF THE INVENTION:

The present invention relates to methods, probes, compositions, and kits that utilize oligonucleotides as biochemical barcodes for detecting multiple analytes in one solution. The approach takes advantage of recognition elements of specific binding pairs functionalized either directly or indirectly with nanoparticles, and the previous observation that hybridization events that result in the aggregation of gold nanoparticles can significantly alter their physical properties (e.g. optical, electrical, mechanical).[8-12] The general idea is that each recognition element of a specific binding pair can be associated with a different oligonucleotide sequence with discrete and tailorable hybridization and melting properties and a physical signature associated with the nanoparticles. The discrete hybridization and melting properties can be used to decode a series of analytes in a multi-analyte assay by creating a change in a physical signature associated with the nanoparticles or by detection of oligonucleotide sequence(s), through hybridization/dehybridization or melting/annealing events.

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

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

immobilizing the target analytes onto the substrate;

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

washing the substrate to remove unbound particle probes; and

optionally amplifying the DNA barcode; and

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

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

In another aspect of the invention, DNA barcode is amplified by PCR.

In another aspect of the invention, the particle is labeled with at least two DNA barcodes.

In another aspect of the invention, the substrate is arrayed with one or more types of capture probes for the target analytes.

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

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

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

contacting the sample, the capture probe, and the detection probe under

conditions effective to allow specific binding interactions between the target analyte and the probes and to form an aggregate complex in the presence of the target analyte;

washing the substrate to remove any unbound detection probes;

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

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

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

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

amplifying the DNA barcode prior to said detecting.

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

In another aspect of this embodiment, the capture probe is bound to a magnetic substrate such as a magnetic particle.

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

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

In another aspect of this embodiment, the detection probe comprises a dendrimer.

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

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

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

contacting the sample with the capture probe and the detection probe under

conditions effective to allow specific binding interactions between the target analyte and the probes and to form an aggregated complex bound to the magnetic particle in the presence of the target analyte;

washing any unbound detection probes from the magnetic particle; and

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

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

isolating the aggregated complex by applying a magnetic field;

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

isolating the released DNA barcodes.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

observing whether aggregate complex formation occurred.

Another embodiment of the invention provides for a method for detecting the presence or absence of one or more target analytes, each target analyte having at least two binding sites. The method comprises at least one type of capture probe and at least one type of detection probe for each target analyte used. These probes may be generated prior to conducting the actual assay or in situ while conducting the assay. The capture probe comprises a first member of a first specific binding pair, wherein the first member of the first specific binding pair binds to the first binding site of the

target analyte, and wherein the first member of the first specific binding pair optionally binds to a substrate. In one preferred embodiment, the substrate comprises a magnetic particle. The detection probe comprises (1) a nanoparticle; (2) a first member of a second specific binding pair attached to the nanoparticle, wherein the first member of the second specific binding pair binds to a second binding site of the target analyte; and (3) at least one type of oligonucleotides bound to the nanoparticle; and (4) at least one type of DNA barcodes, each type of DNA barcode having a predetermined sequence that is complementary to at least a portion of a specific type of oligonucleotides. When employed in a sample containing the target analyte, the first member of a first specific binding pair on the capture probe binds to the first binding site of the target analyte, and the first member of a second specific binding pair on the detection probe binds to the second binding site of the target analyte. Aggregation occurs when capture probes and detection probes are brought together by the target analyte. The aggregates may be isolated and subjected to further melting analysis to identify the particular target analyte where multiple targets are present as discussed above. Alternatively, the aggregates can be dehybridized to release the DNA barcode.

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

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

isolating aggregated complexes; and

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

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

isolating the aggregated complex;

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

isolating the DNA barcode; and

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

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

isolating the aggregated complex;

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

isolating the DNA barcode;

amplifying the isolated DNA barcode; and

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

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

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

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

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

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

substrate and a second portion of the DNA barcodes with some of the oligonucleotides bound to the nanoparticles; and

observing a detectable change.

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

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

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

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

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

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

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

In another aspect of this embodiment, at least two types of particle complex probes are provided, a first type of probe having a specific binding complement to a

first binding site of the target analyte and a second type of probe having a specific binding complement to a second binding site of the target analyte.

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

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

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

In yet another embodiment of the invention, a particle complex probe is provided. Thus in one embodiment of the invention, a particle complex probe is provided which comprises a particle having oligonucleotides bound thereto, a DNA barcode, and an oligonucleotide having bound thereto a specific binding complement

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

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

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

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

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

These and other embodiments of the invention will become apparent in light of the detailed description below.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates a DNA/Au nanoparticle-based protein detection scheme. (A) Preparation of hapten-modified nanoparticle probes. (B) Protein detection using protein binding probes. Notice that there are nine G,C pairs in sequence A and there are only two G,C pairs in sequence B.

Figure 2 illustrates thermal denaturation profiles for Au nanoparticle aggregates linked by DNA and proteins. Extinction at 260 nm was monitored as a function of increasing temperature (1 °C/min, 1 min holding time). Each UV-Vis spectrum was measured under constant stirring to suspend the aggregates. All the aggregates were suspended in 1 ml of 0.3 M PBS prior to performing the melting analyses. A) Two probes with one target antibody present IgE (——), IgG1 (---)); all data have been normalized; (B) Two probes with both target antibodies present. Inset; first derivative of the thermal denaturation curve.

Figure 3 illustrates an array-based protein detection scheme using DNA as a biobarcode for the protein.

Figure 4 illustrates scanometric DNA array detection of the DNA biobarcodes. Left column is for the detection of the biobarcode associated with IgG1 and the right column is for the biobarcode associated with IgE. The capture oligonucleotides are 5' -thiol-modified ataactagaacttga (SEQ ID NO:1)for the IgG1 system and 5' –thiol-modified ttatctattatt (SEQ ID NO:2) for the IgE system. Each spot is approximately 250 um in diameter and read via gray-scale with an Epson Expression 1640XL flatbed scanner (Epson America, Longbeach, California). These assays have been studied and work comparably well over the 20 nM to 700 nM target concentration range.

Figure 5 illustrates a common type of immuno-PCR-based analyte detection scheme.

Figure 6 depicts the use of Barcode PCR (*B*PCR) protocol to detect a target analyte, prostate specific antigen (PSA). Panel A illustrates probe design and preparation. Panel B depicts PSA detection and barcode DNA amplification and identification.

Figure 7 illustrates the control experiment to assess primer-dimer formation, DNA barcode amplification, and the effect of increasing DMSO concentration using 25 thermal cycles. Lanes 1 through 5 are those with DNA barcode present in the PCR reaction mixture while there is no DNA barcode in lanes 6 through 10. Note that DMSO is increased from lane 1 to 5 and 6 to 10 (0 to 2 % in 0.5 % increments).

Figure 8 shows gel electrophoresis images and relative band intensity graph of barcode DNA amplified by PCR after PSA detection. Panel A, lanes 1 and 2 are control experiments (lane 1: with background proteins anti-dinitrophenyl and β-galactosidase without PSA, lane 2: with no protein). From lanes 3 to 8, PSA concentrations in the sample (10μl) are 300 aM, 3 fM, 30 fM, 300 fM, 3 pM, and 30 pM, respectively. The standard biobarcode DNA 40-mer for PSA is run in lane 9 to compare with other gel bands after PCR. Panel B, relative gel electrophoresis band intensity graph after *B*PCR. Panel C, low concentration detection of PSA. Concentrations are from 3 aM to 300 fM in 10x dilutions from lane 2 (3 aM) to lane 7 (300 fM). A negative control with only background proteins is shown in lane 1, and the standard biobarcode 40-mer (6 μM biobarcode duplex) is shown in the fist lane (lane C). Panel D, relative gel electrophoresis band intensity after *B*PCR.

Figure 9 illustrates scanometric detection of PSA-specific barcode DNA. PSA concentration (sample volume of 10 ml) was varied from 300 fM to 3 aM and a negative control sample where no PSA was added (control) is shown. For all seven samples, 2 ml of anti-dinitrophenyl (10 pM) and 2 ml of □-galactosidase (10 pM) were added as background proteins. Also shown is PCR-less detection of PSA (30 aM and control) with 30 nm NP probes (inset). Chips were imaged with the Verigene ID system.

Figure 10 illustrates theoretical detection limit of *B*PCR. Left panel, The gel image shows bands after PCR at decreasing starting barcode DNA concentrations. Lane 1: 3×10^9 copies, lane 2: 3×10^8 copies, lane 3: 3×10^7 copies, lane 4: 3×10^6 copies, lane 5: 3×10^5 copies, lane 6: 3×10^4 copies, lane 7: 3×10^3 copies, lane 8: 3×10^2 copies, lane 9: 3×10^1 copies, and lane 10: no barcode DNA. Right panel, relative gel electrophoresis band intensity graph.

Figure 11 illustrates detection of PSA-specific barcode DNA where the PSA is dissolved in a complex goat serum medium. Each panel shows the signal generated by the *B*PCR amplified barcode DNA at various concentrations of analyte (3 pM to 3 aM).

Figure 12 illustrates PCR-less detection of PSA with 30 nm NP probes. Each panel and associated relative intensity value on the bar graph shows the signal generated by direct detection of barcode DNA (*i.e.*, non-*B*PCR amplified) at various concentrations (30 aM to 3 pM, and control). Chips were imaged with the Verigene ID system (Nanosphere, Inc., Northbrook, IL).

Figure 13 illustrates the DNA-BCA assay. **A.** Nanoparticle and Magnetic Microparticle Probe Preparation. **B.** Nanoparticle-Based PCR-less DNA Amplification Scheme.

Figure 14 illustrates amplified Anthrax Bar-Code DNA Detection with Verigene ID system. **A.** Anthrax Bar-Code DNA Detection with 20 nm NP Probes. **B.** Anthrax Bar-Code DNA Detection with 30 nm NP Probes.

Figure 15 illustrates intensity graph of the bar-code DNA and NP probe sandwiched spots after silver enhancement for 20 nm and 30 nm NP probes.

Figure 16 illustrates one embodiment of the "universal" nanoparticle probe detection scheme. **A.** The universal probes are synthesized for specificity to one or more target nucleic acid sequences. The target recognition DNA can be used to control specificity of the probe for a target. The probes can be used in assay systems where single or multiple target nucleic acid sequences are present in a given test solution. **B.** The universal probes are used in conjunction with a second type of recognition oligonucleotide bound to a substrate, such as a magnetic microparticle or glass slide. The second type of recognition oligonucleotide, the universal probe, and the test solution thought to contain the target nucleic acid are mixed and reacted under conditions that allow for hybridization and complex formation. The complex is separated from the unreacted universal probes and test solution components, and the reporter oligonucleotides are detected.

Figure 17 illustrates another embodiment of the universal nanoparticle probe with dendrimer probes, amplified dendrimer probes, and dendrimer-nanoparticle

hybrid probes. The first type of dendrimer probes comprise a recognition oligonucleotide sequence and a nucleic acid sequence that is complementary to a reporter oligonucleotide, such as a barcode DNA. The recognition oligonucleotide sequence on the first type of dendrimer probe can hybridize to a second type of dendrimer probe. Under hybridization conditions, this generates a dendrimer probe complex (or matrix) that can be extended as desired. The second type of dendrimer probe can bind a plurality of dendrimer probes and functions to amplify the amount of reporter oligonucleotide contained within the entire matrix. Similarly, the amount of recognition oligonucleotide present on the first type of dendrimer can be increased or decreased, in order to provide more sites of complexation with the second type of dendrimer, or in order to provide more reporter oligonucleotide. The first or second type of dendrimer probes can be used with other particle probes, such as gold nanoparticle probes, or magnetic particle probes in order to generate a hybrid probe complex (or matrix) system, as required by the particular assay.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, a "type of" nanoparticles, conjugates, particles, latex microspheres, etc. having oligonucleotides attached thereto refers to a plurality of that item having the same type(s) of oligonucleotides attached to them. "Nanoparticles having oligonucleotides attached thereto" or "Nanoparticles having oligonucleotides attached thereto" are also sometimes referred to as "nanoparticle-oligonucleotide conjugates" or, in the case of the detection methods of the invention, "nanoparticle-oligonucleotide probes," "nanoparticle probes," or just "probes."

As used throughout the invention "barcode", "biochemical barcode", "biobarcode", "barcode DNA", "DNA barcode", "reporter barcode", "reporter barcode DNA", etc. are all interchangeable with each other and have the same meaning. The DNA barcode may be a nucleic acid such as deoxynucleic acid or ribonucleic acid. Preferably, the DNA barcode is an oligonucleotide of a predefined sequence. If desired, the DNA barcode may be labeled, for instance, with biotin, a radiolabel, or a fluorescent label.

The term "nanoparticle complex" or "nanoparticle complex probe" refers to a conjugate comprised of nanoparticle-oligonucleotide conjugates, a reporter oligonucleotide, and an oligonucleotide having bound thereto a specific binding complement to a target analyte.

The term "analyte" or "target analyte" refers to the compound or composition to be detected, including drugs, metabolites, pesticides, pollutants, and the like. The analyte can be comprised of a member of a specific binding pair (sbp) and may be a ligand, which is monovalent (monoepitopic) or polyvalent (polyepitopic), preferably antigenic or haptenic, and is a single compound or plurality of compounds, which share at least one common epitopic or determinant site. The analyte can be a part of a cell such as bacteria or a cell bearing a blood group antigen such as A, B, D, etc., or an HLA antigen or a microorganism, e.g., bacterium, fungus, protozoan, or virus. If the analyte is monoepitopic, the analyte can be further modified, e.g. chemically, to provide one or more additional binding sites. In practicing this invention, the analyte has at least two binding sites.

The polyvalent ligand analytes will normally be larger organic compounds, often of polymeric nature, such as polypeptides and proteins, polysaccharides, nucleic acids, and combinations thereof. Such combinations include components of bacteria, viruses, chromosomes, genes, mitochondria, nuclei, cell membranes and the like.

For the most part, the polyepitopic ligand analytes to which the subject invention can be applied will have a molecular weight of at least about 5,000, more usually at least about 10,000. In the polymeric molelecule category, the polymers of interest will generally be from about 5,000 to 5,000,000 molecular weight, more usually from about 20,000 to 1,000,000 molecular weight; among the hormones of interest, the molecular weights will usually range from about 5,000 to 60,000 molecular weight.

A wide variety of proteins may be considered as belonging to the family of proteins having similar structural features, proteins having particular biological functions, proteins related to specific microorganisms, particularly disease causing microorganisms, etc. Such proteins include, for example, immunoglobulins,

cytokines, enzymes, hormones, cancer antigens, nutritional markers, tissue specific antigens, etc.

The types of proteins, blood clotting factors, protein hormones, antigenic polysaccharides, microorganisms and other pathogens of interest in the present invention are specifically disclosed in U.S. Pat. No. 4,650,770, the disclosure of which is incorporated by reference herein in its entirety.

The monoepitopic ligand analytes will generally be from about 100 to 2,000 molecular weight, more usually from 125 to 1,000 molecular weight.

The analyte may be a molecule found directly in a sample such as a body fluid from a host. The sample can be examined directly or may be pretreated to render the analyte more readily detectible. Furthermore, the analyte of interest may be determined by detecting an agent probative of the analyte of interest such as a specific binding pair member complementary to the analyte of interest, whose presence will be detected only when the analyte of interest is present in a sample. Thus, the agent probative of the analyte becomes the analyte that is detected in an assay. The body fluid can be, for example, urine, blood, plasma, serum, saliva, semen, stool, sputum, cerebral spinal fluid, tears, mucus, and the like.

The term "specific binding pair (sbp) member" refers to one of two different molecules, which specifically binds to and can be defined as complementary with a particular spatial and/or polar organization of the other molecule. The members of the specific binding pair can be referred to as ligand and receptor (antiligand). These will usually be members of an immunological pair such as antigen-antibody, although other specific binding pairs such as biotin-avidin, enzyme-substrate, enzyme-antagonist, enzyme–agonist, drug-target molecule, hormones-hormone receptors, nucleic acid duplexes, IgG-protein A/protein G, polynucleotide pairs such as DNA-DNA, DNA-RNA, protein-DNA, lipid-DNA, lipid-protein, polysaccharide-lipid, protein-polysaccharide, nucleic acid aptamers and associated target ligands (*e.g.*, small organic compounds, nucleic acids, proteins, peptides, viruses, cells, etc.), and the like are not immunological pairs but are included in the invention and the definition of sbp member. A member of a specific binding pair can be the entire

molecule, or only a portion of the molecule so long as the member specifically binds to the binding site on the target analyte to form a specific binding pair.

The term "ligand" refers to any organic compound for which a receptor naturally exists or can be prepared. The term ligand also includes ligand analogs, which are modified ligands, usually an organic radical or analyte analog, usually of a molecular weight greater than 100, which can compete with the analogous ligand for a receptor, the modification providing means to join the ligand analog to another molecule. The ligand analog will usually differ from the ligand by more than replacement of a hydrogen with a bond, which links the ligand analog to a hub or label, but need not. The ligand analog can bind to the receptor in a manner similar to the ligand. The analog could be, for example, an antibody directed against the idiotype of an antibody to the ligand.

The term "receptor" or "antiligand" refers to any compound or composition capable of recognizing a particular spatial and polar organization of a molecule, e.g., epitopic or determinant site. Illustrative receptors include naturally occurring receptors, e.g., thyroxine binding globulin, antibodies, enzymes, Fab fragments, lectins, nucleic acids, nucleic acid aptamers, avidin, protein A, barstar, complement component C1q, and the like. Avidin is intended to include egg white avidin and biotin binding proteins from other sources, such as streptavidin.

The term "specific binding" refers to the specific recognition of one of two different molecules for the other compared to substantially less recognition of other molecules. Generally, the molecules have areas on their surfaces or in cavities giving rise to specific recognition between the two molecules. Exemplary of specific binding are antibody-antigen interactions, enzyme-substrate interactions, polynucleotide interactions, and so forth.

The term "non-specific binding" refers to the binding between molecules that is relatively independent of specific surface structures. Non-specific binding may result from several factors including hydrophobic interactions between molecules.

The term "antibody" refers to an immunoglobulin which specifically binds to and is thereby defined as complementary with a particular spatial and polar organization of another molecule. The antibody can be monoclonal or polyclonal and

can be prepared by techniques that are well known in the art such as immunization of a host and collection of sera (polyclonal) or by preparing continuous hybrid cell lines and collecting the secreted protein (monoclonal), or by cloning and expressing nucleotide sequences or mutagenized versions thereof coding at least for the amino acid sequences required for specific binding of natural antibodies. Antibodies may include a complete immunoglobulin or fragment thereof, which immunoglobulins include the various classes and isotypes, such as IgA, IgD, IgE, IgG1, IgG2a, IgG2b and IgG3, IgM, etc. Fragments thereof may include Fab, Fv and F(ab').sub.2, Fab', and the like. In addition, aggregates, polymers, and conjugates of immunoglobulins or their fragments can be used where appropriate so long as binding affinity for a particular molecule is maintained.

The present invention relates to a method that utilizes oligonucleotides as biochemical barcodes for detecting multiple analytes in a sample. The approach takes advantage of recognition elements (e.g., proteins or nucleic acids) functionalized either directly or indirectly with nanoparticles and the previous observation that hybridization events that result in the aggregation of gold nanoparticles can significantly alter their physical properties (e.g. optical, electrical, mechanical).[8-12] The general idea is that each recognition element can be associated with a different oligonucleotide sequence (a DNA barcode) with discrete and tailorable hybridization and melting properties and a physical signature associated with the nanoparticles that changes upon melting to decode a series of analytes in a multi-analyte assay. Therefore, one can use the melting temperature of a DNA-linked aggregate and a physical property associated with the nanoparticles that changes upon melting to decode a series of analytes in a multi-analyte assay. The barcodes herein are different from the ones based on physical diagnostic markers such as nanorods,[23] flourophore-labeled beads,[24] and quantum dots,[25] in that the decoding information is in the form of chemical information stored in a predesigned oligonucleotide sequence.

The present invention provides several broadly applicable strategies for using nanoparticle probes (preferably gold nanoparticle probes), heavily functionalized with oligonucleotides, to detect a variety of biomolecules, for example, single or multiple polyvalent proteins, in one sample. In particular known methods for detection of

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

The present invention contemplates the use of any suitable particle having oligonucleotides attached thereto that are suitable for use in detection assays. In practicing this invention, however, nanoparticles are preferred. The size, shape and chemical composition of the particles will contribute to the properties of the resulting probe including the DNA barcode. These properties include optical properties, optoelectronic properties, electrochemical properties, electronic properties, stability in various solutions, pore and channel size variation, ability to separate bioactive molecules while acting as a filter, etc. The use of mixtures of particles having different sizes, shapes and/or chemical compositions, as well as the use of nanoparticles having uniform sizes, shapes and chemical composition, are contemplated. Examples of suitable particles include, without limitation, nano- and microsized core particles, aggregate particles, isotropic (such as spherical particles) and anisotropic particles (such as non-spherical rods, tetrahedral, prisms) and core-shell particles such as the ones described in U.S. Patent application no. 10/034,451, filed December 28, 2002 and International application no. PCT/US01/50825, filed December 28, 2002, which are incorporated by reference in their entirety. In

practicing the invention, the detection probes are preferably generated prior to conducting the actual assay. Alternatively, the detection probes may be generated in situ while conducting the assay.

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

Methods of making metal, semiconductor and magnetic nanoparticles are well known in the art. See, *e.g.*, Schmid, G. (ed.) *Clusters and Colloids* (VCH, Weinheim, 1994); Hayat, M.A. (ed.) *Colloidal Gold: Principles, Methods, and Applications* (Academic Press, San Diego, 1991); Massart, R., *IEEE Taransactions On Magnetics*, 17, 1247 (1981); Ahmadi, T.S. et al., *Science*, 272, 1924 (1996); Henglein, A. et al., *J. Phys. Chem.*, 99, 14129 (1995); Curtis, A.C., et al., *Angew. Chem. Int. Ed. Engl.*, 27, 1530 (1988).

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

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

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

The nanoparticles, the oligonucleotides or both are functionalized in order to attach the oligonucleotides to the nanoparticles. Such methods are known in the art. For instance, oligonucleotides functionalized with alkanethiols at their 3'-termini or 5'-termini readily attach to gold nanoparticles. See Whitesides, *Proceedings of the Robert A. Welch Foundation 39th Conference On Chemical Research Nanophase Chemistry*, Houston, TX, pages 109-121 (1995). See also, Mucic et al. *Chem. Commun.* 555-557 (1996) (describes a method of attaching 3' thiol DNA to flat gold surfaces; this method can be used to attach oligonucleotides to nanoparticles). The alkanethiol method can also be used to attach oligonucleotides to other metal, semiconductor and magnetic colloids and to the other nanoparticles listed above. Other functional groups for attaching oligonucleotides to solid surfaces include phosphorothioate groups (see, *e.g.*, U.S. Patent No. 5,472,881 for the binding of oligonucleotide-phosphorothioates to gold surfaces), substituted alkylsiloxanes (see, *e.g.* Burwell, *Chemical Technology*, 4, 370-377 (1974) and Matteucci and Caruthers, *J. Am. Chem. Soc.*, 103, 3185-3191 (1981) for binding of oligonucleotides to silica and glass surfaces, and Grabar et al., *Anal. Chem.*, 67, 735-743 for binding of aminoalkylsiloxanes and for similar binding of mercaptoaklylsiloxanes). Oligonucleotides terminated with a 5' thionucleoside or a 3' thionucleoside may also be used for attaching oligonucleotides to solid surfaces. The following references describe other methods which may be employed to attached oligonucleotides to nanoparticles: Nuzzo et al., *J. Am. Chem. Soc.*, 109, 2358 (1987) (disulfides on gold);

Allara and Nuzzo, *Langmuir*, 1, 45 (1985) (carboxylic acids on aluminum); Allara and Tompkins, *J. Colloid Interface Sci.*, 49, 410-421 (1974) (carboxylic acids on copper); Iler, The Chemistry Of Silica, Chapter 6, (Wiley 1979) (carboxylic acids on silica); Timmons and Zisman, *J. Phys. Chem.*, 69, 984-990 (1965) (carboxylic acids on platinum); Soriaga and Hubbard, *J. Am. Chem. Soc.*, 104, 3937 (1982) (aromatic ring compounds on platinum); Hubbard, *Acc. Chem. Res.*, 13, 177 (1980) (sulfolanes, sulfoxides and other functionalized solvents on platinum); Hickman et al., *J. Am. Chem. Soc.*, 111, 7271 (1989) (isonitriles on platinum); Maoz and Sagiv, *Langmuir*, 3, 1045 (1987) (silanes on silica); Maoz and Sagiv, *Langmuir*, 3, 1034 (1987) (silanes on silica); Wasserman et al., *Langmuir*, 5, 1074 (1989) (silanes on silica); Eltekova and Eltekov, *Langmuir*, 3, 951 (1987) (aromatic carboxylic acids, aldehydes, alcohols and methoxy groups on titanium dioxide and silica); Lec et al., *J. Phys. Chem.*, 92, 2597 (1988) (rigid phosphates on metals).

In one aspect of this embodiment of the invention, nanoparticles conjugated with dendrimers labeled with at least two types of oligonucleotides are provided. Dendritic molecules are structures comprised of multiple branching unit monomers, and are used in various applications. See, e.g., Barth et al., Bioconjugate Chemistry 5:58-66 (1994); Gitsov & Frechet, Macromolecules 26:6536-6546 (1993); Hawker & Frechet, J. Amer. Chem. Soc. 112:7638-7647 (1990a); Hawker & Frechet, Macromolecules 23:4726-4729 (1990b); Hawker et al., J. Chem. Soc. Perkin Trans. 1:1287-1297 (1993); Lochmann et al. J. Amer. Chem. Soc. 115:7043-7044 (1993); Miller et al., J. Amer. Chem. Soc. 114:1018-1025 (1992); Mousy et al., Macromolecules 25:2401-2406 (1992); Naylor et al., J. Amer. Chem. Soc. 111:2339-2341 (1989); Spindler & Frechet, Macromolecules Macromolecules 26:4809-4813 (1993); Turner et al., Macromolecules 26:4617-4623 (1993); Wiener et al., Magnetic Resonance Med. 31(1):1-8 (1994); Service, 267:458-459 (1995); Tomalia, Sci. Amer. 62-66 (1995); and U.S. Pat. Nos. 4,558,120; 4,507,466; 4,568,737; 4,587,329; 4,857,599; 5,527,524; 5,338,532 to Tomalia, and U.S. Patent 6,274,723 to Nilsen, all of which are incorporated herein, in their entirety. Dendritic molecules provide important advantages over other types of supermolecular architectures, such as contacting a maximum volume a minimum of structural units, ability to more easily

control size, weight, and growth properties, and the multiple termini can be derivatized to yield highly labeled molecules with defined spacing between the labels, or provide sites of attachment for other molecules, or mixtures thereof. See generally U.S. Patent 6,274,723 and the above cited references for methods of synthesis.

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

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

Contacting the universal probe with a solution comprising the reporter oligonucleotide (barcode DNA) and the target recognition oligonucleotide under conditions that allow for hybridization create a universal probe that is "activated" for contacting with a solution that may contain the target nucleic acid (Fig. 16A, top

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

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

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

Preferably, the linker further comprises a hydrocarbon moiety attached to the cyclic disulfide. Suitable hydrocarbons are available commercially, and are attached to the cyclic disulfides. Preferably the hydrocarbon moiety is a steroid residue. Oligonucleotide-nanoparticle conjugates prepared using linkers comprising a steroid residue attached to a cyclic disulfide have unexpectedly been found to be remarkably stable to thiols (e.g., dithiothreitol used in polymerase chain reaction (PCR) solutions) as compared to conjugates prepared using alkanethiols or acyclic disulfides as the linker. Indeed, the oligonucleotide-nanoparticle conjugates of the invention have

been found to be 300 times more stable. This unexpected stability is likely due to the fact that each oligonucleotide is anchored to a nanoparticle through two sulfur atoms, rather than a single sulfur atom. In particular, it is thought that two adjacent sulfur atoms of a cyclic disulfide would have a chelation effect which would be advantageous in stabilizing the oligonucleotide-nanoparticle conjugates. The large hydrophobic steroid residues of the linkers also appear to contribute to the stability of the conjugates by screening the nanoparticles from the approach of water-soluble molecules to the surfaces of the nanoparticles.

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

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

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

Oligonucleotides of defined sequences are used for a variety of purposes in the practice of the invention. Methods of making oligonucleotides of a predetermined sequence are well known. See, *e.g.*, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (2nd ed. 1989) and F. Eckstein (ed.) *Oligonucleotides and Analogues*, 1st Ed. (Oxford University Press, New York, 1991). Solid-phase

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

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

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

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

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

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

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

this "aging" step can also be determined empirically. For instance, incubation at room temperature and pH 7.0 gives good results.

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

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

It has been found that the hybridization efficiency of nanoparticle-oligonucleotide conjugates can be increased dramatically by the use of recognition oligonucleotides which comprise a recognition portion and a spacer portion. "Recognition oligonucleotides" are oligonucleotides which comprise a sequence complementary to at least a portion of the sequence of a nucleic acid or oligonucleotide target. In this embodiment, the recognition oligonucleotides comprise a recognition portion and a spacer portion, and it is the recognition portion which hybridizes to the nucleic acid or oligonucleotide target. The spacer portion of the recognition oligonucleotide is designed so that it can bind to the nanoparticles. For instance, the spacer portion could have a moiety covalently bound to it, the moiety

comprising a functional group which can bind to the nanoparticles. These are the same moieties and functional groups as described above. As a result of the binding of the spacer portion of the recognition oligonucleotide to the nanoparticles, the recognition portion is spaced away from the surface of the nanoparticles and is more accessible for hybridization with its target. The length and sequence of the spacer portion providing good spacing of the recognition portion away from the nanoparticles can be determined empirically. It has been found that a spacer portion comprising at least about 10 nucleotides, preferably 10-30 nucleotides, gives good results. The spacer portion may have any sequence which does not interfere with the ability of the recognition oligonucleotides to become bound to the nanoparticles or to a nucleic acid or oligonucleotide target. For instance, the spacer portions should not have sequences complementary to each other, to that of the recognition olignucleotides, or to that of the nucleic acid or oligonucleotide target of the recognition oligonucleotides. Preferably, the bases of the nucleotides of the spacer portion are all adenines, all thymines, all cytidines, or all guanines, unless this would cause one of the problems just mentioned. More preferably, the bases are all adenines or all thymines. Most preferably the bases are all thymines.

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

oligonucleotides can bind to their nucleic acid or oligonucleotide targets. If the recognition oligonucleotides comprise spacer portions, the diluent oligonulceotides are, most preferably, about the same length as the spacer portions. In this manner, the diluent oligonucleotides do not interefere with the ability of the recognition portions of the recognition oligonucleotides to hybridize with nucleic acid or oligonucleotide targets. Even more preferably, the diluent oligonucleotides have the same sequence as the sequence of the spacer portions of the recognition oligonucleotides.

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

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

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

to at least a portion of the sequence of the oligonucleotide having a specific binding complement.

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

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

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

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

complementary to at least a portion of the sequence of the DNA barcode and where the DNA barcode serves as an identifier for a specific target analyte.

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

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

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

providing a substrate;

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

and the DNA barcode for each type of particle probe serves as a marker for the particular target analyte;

immobilizing the target analytes onto the substrate;

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

washing the substrate to remove unbound particle probes; and

optionally amplifying the DNA barcode; and

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

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

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

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

DNA barcode can optionally be amplified by any suitable means including PCR amplification, and then be detected by any suitable DNA detection system using any suitable detection probes. The particle is preferably labeled with a sufficient amount of DNA barcodes to provide sufficient signal amplification and eliminate the need for DNA barcode amplification. In practicing this invention, amplification by the PCR method is preferred. PCR amplification (herein also referred to as *B*PCR) of the DNA barcode allows one to detect a protein target at attomolar level. The assay as illustrated in Figure 6, utilizes a new type of nanoparticle heavily functionalized with hybridized oligonucleotides (biobarcodes)[36] and polyclonal detection antibodies to recognize a target analyte, the prostate specific antigen (PSA) (Example 5). In

addition, polyamine microparticles (1μm diameter) with magnetic iron oxide cores are funtionalized with PSA monoclonal antibodies (Figure 6 and Example 5). The gold nanoparticles and the polyamine microparticles sandwich the PSA target, generating a complex with a large ratio of barcode DNA to protein target (for 13 nm particles, each particle can support up to 200 strands of DNA; this represents the upper limit for this size particle). Application of a magnetic field draws the magnetic particles to the wall of the reaction vessel in a matter of seconds, allowing one to separate both reacted and unreacted microparticles but only reacted nanoparticles from the reaction mixture. Washing the aggregate structures in Nanopure water (18 MOhms) dehybridizes barcode DNA from nanoparticle-immobilized complements. Using the magnetic separator, the aggregate can be easily removed from the assay solution, leaving the barcode DNA, which can be amplified using PCR, and subsequently and quickly identified by standard DNA detection methodologies (scanometric[11], gel electrophoresis, or fluorophore-labeling approaches). PSA was chosen as the initial target for these studies because of its importance in the early detection of prostate cancer, one of the most common cancers and second leading cause of cancer death in American men [47, 48]. Importantly, identification of disease relapse following the surgical treatment of prostate cancer using PSA as a marker present at low levels (10s of copies), could be extremely beneficial and enable the delivery of curative adjuvant therapies [46, 49].

Examples 5-6 demonstrate that *B*PCR is an extremely powerful method for detecting protein analytes, namely PSA, at low attomolar concentrations in the presence of background proteins using either gel electrophoresis or scanometric microarray detection. The work demonstrates several advantages over current protein detection methods. First, the target binding protein of the assay is homogeneous. Therefore, one can add a large quantity of magnetic particles to the reaction vessel to facilitate the binding kinetics between the detection antibody and target analyte. This leads to an assay that is faster than heterogeneous systems and also allows one to increase sensitivity because the capturing step is more efficient. Second, the use of the nanoparticle biobarcodes provides a high ratio of PCR-amplifiable DNA to labeling antibody serving to substantially increase assay sensitivity. For example, the *B*PCR

assay reported herein was able to detect PSA at 3 aM concentration while a PCR-based immunoassay has been reported to have a detection limit of 3 fM for the same target analyte [43]. Third, this assay obviates the need for complicated conjugation chemistry for attaching DNA to the labeling antibodies. Barcode DNA is bound to the nanoparticle probe through hybridization at the start of the labeling reaction and liberated for PCR amplification using a simple wash step. Ad the labeling antibody and DNA are present on the same particle, there is no need for the addition of further antibodies or DNA-protein conjugates prior to the PCR amplification of barcode DNA. In addition, the barcode DNA is removed from the detection assay, and PCR is carried out on samples of barcode DNA that is free from PSA, most of the biological sample, the microparticles, and nanoparticles. This substantially reduces background signal. Finally, this protein detection scheme has the potential for massive multiplexing and the simultaneous detection of many analytes in one solution. Although the PSA system is used for proof-of-concept, the approach should be general for almost any target with known binding partners, and by using the nanoparticle-based biobarcode approach [36], one can prepare a unique identifiable barcode for virtually every target of interest.

Example 9 demonstrates that the probes of the invention can be used to detect and measure directly the amount of target analyte in a sample. Thus, a step comprising *B*PCR to amplify the barcode DNA is not required to achieve excellent sensitivity and detection limits (Figs. 6B (step 4), 9 (inset), and 12).

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

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

Analyte detection occurs indirectly by ascertaining for the presence of reporter oligonucleotide or biobarcode by any suitable means such as a DNA chip.

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

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

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

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

In one aspect of the invention, the reporter oligonucleotide bound to the oligonucleotides on the substrate is contacted with a first type of nanoparticles having

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

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

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

The combination of hybridizations produces a detectable change. The detectable changes are the same as those described above, except that the multiple hybridizations result in an amplification of the detectable change. In particular, since each of the first type of nanoparticles has multiple oligonucleotides (having the same or different sequences) attached to it, each of the first type of nanoparticle-oligonucleotide conjugates can hybridize to a plurality of the second type of nanoparticle-oligonucleotide conjugates. Also, the first type of nanoparticle-oligonucleotide conjugates may be hybridized to more than one portion of the reporter

oligonucleotide to be detected. The amplification provided by the multiple hybridizations may make the change detectable for the first time or may increase the magnitude of the detectable change. This amplification increases the sensitivity of the assay, allowing for detection of small amounts of reporter oligonucleotide.

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

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

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

In yet another aspect, the methods of analyte detection by *B*PCR, as well as direct detection, can be adapted for use with methods that comprise analyte detection on a substrate, for example, glass, gold, silicon, nickel, plastics, and the like. These methods can also be adapted to detect other biological and chemical recognition events such as DNA-protein binding events, physiological protein-protein binding or dimerization, and other biomolecular interactions previously described above.

In one embodiment of this aspect, the method comprises attaching one or more types of capture probe for each target analyte to a substrate, contacting the substrate with a test solution, optionally washing the test solution from the substrate, subsequently contacting the substrate with one or more types of detection probe for

each target analyte, removing any unbound detection probe, and detecting an observable signal, wherein the detection of an observable signal indicates the presence of the target analyte in the test solution.

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

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

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

In assays for detecting reporter oligonucleotides performed on a substrate, the detectable change can be observed with an optical scanner. Suitable scanners include those used to scan documents into a computer which are capable of operating in the reflective mode (*e.g.,* a flatbed scanner), other devices capable of performing this function or which utilize the same type of optics, any type of greyscale-sensitive measurement device, and standard scanners which have been modified to scan substrates according to the invention (*e.g.*, a flatbed scanner modified to include a

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

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

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

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

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

washing the substrate to remove any unbound detection probes;

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

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

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

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

optionally amplifying the DNA barcode prior to said detecting.

In another aspect of the invention, the capture probe is bound to a magnetic substrate such as a magnetic particle, e.g., a polystyrene MMP with a magnetic iron oxide. This allows for facile removal of complexes from solution. DNA barcodes

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

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

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

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

particular assay conditions (e.g., buffer system, target nucleic acid sequence, temperature, etc.).

In another embodiment, the second probe comprises a nanoparticle modified with two types of oligonucleotides, one that comprises a sequence that is complementary to at least a portion of a target sequence that is different from the region on the target that is recognized by the MMP; and the other comprises a sequence that is complementary to at least a portion of barcode DNA sequence, which barcode DNA provides a unique identification tag for the particular target sequence. In certain embodiments the nanoparticle is a gold nanoparticle. In further embodiments the gold nanoparticle comprises 13, 20, or 30 nm gold nanoparticles.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

washing any unbound detection probes from the magnetic particle; and

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

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

isolating the aggregated complex by applying a magnetic field;

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

isolating the released DNA barcodes.

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

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

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

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

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

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

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

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

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

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

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

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

complex probes are provided, each type of probe having a specific binding complement to different binding sites on the target analyte.

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

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

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

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

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

The methods may further comprise a filtration step to remove aggregate complexes, wherein the filtration is performed prior to analyzing the aggregated

complex. The filtration step comprises a membrane that removes sample components that do not comprise DNA barcodes.

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

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

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

observing whether aggregate complex formation occurred.

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

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

thin. Preferred are transparent substrates such as glass (*e.g.*, glass slides) or plastics (*e.g.*, wells of microtiter plates).

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

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

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

In practicing the invention, a nanoparticle complex probes are prepared by hybridizing the nanoparticles having oligonucleotides bound thereto with an oligonucleotide modified with a specific binding complement to a target analyte, and a reporter oligonucleotide. At least some of the oligonucleotides attached to the nanoparticle have a sequence that is complementary to a first portion of a reporter

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

A typical assay for detecting the presence of a target analyte, e.g, antibody, in a sample is as follows: a solution containing nanoparticle complex probe comprising nanoparticles having oligonucleotides bound thereto, a reporter oligonucleotide, and an oligonucleotide having a specific binding complement to the target analyte, is admixed with an aqueous sample solution believed to contain target protein. The total protein content in the aqueous sample solution generally ranges between about 5 and about 100, usually about 43 ug/ml. The concentration of nanoparticles in the reaction mixture generally ranges between about 2 nM and about 20 nM, usually about ~13 nM. The total volume of the resulting mixture generally ranges between about 100 uL and about 1000 uL, preferably about 400 uL. Any suitable solvent may be

employed in preparing the aqueous sample solution believed to contain target analyte, preferably PBS comprising 0.3 M NaCl and 10 mM phosphate buffer (pH 7-7.4).

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

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

To determine which proteins are present, a melting analysis of the aggregates which monitors the extinction at 260 nm as a function of temperature may carried out in the solution. See, for instance, Figure 2 in Example 3 which describes analysis of a sample containing one or two known target analytes: IgG1 and IgE. As discussed in Example 3, when IgG1 is treated with the probes via the aforementioned protocol, the solution turns pinkish-blue, indicating the formation of nanoparticle aggregates. In a control experiment where no target but background proteins are present, there is no discernible precipitation. A melting analysis of the solution shows a sharp transition with a melting temperature (Tm) of 55 °C. This is the expected transition for the IgG1 target, Figure 2A (---). If IgE is added to a fresh solution of probes, the same color change is observed but the melting analysis provides a curve with a Tm of 36

°C, the expected transition for this target, Figure 2A (——). Significantly, when both protein targets are added to the solution of probes, the solution turns dark purple, and the melting analysis exhibits two distinct transactions. The first derivative of this curve shows two peaks centered at 36 and 55 °C, respectively, Figure 2B. This demonstrates that two distinct assemblies form and their melting properties, which derive from the oligonucleotide barcodes, can be used to distinguish two protein targets.

In another aspect of the invention, a variation of the above aggregation method strategy can be used to increase the sensitivity of the aforementioned system and to increase the number of targets that can be interrogated in one solution. See, for instance, Figure 3 in Example 4. With this strategy, the protein targets can be detected indirectly via the DNA biobarcodes or unique reporter oligonucleotides assigned to specific target analytes. Generally, the suitable length, GC content, and sequence, and selection of the reporter oligonucleotide for the target analyte is predetermined prior to the assay. For instance, a 12-mer oligonucleotide has 4^{12} different sequences, many of which can be used to prepare a barcode for a polyvalent protein of interest as shown in Figure 1A. In this variation of the assay, the melting properties of the aggregates that form are not measured in solution but rather the reporter oligonucleotides or DNA biobarcodes within the aggregates are separated via centrifugation (e.g., 3000 rpm for 2 minutes) from the unreacted probes and target molecules. The aggregates are then denatured by any suitable means, e.g., by adding water to the solution, to free the reporter oligonucleotides or biobarcodes. If the reporter oligonucleotide is present in small amounts, it may be amplified by methods known in the art. See, *e.g.*, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (2nd ed. 1989) and B.D. Hames and S.J. Higgins, Eds., *Gene Probes 1* (IRL Press, New York, 1995). Preferred is polymerase chain reaction (PCR) amplification. The particles and proteins can be separated from the reporter oligonucleotides by any suitable means, e.g., a centrifugal filter device (Millipore Microcon YM-100, 3500 rpm for 25 min. Once the reporter oligonucleotides are isolated, they can be captured on an oligonucleotide array and can be identified using one of the many suitable DNA detection assays (Figure 3). For the examples described herein involving IgG1 and

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

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

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

isolating aggregated complexes; and

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

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

isolating the aggregated complex;

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

isolating the DNA barcode; and

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

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

isolating the aggregated complex;

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

isolating the DNA barcode;

amplifying the isolated DNA barcode; and

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

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

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

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

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

In another aspect of this embodiment, the target analyte may be a sequence from a genomic DNA sample and the specific binding complements are oligonucleotides, the oligonucleotides having a sequence that is complementary to at least a portion of the genomic sequence.

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

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

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

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

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

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

observing a detectable change.

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

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

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

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

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

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

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

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

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

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

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

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

In another aspect of this embodiment, the kit comprises:

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

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

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

In another aspect of this embodiment, the kit comprises:

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

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

In another aspect of this embodiment, the kit comprises:

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

In another aspect of this embodiment, the kit comprises:

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

In another aspect of this embodiment, the kit comprises:

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

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

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

In another aspect of this embodiment, the kit comprises:

at least two or more pairs of containers,

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

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

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

In another aspect of this embodiment, the kit comprises:

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

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

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

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

wherein the DNA barcode present in the first container of each pair of containers serves as an identifier for a target analyte and has a sequence that is

different from a DNA barcode in another pair of containers, and wherein the kite optionally include a substrate for observing a detectable change.

In another aspect of this embodiment, the kit comprises:

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

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

It is to be noted that the term "a" or "an" entity refers to one or more of that entity. For example, "a characteristic" refers to one or more characteristics or at least one characteristic. As such, the terms "a" (or "an"), "one or more" and "at least one" are used interchangeably herein. It is also to be noted that the terms "comprising", "including", and "having" have been used interchangeably. The following examples are intended for illustration purposes only, and should not be construed as limiting the spirit or scope of the invention in any way.

EXAMPLES

Example 1: Preparation of Oligonucleotide-Modified Gold Nanoparticles

A. Preparation Of Gold Nanoparticles

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

B. Synthesis Of Oligonucleotides

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

For 3'-thiol-oligonucleotides, Thiol-Modifier C3 S-S CPG support was purchased from Glen Research and used in the automated synthesizer. The final dimethoxytrityl (DMT) protecting group was not removed to aid in purification. After synthesis, the supported oligonucleotide was placed in 1 mL of concentrated ammonium hydroxide for 16 hours at 55 °C to cleave the oligonucleotide from the solid support and remove the protecting groups from the bases.

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

5'-Alkylthiol modified oligonucleotides were prepared using the following protocol: 1) a CPG-bound, detritylated oligonucleotide was synthesized on an automated DNA synthesizer (Expedite) using standard procedures; 2) the CPG-

cartridge was removed and disposable syringes were attached to the ends; 3) 200 uL of a solution containing 20 umole of 5-Thiol-Modifier C6-phosphoramidite (Glen Research) in dry acetonitrile was mixed with 200 uL of standard "tetrazole activator solution" and, *via* one of the syringes, introduced into the cartridge containing the oligonucleotide-CPG; 4) the solution was slowly pumped back and forth through the cartridge for 10 minutes and then ejected followed by washing with dry acetonitrile (2 x 1 mL); 5) the intermediate phosphite was oxidized with 700 uL of 0.02 M iodine in THF/pyridine/water (30 seconds) followed by washing with acetonitrile/pyridine (1:1; 2 x 1 mL) and dry acetonitirile. The trityloligonucleotide derivative was then isolated and purified as described by the 3'-alkylthiol oligonucleotides; then the trityl protecting group was cleaved by adding 15 uL (for 10 OD's) of a 50 mM $AgNO_3$ solution to the dry oligonucleotide sample for 20 minutes, which resulted in a milky white suspension. The excess silver nitrate was removed by adding 20 uL of a 10 mg/mL solution of DTT (five minute reaction time), which immediately formed a yellow precipitate that was removed by centrifugation. Aliquots of the oligonucleotide solution (< 10 OD's) were then transferred onto a desalting NAP-5 column for purification. The final amount and the purity of the resulting 5' alkylthiol oligonucleotides were assessed using the techniques described above for 3' alkylthiol oligonucleotides. Two major peaks were observed by ion-exchange HPLC with retention times of 19.8 minutes (thiol peak, 16 % by area) and 23.5 minutes (disulfide peak, 82 % by area).

C. Attachment Of Oligonucleotides To Gold Nanoparticles

A 1 mL solution of the gold colloids (17nM) in water was mixed with excess (3.68 uM) thiol-oligonucleotide (22 bases in length) in water, and the mixture was allowed to stand for 12-24 hours at room temperature. Then, the solution was brought to 0.1 M NaCl, 10 mM phosphate buffer (pH 7) and allowed to stand for 40 hours. This "aging" step was designed to increase the surface coverage by the thiol-oligonucleotides and to displace oligonucleotide bases from the gold surface. The solution was next centrifuged at 14,000 rpm in an Eppendorf Centrifuge 5414 for about 25 minutes to give a very pale pink supernatant containing most of the

oligonucleotide (as indicated by the absorbance at 260 nm) along with 7-10% of the colloidal gold (as indicated by the absorbance at 520 nm), and a compact, dark, gelatinous residue at the bottom of the tube. The supernatant was removed, and the residue was resuspended in about 200 μL of buffer (10 mM phosphate, 0.1 M NaCl) and recentrifuged. After removal of the supernatant solution, the residue was taken up in 1.0 mL of buffer (10 mM phosphate, 0.3 M NaCl, 0.01% NaN_3). The resulting red master solution was stable (*i.e.*, remained red and did not aggregate) on standing for months at room temperature, on spotting on silica thin-layer chromatography (TLC) plates (see Example 4), and on addition to 1 M NaCl, 10 mM $MgCl_2$, or solutions containing high concentrations of salmon sperm DNA.

Example 2: Preparation of Hapten-modified oligonucleotides

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

Biotin modified oligonucleotides were prepared using the following protocol: A CPG-bound, detritylated oligonucleotide was synthesized on an automated DNA synthesizer (Expedite) using standard procedures[21]. The CPG-cartridge was then removed and disposable syringes were attached to the ends. 200 uL of a solution containing 20 umole of biotin-triethylene glycol phosphoramidite in dry acetonitrile was then mixed with 200 uL of standard "tetrazole activator solution" and, *via* one of the syringes, introduced into the cartridge containing the oligonucleotide-CPG. The solution then was slowly pumped back and forth through the cartridge for 10 minutes and then ejected followed by washing with dry acetonitrile (2 x 1 mL). Thereafter, the intermediate phosphite was oxidized with 700 uL of 0.02 M iodine in THF/pyridine/ water (30 seconds) followed by washing with acetonitrile/pyridine (1:1; 2 x 1 mL) and dry acetonitirile with subsequent drying of the column with a stream of nitrogen. The trityl protecting group was not removed, which aids in purification. The supported oligonucleotide was placed in 1 mL of concentrated ammonium hydroxide for 16 hours at 55 °C to cleave the oligonucleotide from the

solid support and remove the protecting groups from the bases. After evaporation of the ammonia, the oligonucleotides were purified by preparative reverse-phase HPLC using an HP ODS Hypersil column (5 um, 250 x 4 mm) with 0.03 M triethyl ammonium acetate (TEAA), pH 7 and a 1%/minute gradient of 95% CH_3CN/5% 0.03 M TEAA at a flow rate of 1 mL/minute, while monitoring the UV signal of DNA at 254 nm. The retention time of the DMT protected oligonucleotides was ~32 minutes. The DMT was subsequently cleaved by soaking the purified oligonucleotide in an 80% acetic acid solution for 30 minutes, followed by evaporation; the oligonucleotide was redispersed in 500 uL of water, and the solution was extracted with ethyl acetate (3 x 300 uL) and dried. The same protocol was used to synthesize DNP modified oligonucleotide using 2, 4-dinitrophenyl-triethylene glycol phosphoramidite.

Example 3: Assay Using Nanoparticle Complex Probes

The Oligonucleotide-modified 13 nm gold particles were prepared as described in Example 1. Hapten-modified oligonucleotides were prepared as described in Example 2 with a biotin-triethylene glycol phosphoramidite for **A1** and 2, 4-dinitrophenyl-triethylene glycol phosphoramidite for **B1** (Glen research) using standard solid-phase DNA synthesis procedures.[21] The PBS buffer solution used in this research consists of 0.3 M NaCl and 10 mM phosphate buffer (pH 7). IgE and IgG1 were purchased from Sigma Aldrich (Milwaukee, WI) and dissolved in 0.3 M PBS buffer with 0.05% Tween 20 (final concentration: $4.3x10^{-8}$ b/μl) and background proteins (10 ug/ml of lysozyme, 1% bovine serum albumin, and 5.3 ug/ml of anti-digoxin; 10 uL of each) prior to use.

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

In a typical assay for IgE and/or IgG1, the target proteins (40 μl of 43 μg/ml for each) were added to the solution containing the probes (~13 nM), and the mixture

was incubated at 37 °C for 50 minutes to facilitate protein-hapten complexation. To ensure complete reaction among all the components, especially the complementary DNA strands, the solution was incubated to expedite hybridization at –15 °C for 20 minutes (Boekel Tropicooler Hot/Cold Block Incubator) and stored at 4 °C for 24 hours. If the target protein is present, particle aggregation takes place affecting a shift in the gold nanoparticle plasmon band and a red-to-purple color change along with precipitation. The hybridized products were centrifuged (3000 rpm for 2 minutes), and the supernatant containing unreacted elements was decanted prior to analysis. To determine which proteins are present, a melting analysis, which monitors the extinction at 260 nm as a function of temperature is carried out in the solution, Figure 2. When IgG1 is treated with the probes via the aforementioned protocol, the solution turns pinkish-blue, indicating the formation of nanoparticle aggregates. In a control experiment where no target but background proteins are present, there is no discernible precipitation. A melting analysis of the solution shows a sharp transition with a melting temperature (Tm) of 55 °C. This is the expected transition for the IgG1 target, Figure 2A (---). If IgE is added to a fresh solution of probes, the same color change is observed but the melting analysis provides a curve with a Tm of 36 °C, the expected transition for this target, Figure 2A (——). Significantly, when both protein targets are added to the solution of probes, the solution turns dark purple, and the melting analysis exhibits two distinct transactions. The first derivative of this curve shows two peaks centered at 36 and 55 °C, respectively, Figure 2B. This demonstrates that two distinct assemblies form and their melting properties, which derive from the oligonucleotide barcodes, can be used to distinguish two protein targets.

Example 4: Assay Using Nanoparticle Complex Probes

A variation of this strategy can be used to increase the sensitivity of the aforementioned system and to increase the number of targets that can be interrogated in one solution (Figure 3). With this strategy, the protein targets can be detected indirectly via the DNA biobarcodes. A 12-mer oligonucleotide has 4^{12} different sequences, many of which can be used to prepare a barcode for a polyvalent protein of

interest via Figure 1A. In this variation of the assay, the melting properties of the aggregates that form are not measured in solution but rather the DNA biobarcodes within the aggregates are separated via centrifugation (3000 rpm for 2 minutes) from the unreacted probes and target molecules. The aggregates are then denatured by adding water to the solution, freeing the complexed DNA. The particles and proteins can be separated from the DNA barcodes with a centrifugal filter device (Millipore Microcon YM-100, 3500 rpm for 25 min). Once the DNA barcodes are isolated, they can be captured on an oligonucleotide array and can be identified using one of the many DNA detection assays (Figure 3). For the examples described herein involving IgG1 and IgE, the barcodes are captured on a microscope slide that has been functionalized with oligonucleotides (250 μm diameter spots) that are complementary to one half of the barcode of interest (**A3** and **B3** in Figure 1). If the barcode is captured by the oligonucleotide array, a DNA-modified particle that is complementary to the remaining portion of the barcode can be hybridized to the array (see experimental section). When developed via the standard scanometric approach [11] (which involves treatment with photographic developing solution), a flat bed scanner can be used to quantify the results, Figure 4.[11] If IgG1 is present, only the spot designed for IgG1 shows measurable signal. Similarly if IgE is the only protein present, the spot designed for it only exhibits signal. Finally, if both proteins are present, both spots exhibit intense signals.

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

(pH 7) and submerged in Silver Enhancer Solution (Sigma) for three minutes at room temperature. The slide was washed with water and then analyzed with a flat bed scanner.

Example 5: Assay using nanoparticle complex probe

The Biobarcode PCR (*B*PCR) protocol was performed to detect a protein target, free prostate specific antigen (PSA), at 3 aM sensitivity (Figure 6), which is six orders of magnitude more sensitive than the current conventional clinical assay for detecting PSA (46). The nanoparticle detection probes were prepared by adding polyclonal anti-PSA antibody (7 μg) to an aqueous solution of 13 nm Au nanoparticles (10 ml of 13 nM solution in colloidal suspension, citrate (~38 mM) stabilized) at pH 9.0. After 20 minutes, the anti-PSA modified nanoparticles were reacted with alkylthiol-capped barcode DNA capture strands (0.2 OD; 5' CAA CTT CAT CCA CGT TCA ACG CTA GTG AAC ACA GTT GTG T-A_{10}-SH 3' SEQ ID NO:9) for 12 hours and then salt-stabilized to 0.1 M NaCl/0.01 M phosphate buffer, pH 7. Next, the solution was treated with 1 ml of a 10 % BSA solution for 20 minutes to passivate and stabilize the gold nanoparticles. The final solution was centrifuged for 1 hour at 4 °C (20,000g), and the supernatant was removed. This centrifugation procedure was repeated for further purification. The PSA-specific barcode DNA strands (1 OD; 5' ACA CAA CTG TGT TCA CTA GCG TTG AAC GTG GAT GAA GTT G 3' SEQ ID NO:10) were then hybridized with the barcode DNA capture strands coordinated to the nanoparticles and purified using a similar centrifugation procedure.

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

In a typical PSA detection experiment (Figure 6B), an aqueous dispersion of magnetic capture probes functionalized with monoclonal anti-PSA antibodies (50 μl

of 3 mg/ml magnetic probe solution) is mixed with an aqueous solution (0.1 M PBS) of free PSA (10 μl of PSA) and stirred at 37 °C for 30 minutes (Step 1 of Figure 6B). PSA bound magnetic detection probe can be easily separated from unbound PSA by applying a magnetic field. To effect magnetic separation, a 1.5 ml tube containing the assay solution is placed in a BioMag® microcentrifuge tube separator (Polysciences, Inc.) at room temperature. After 15 seconds, the magnetic capture probe-PSA hybrids are concentrated on the wall of the tube. The supernatant (solution of unbound PSA molecules) is removed, and the magnetic capture probes, now in the form of a pellet on the side of the tube, are re-suspended in 50 μl of 0.1 M PBS (repeated 2X). Nanoparticle detection probes (50 μl at 1 nM), functionalized with polyclonal anti-PSA antibodies and hybrid barcode DNA strands, are then added. The detection probes react with the PSA immobilized on the capture probes and provide DNA strands for signal amplification and protein identification (Step 2 of Figure 6B). The solution is vigorously stirred at 37 °C for 30 minutes. The magnetic particles were then washed with 0.3 M PBS using the magnetic separator to isolate the magnetic particles. This step is repeated 4 times, each time requiring one minute, removing everything but the magnetic capture probes (along with PSA bound nanoparticle detection probes). After the final wash step, the magnetic capture probes are resuspended in Nanopure (18 M Ohm) water (50 μl) to dehybridize barcode DNA strands from the nanoparticle detection probe surface for 2 minutes. Dehybridized barcode DNA is then easily separated and collected from the probes using the magnetic separator (Step 3 of Figure 6B).

For barcode DNA amplification (Step 4, figure 6B), isolated barcode DNA is added to a PCR reaction mixture (20 μl, final volume) containing the appropriate primers, and the solution is then thermally cycled according to the following procedure. An aliquot of free barcode DNA (8.9μl) is added to the top wax layer of an EasyStart™ Micro 20 PCR Mix-in-a-Tube (Molecular Bio-Products, San Diego, CA) along with 0.3 μl of the appropriate primers (each at 25 μM, Primer 1: 5'CAA CTT CAT CCA CGT TCA AC 3' SEQ ID NO:11, primer 2: 5'ACA CAA CTG TGT TCA CTA GC 3' SEQ ID NO:12), 0.4 μl DMSO (2 % final concentration), and 0.1 μL of

Taq DNA polymerase, a polymerase shown to be compatible with the EasyStart™ system (5U/μl, Fisher Scientific), for a final volume of 20 μl. The final concentrations of the PCR reaction mix are as follows: Primers 1 and 2, 0.37 μM; dNTP mix, 0.2 mM; PCR buffer, 1X; and $MgCl_2$, 2 mM. The PCR tubes are then loaded into a thermal cycler (GeneAmp 9700, Applied Biosystems) and subjected to a 7 minute "hot start" at 94 °C, cycled 25 times at 94 °C for 30 seconds, 58 °C for 30 seconds and 72 °C for 1 minute, with a final extension of 72 °C for seven minutes followed by a 4 °C soak.

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

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

(0.5 μg/ml in 1X TAE running buffer) and qualitatively similar results to those where ethidium bromide was added to the PCR reaction prior to electrophoresis were obtained.

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

Example 6: Assay using nanoparticle complex probe

Although gel electrophoresis was routinely used to analyze the results of the assay, in general, the scanometric method provides higher sensitivity and is easier to implement than the gel-based method. Therefore, the results of the scanometric assay are reported herein. Chip-immobilized DNA 20-mers, which are complementary with half of the target barcode sequence (40-mer), were used to capture the amplified barcode DNA sequences, and gold nanoparticles were used to label the other half of the sequence in a sandwich assay format. The amplified duplex barcode DNA must be first denatured in order to effect hybridization between the barcode DNA, the chip surface, and gold nanoparticle probes. Thus, the barcode DNA amplicons were removed from the original PCR tube and added (5 μl) to a solution of gold nanoparticle probes (5 μl, 10 nM in 0.3 M PBS). The solution was diluted with 0.3 M PBS (90 μl) to a final volume of 100 μl in a clean 0.2 ml PCR tube. In order to hybridize barcode DNA single strands (40-mer) and nanoparticle bound complements (20-mer), the PCR tubes were added to a thermal cycler, heated to 95 °C for 3 minutes to denature the barcode DNA duplexes, and then cooled to a hybridization

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

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

To examiner the theoretical lower-limit of protein detection using *B*PCR, PCR/gel electrophoresis was performed with a dilution series of barcode DNA concentrations for PCR amplification (Figure 10). The signal when barcode DNA amplicons were present is quite discernible from the control band (lane 10) even when only 30 copies of barcode DNA were added to the PCR reaction (lane 9). Assuming each nanoparticle probe has about 50 barcode DNA strands, *B*PCR can, in theory, generate a detection signal with a single bound nanoparticle probe.

Example 8: Detection of PSA in complex medium

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

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

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

The gold nanoparticle (NP) probes were prepared essentially as described above in Example 5. Briefly, polyclonal antibodies (Abs) to PSA (3.5 μg) were added to an aqueous solution of 30 nm gold NPs (1 ml of 40 pM NP solution) at pH 9.0. After 20 minutes, the Ab modified NPs were reacted with alkylthiol-capped barcode DNA capture strands (1 OD for 30 nm gold particles; 5'- CAA CTT CAT CCA CGT TCA ACG CTA GTG AAC ACA GTT GTGT-A_{10}-$(CH_2)_3$-SH 3') for 16 hours and then salt-stabilized to 0.1 M NaCl. This solution was treated with 0.3 ml of a 10 % BSA solution for 30 minutes to passivate and stabilize the gold NPs. The final solution was centrifuged for 1 hour at 4 °C (20,000g), and the supernatant was removed. This centrifugation procedure was repeated for further purification. The final NP probes were re-dispersed in 0.1 M NaCl/0.01 M phosphate buffer solution at pH 7.4. The PSA-specific barcode DNA strands (1 OD; 5'-ACA CAA CTG TGT

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

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

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

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

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

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

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

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

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

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

assay. Figure 14A illustrates 20-nm NP probes used in the detection of "amplified" barcode DNA. The spot intensities for target DNA concentrations from 5 fM to 50 aM are clearly stronger than control spots. To measure spot intensity of each concentration, three spots were patterned on a chip and imaged with the Verigene ID system. The gray levels of the spots were calculated with graphic software (Adobe Photoshop) and the spot intensity graph is shown in Figure 15. This graph shows that 20-nm NP probes can clearly detect target DNA at about 50 aM, but cannot differentiate signal from background at a concentration of 5 aM.

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

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WHAT IS CLAIMED:

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

providing a substrate;

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

immobilizing the target analytes onto the substrate;

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

washing the substrate to remove unbound particle probes; and

optionally amplifying the DNA barcode; and

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

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

3. The method of claim 1 wherein the DNA barcode is amplified by PCR.

4. The method of claim 1 wherein the particle is labeled with at least two DNA barcodes.

5. The method of claim 1 wherein the substrate is arrayed with one or more types of capture probes for the target analytes.

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

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

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

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

washing the substrate to remove any unbound detection probes;

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

7. The method of claim 6 wherein the the detection probe comprises (i) one or more specific binding complements to the second binding site of a specific target analyte, (ii) at least one type of oligonucleotides bound to the nanoparticle, and a DNA barcode having a predetermined sequence that is complementary to at least a portion of at least one type of oligonucleotides, the DNA barcode bound to each type of detection probe serving as a marker for a specific target analyte;

8. The method of claim 6 wherein, prior to said detecting step, further comprising the steps of:

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

amplifying the DNA barcode prior to said detecting.

9. The method of claim 8, wherein the DNA barcode is amplified by PCR.

10. The method of claim 6, wherein the capture probe is bound to a magnetic substrate.

11. The method of claim 10, wherein the substrate is a magnetic particle.

12. The method of claim 6 wherein the specific binding complement bound to the nanoparticle is a monoclonal or polyclonal antibody.

13. The method of claims 12 wherein the antibody is an anti-PSA antibody.

14. The method of claim 11, wherein, prior to said washing step, further comprising the step of:

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

15. The method according to claim 14 further comprising the step of:

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

16. The method according to claim 15 wherein said released DNA barcode is amplified.

17. The method according to claim 16 wherein said released DNA barcode is amplified by PCR.

18. The method of claim 6 wherein the target analyte is a nucleic acid having at least two portions.

19. The method of claim 6 wherein the target analyte is a target nucleic acid having a sequence of at least two portions, the detection probe comprises a nanoparticle having oligonucleotides bound thereto, a least a portion of the oligonucleotides bound to the nanoparticle having a sequence that is complementary to the DNA bar code, the specific binding complement of the detection probe comprising a first target recognition oligonucleotide having a sequence that is complementary to a first portion of the target nucleic acid, and the specific binding complement of the capture probes comprises second target recognition oligonucleotide having a sequence that is complementary to at least a second portion of the target nucleic acid.

20. The method of claim 6 wherein the target analyte is a target nucleic acid having a sequence of at least two portions, the detection probe comprising a nanoparticle having oligonucleotides bound thereto, the DNA barcode having a sequence that is complementary to at least a portion of the oligonucleotides bound to the detection probe, the specific binding complement comprises a target recognition oligonucleotide having a sequence of at least first and second portions, the first portion is complementary to a first portion of the target nucleic acid and the second portion is complementary to a least a portion of the oligonucleotides bound to the nanoparticles, the specific binding complement of the substrate comprising a target recognition oligonucleotide having at least a portion that is complementary to a second portion of the target nucleic acid..

21. The method of claim 6 wherein the detection probe comprises a dendrimer.

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

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

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

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

washing any unbound detection probes from the magnetic particle; and

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

23. The method of claim 22, further comprising prior to said detecting step, the steps of:

isolating the aggregated complex by applying a magnetic field;

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

isolating the released DNA barcodes.

24. The method of claim 23, further comprising amplifying the released DNA barcodes.

25. The method of claims 22 or 23, further comprising:

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

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

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

26. The method of claim 25, wherein the DNA barcode is amplified by PCR prior to detection.

27. The method of claim 22, further comprising isolating the aggregated complexes prior to analyzing the aggregated complex.

28. The method of claim 27, wherein the aggregated complex is isolated by applying a magnetic field to the aggregated complex.

29. The method of claim 22, wherein the nanoparticles are metal nanoparticles or semiconductor nanoparticles.

30. The method of claim 29, wherein the nanoparticles are gold nanoparticles.

31. The method of claim 22, wherein the specific binding pair is an antibody and an antigen.

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

33. The method of claim 22, wherein the specific binding pair is an enzyme and a substrate.

34. The method of claim 22, wherein the specific binding pair is a drug and a target molecule.

35. The method of claim 22, wherein the specific binding pair is two strands of at least partially complementary oligonucleotides.

36. The method of claim 22, wherein the DNA barcode is biotinylated.

37. The method of claim 22, wherein the DNA barcode is radioactively labeled.

38. The method of claim 22, wherein the DNA barcode is fluorescently labeled.

39. The method of any one of claims 1, 6, and 22 wherein the target has more than two binding sites.

40. The method of claim 39 wherein at least two types of particle complex probes are provided, the first type of probe having a specific binding complement to a first binding site on the target analyte and the second type of probe having a specific binding complement to a second binding site on the probe.

41. The method of claim 39 wherein a plurality of particle complex probes are provided, each type of probe having a specific binding complement to different binding sites on the target analyte.

42. The method of any one of claims 1,6 or 22 wherein the specific binding complement and the target analyte are members of a specific binding pair.

43. The method of claim 42 wherein members of a specific binding pair comprise nucleic acid, oligonucleotide, peptide nucleic acid, polypeptide, antibody, antigen, carbohydrate, protein, peptide, amino acid, hormone, steroid, vitamin, drug, virus, polysaccharides, lipids, lipopolysaccharides, glycoproteins, lipoproteins, nucleoproteins, oligonucleotides, antibodies, immunoglobulins, albumin, hemoglobin, coagulation factors, peptide and protein hormones, non-peptide hormones, interleukins, interferons, cytokines, peptides comprising a tumor-specific epitope, cells, cell-surface molecules, microorganisms, fragments, portions, components or products of microorganisms, small organic molecules, nucleic acids and oligonucleotides, metabolites of or antibodies to any of the above substances.

44. The method of claim 43 wherein nucleic acid and oligonucleotide comprise genes, viral RNA and DNA, bacterial DNA, fungal DNA, mammalian DNA, cDNA, mRNA, RNA and DNA fragments, oligonucleotides, synthetic oligonucleotides, modified oligonucleotides, single-stranded and double-stranded nucleic acids, and natural and synthetic nucleic acids.

45. The method according to any one of claims 1, 6, or 22 wherein the target analyte is a nucleic acid and the specific binding complement is an oligonucleotide.

46. The method according to any one of claims 1, 6, or 22 wherein the target analyte is a protein or hapten and the specific binding complement is an antibody comprising a monoclonal or polyclonal antibody..

47. The method according to any one of claims 1, 6, or 22 wherein the target analyte is a sequence from a genomic DNA sample and the specific binding complements are oligonucleotides, the oligonucleotides having a sequence that is complementary to at least a portion of the genomic sequence.

48. The method of claim 47, wherein the genomic DNA is eukaryotic, bacterial, fungal or viral DNA.

49. The method according to any one of claims 1, 6, or 22 wherein the specific binding complement and the target analyte are members of an antibody-ligand pair.

50. The method according to any one of claims 1, 6, or 22 wherein in addition to its first binding site, the target analyte has been modified to include a second binding site.

51. The method of claim 22 further comprising a filtration step, wherein the filtration is performed prior to analyzing the aggregated complex.

52. The method of claim 51 wherein the filtration step comprises a membrane that removes sample components that do not comprise DNA barcodes.

53. A method for detecting for the presence or absence of one or more target analytes in a sample comprising:

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

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

observing whether aggregate complex formation occurred.

54. The method according to claim 53 wherein the DNA barcode in each type of particle complex probe has a sequence that is different and that serves as an identifier for a particular target analyte.

55. The method according to claim 53 further comprising the steps of:

isolating aggregated complexes; and

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

56. The method according to claim 53 further comprising the steps of:

isolating the aggregated complex;

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

isolating the DNA barcode; and

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

57. The method according to claim 53 further comprising the steps of:

isolating the aggregated complex;

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

isolating the DNA barcode;

amplifying the isolated DNA barcode; and

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

58. The method of claim 53 wherein the target has at least two binding sites.

59. The method of claim 58 wherein at least two types of particle complex probes are provided, the first type of probe having a specific binding complement to a first binding site on the target analyte and the second type of probe having a specific binding complement to a second binding site on the probe.

60. The method of claim 58 wherein a plurality of particle complex probes are provided, each type of probe having a specific binding complement to different binding sites on the target analyte.

61. The method of claim 53 wherein the specific binding complement and the target analyte are members of a specific binding pair.

62. The method of claim 61 wherein members of a specific binding pair comprise nucleic acid, oligonucleotide, peptide nucleic acid, polypeptide, antibody, antigen, carbohydrate, protein, peptide, amino acid, hormone, steroid, vitamin, drug, virus, polysaccharides, lipids, lipopolysaccharides, glycoproteins, lipoproteins, nucleoproteins, oligonucleotides, antibodies, immunoglobulins, albumin,

hemoglobin, coagulation factors, peptide and protein hormones, non-peptide hormones, interleukins, interferons, cytokines, peptides comprising a tumor-specific epitope, cells, cell-surface molecules, microorganisms, fragments, portions, components or products of microorganisms, small organic molecules, nucleic acids and oligonucleotides, metabolites of or antibodies to any of the above substances.

63. The method of claim 62 wherein nucleic acid and oligonucleotide comprise genes, viral RNA and DNA, bacterial DNA, fungal DNA, mammalian DNA, cDNA, mRNA, RNA and DNA fragments, oligonucleotides, synthetic oligonucleotides, modified oligonucleotides, single-stranded and double-stranded nucleic acids, and natural and synthetic nucleic acids.

64. The method of claim 53 wherein the target analyte is a nucleic acid and the specific binding complement is an oligonucleotide.

65. The method of claim 53 wherein the target analyte is a protein or hapten and the specific binding complement is an antibody comprising a monoclonal or polyclonal antibody.

66. The method of claim 53 wherein the target analyte is a sequence from a genomic DNA sample and the specific binding complements are oligonucleotides, the oligonucleotides having a sequence that is complementary to at least a portion of the genomic sequence.

67. The method of claim 65, wherein the genomic DNA is eukaryotic, bacterial, fungal or viral DNA.

68. The method of claim 53 wherein the specific binding complement and the target analyte are members of an antibody-ligand pair.

69. The method of claim 53 wherein in addition to its first binding site, the target analyte has been modified to include a second binding site.

70. The method of claim 51 wherein the particle comprise a nanoparticle.

71. The method of claim 70 wherein the particle comprises metal, semiconductor, insulator, or magnetic nanoparticles.

72. The method of claim 71 wherein the particle comprises gold nanoparticles.

73. The method according to any one of claims 58 or 59 wherein the detecting for the presence of one or more DNA barcodes comprises:

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

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

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

observing a detectable change.

74. The method according to claim 73, wherein the substrate comprises a plurality of types of oligonucleotides attached thereto in an array to allow for the detection of one or more different types of DNA barcodes.

75. The method according to claim 73 wherein the detectable change is the formation of dark areas on the substrate.

76. The method according to claim 73 wherein the detectable change is observed with an optical scanner.

77. The method according to claim 73 wherein the substrate is contacted with a silver stain to produce the detectable change.

78. The method according to claim 73 wherein the DNA barcodes are contacted with the substrate under conditions effective to allow the DNA barcodes to hybridize with complementary oligonucleotides bound to the substrate and subsequently contacting the DNA barcodes bound to the substrate with the nanoparticles having oligonucleotides bound thereto under conditions effective to allow at least some of the oligonucleotides bound to the nanoparticles to hybridize with a portion of the sequence of the DNA barcodes on the substrate.

79. The method according to claim 73 wherein the DNA barcodes are contacted with the nanoparticles having oligonucleotides bound thereto under conditions effective to allow the DNA barcodes to hybridize with at least some of the oligonucleotides bound to the nanoparticles; and subsequently contacting the DNA barcodes bound to the nanoparticles with the substrate under conditions effective to allow at least a portion of the sequence of the DNA barcodes bound to the nanoparticles to hybridize with complementary oligonucleotides bound to the substrate.

80. The method according to claim 73 wherein the DNA barcode is amplified prior to the contacting step.

81. The method of claim 53 wherein the target analyte has at least two binding sites.

82. The method of claim 81 wherein at least two types of particle complex probes are provided, a first type of probe having a specific binding complement to a first binding site of the target analyte and a second type of probe having a specific binding complement to a second binding site of the target analyte.

83. The method according to claim 53 wherein the particle complex probe comprises a particle having oligonucleotides bound thereto, one or more DNA barcodes, and an oligonucleotide having bound thereto a specific binding complement to a specific target analyte, wherein (i) the DNA barcode has a sequence having at least two portions; (ii) at least some of the oligonucleotides attached to the particle have a sequence that is complementary to a first portion of a DNA barcode; (iii) the oligonucleotide having bound thereto a specific binding complement have a sequence that is complementary to a second portion of a DNA barcode; and (iv) the DNA barcode in each type of particle complex probe has a sequence that is different and that serves as an identifier for a particular target analyte.

84. The method according to claim 53 wherein the particle complex probe comprises a particle having at least two types of oligonucleotides bound thereto, one or more DNA barcodes, and an oligonucleotide having bound thereto a specific binding complement to a target analyte, wherein a first type of oligonucleotides bound to the probe having a sequence that is complementary to at least a portion of the DNA barcode, the second type of oligonucleotide bound to the probe having a sequence that is complementary to at least a portion of the sequence of the oligonucleotide having a specific binding complement.

85. The method according to claim 53 wherein the particle complex probe comprising a particle having oligonucleotides bound thereto, one or more DNA barcodes, and a specific binding complement to a target analyte, wherein at least a portion of the oligonucleotides bound to the particle have a sequence that is

complementary to at least a portion of the sequence of the DNA barcode and where the DNA barcode serves as an identifier for a specific target analyte.

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

87. A particle complex probe comprising a particle having at least two types of oligonucleotides bound thereto, a DNA barcode, and an oligonucleotide having bound thereto a specific binding complement to a target analyte, wherein a first type of oligonucleotides bound to the probe having a sequence that is complementary to at least a portion of the DNA barcode, the second type of oligonucleotide bound to the probe having a sequence that is complementary to at least a portion of the sequence of the oligonucleotide having a specific binding complement.

88. A particle complex probe comprising a particle having oligonucleotides bound thereto, a DNA barcode, and a specific binding complement to a target analyte, wherein at least a portion of the oligonucleotides bound to the particle have a sequence that is complementary to at least a portion of the sequence of the DNA barcode and where the DNA barcode serves as an identifier for a specific target analyte.

89. A detection probe comprising:

(a) a nanoparticle;

(b) a member of a specific binding pair bound to the nanoparticle;

(c) at least one type of oligonucleotide bound to the nanoparticle; and

(d) at least one type of DNA barcode each having a predetermined sequence, wherein each type of DNA barcode is hybridized to at least a portion of the at least one type of oligonucleotide.

90. The probe according to any one of claims 86-88 wherein the particle comprises a nanoparticle.

91. The probe according any one of claims 86-89 wherein the nanoparticles are metal, semiconductor, insulator, or magnetic nanoparticles.

92. The probe according any one of claims 86-89 wherein the particles are gold nanoparticles.

93. The probe according any one of claims 86-89 wherein the target has at least two binding sites.

94. The probe of claim 93 wherein at least two types of particle complex probes are provided, the first type of probe having a specific binding complement to a first binding site on the target analyte and the second type of probe having a specific binding complement to a second binding site on the probe.

95. The probe of claim 93 wherein a plurality of particle complex probes are provided, each type of probe having a specific binding complement to different binding sites on the target analyte.

96. The probe according any one of claims 86-89 wherein the specific binding complement and the target analyte are members of a specific binding pair.

97. The probe of claim 96 wherein members of a specific binding pair comprise nucleic acid, oligonucleotide, peptide nucleic acid, polypeptide, antibody, antigen, carbohydrate, protein, peptide, amino acid, hormone, steroid, vitamin, drug, virus, polysaccharides, lipids, lipopolysaccharides, glycoproteins, lipoproteins, nucleoproteins, oligonucleotides, antibodies, immunoglobulins, albumin, hemoglobin, coagulation factors, peptide and protein hormones, non-peptide hormones, interleukins, interferons, cytokines, peptides comprising a tumor-specific epitope, cells, cell-surface molecules, microorganisms, fragments, portions, components or products of microorganisms, small organic molecules, nucleic acids and oligonucleotides, metabolites of or antibodies to any of the above substances.

98. The probe of claim 97 wherein nucleic acid and oligonucleotide comprise genes, viral RNA and DNA, bacterial DNA, fungal DNA, mammalian DNA, cDNA, mRNA, RNA and DNA fragments, oligonucleotides, synthetic oligonucleotides, modified oligonucleotides, single-stranded and double-stranded nucleic acids, and natural and synthetic nucleic acids.

99. The probe according any one of claims 86-89 wherein the target analyte is a nucleic acid and the specific binding complement is an oligonucleotide.

100. The probe according any one of claims 86-89 wherein the target analyte is a protein or hapten and the specific binding complement is an antibody comprising a monoclonal or polyclonal antibody.

101. The probe according any one of claims 86-89 wherein the target analyte is a sequence from a genomic DNA sample and the specific binding complements are oligonucleotides, the oligonucleotides having a sequence that is complementary to at least a portion of the genomic sequence.

102. The probe of claim 100, wherein the genomic DNA is eukaryotic, bacterial, fungal or viral DNA.

103. The probe according any one of claims 86-89 wherein the specific binding complement and the target analyte are members of an antibody-ligand pair.

104. The probe according any one of claims 86-89 wherein in addition to its first binding site, the target analyte has been modified to include a second binding site.

105. A kit comprising a probe of any one of claims 86-89.

106. A kit for detecting for the presence or absence of one or more target analytes in a sample, each target analyte having at least two binding sites, the kit comprising:

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

107. A kit for detecting for the presence or absence of one or more target analytes in a sample, each target analyte having at least two binding sites, the kit comprising:

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

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

108. The kit of claim 107, wherein the substrate is a magnetic particle.

109. A kit for detecting for the presence or absence of one or more target analytes in a sample, each target analyte having at least two binding sites, the kit comprising:

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

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

110. A kit for detecting a target analyte in a sample, the kit comprising at least one container including particle complex probes comprising a particle having oligonucleotides bound thereto, a DNA barcode, and an oligonucleotide having bound thereto a specific binding complement to a target analyte, wherein the DNA barcode has a sequence having at least two portions, at least some of the oligonucleotides attached to the particle have a sequence that is complementary to a first portion of a DNA barcode, the oligonucleotides having bound thereto a specific binding complement have a sequence that is complementary to a second portion of a DNA barcode, and wherein the DNA barcode is hybridized to at least to some of the oligonucleotides attached to the particle and to the oligonucleotides having bound thereto the specific binding complement, and an optional substrate for observing a detectable change.

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

112. A kit for the detection of a target analyte, the kit includes at least one pair of containers and an optional substrate for observing a detectable change,

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

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

113. A kit for the detection of multiple target analytes in a sample, the kit includes at least two or more pairs of containers,

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

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

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

114. A kit for the detection of multiple target analytes in a sample, the kit includes a first container and at least two or more pairs of containers,

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

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

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

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

115. The kit of claim 106 wherein the DNA barcode comprises an oligonucleotide sequence that serves as an identifier for the presence of a specific target analyte.

116. The kit of claim 106, wherein the specific target analyte is an antibody.

117. The kit of claim 115, wherein the member of a specific binding pair comprises an antibody or an antigen.

118. The kit of claim 115, wherein the member of a specific binding pair comprises a receptor or a ligand.

119. The kit of claim 115, wherein the member of a specific binding pair comprises an enzyme or a substrate.

120. The kit of claim 115, wherein the member of a specific binding pair comprises a drug or a target molecule.

121. The kit of claim 115, wherein the member of a specific binding pair comprises two strands of at least partially complementary oligonucleotides.

122. The kit of claim 115, wherein the DNA barcode is biotinylated.

123. The kit of claim 115, wherein the DNA barcode is radioactively labeled.

124. The kit of claim 115, wherein the DNA barcode is fluorescently labeled.

125. The kit of claim 115, wherein the oligonucleotide attached to the particle is present on the surface of the particle at a density of at least 10 picomoles/cm^2.

126. The kit of claim 115, wherein the oligonucleotide attached to the particle is present on the surface of the particle at a density of at least 15 picomoles/cm^2.

127. The kit of claim 115, wherein the oligonucleotide attached to the particle is present on the surface of the particle at a density from about 15 picomoles/cm^2 to about 40 picomoles/cm^2

128. The kit of claim 115, wherein the nanoparticle is a metal nanoparticle, or a semiconductor nanoparticle.

129. The kit of claim 115, wherein the nanoparticle is a gold nanoparticle.

130. The kit of claim 115, wherin the semiconductor nanoparticle is made of CdSe/ZnS (core/shell).

FIGURE 1

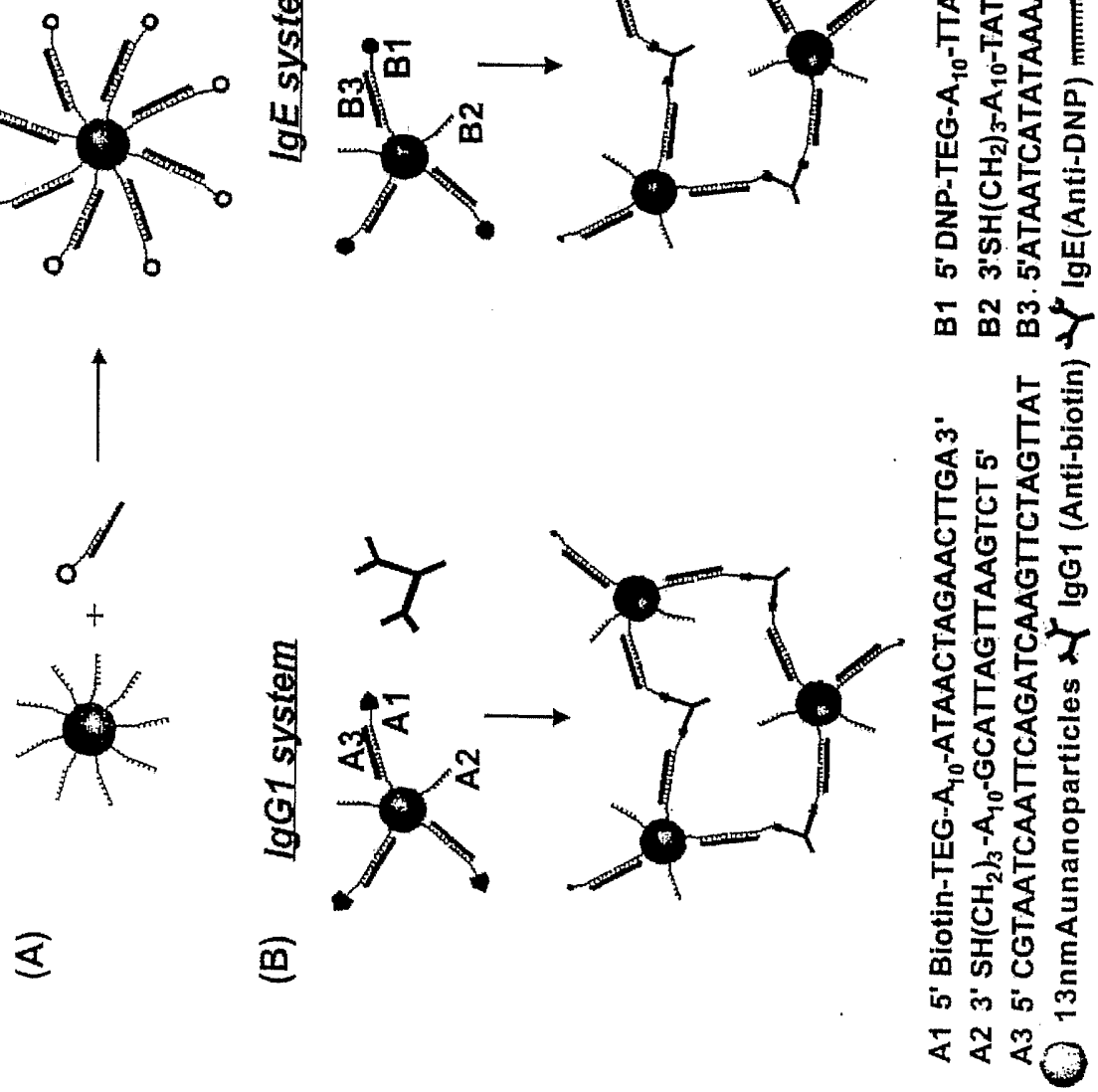

(A)
(B)
IgG1 system
IgE system
A1
A2
A3
B1
B2
B3
A1 5' Biotin-TEG-A10-ATAACTAGAACTTGA 3'
A2 3' SH(CH2)3-A10-GCATTAGTTAAGTCT 5'
A3 5' CGTAATCAATTCAGATCAAGTTCTAGTTAT
B1 5' DNP-TEG-A10-TTATCTATTATT 3'
B2 3'SH(CH2)3-A10-TATTAGTATATT 5'
B3 5'ATAATCATATAAAATAATAGATAA 3'
13nmAunanoparticles
IgG1 (Anti-biotin)
IgE(Anti-DNP)
Biobarcode DNA

FIGURE 2

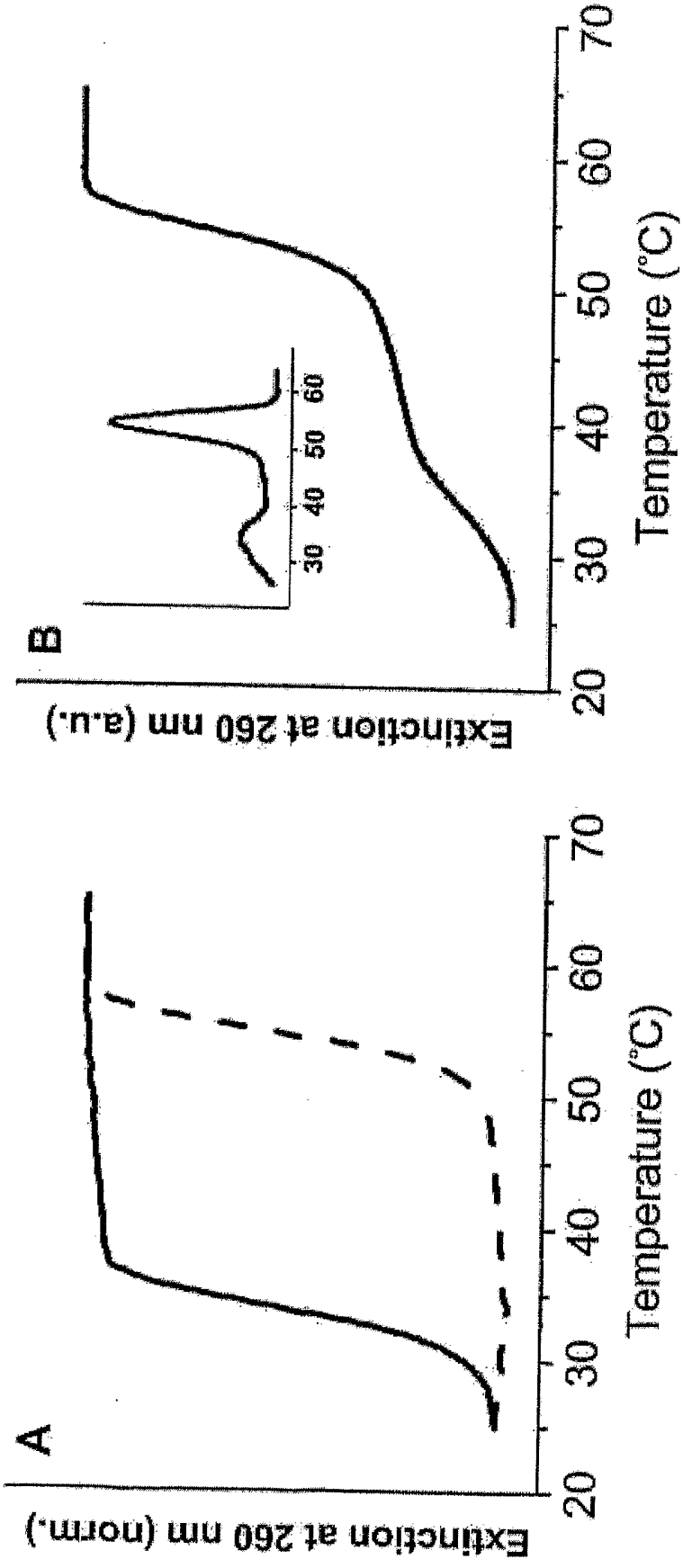

A
Extinction at 260 nm (norm.)
Temperature (°C)
20
30
40
50
60
70
B
Extinction at 260 nm (a.u.)
Temperature (°C)
20
30
40
50
60
70
30
40
50
60

FIGURE 3

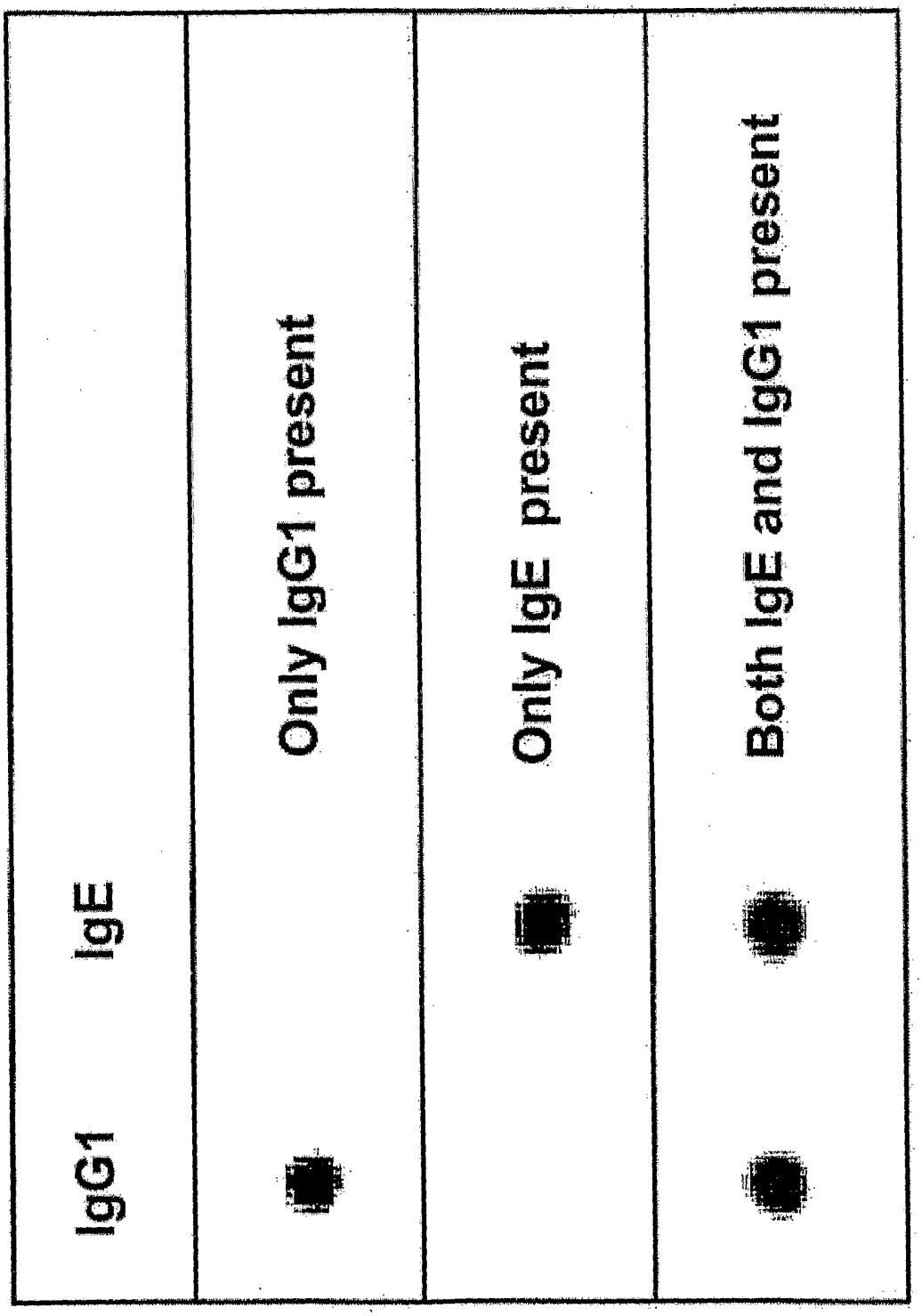
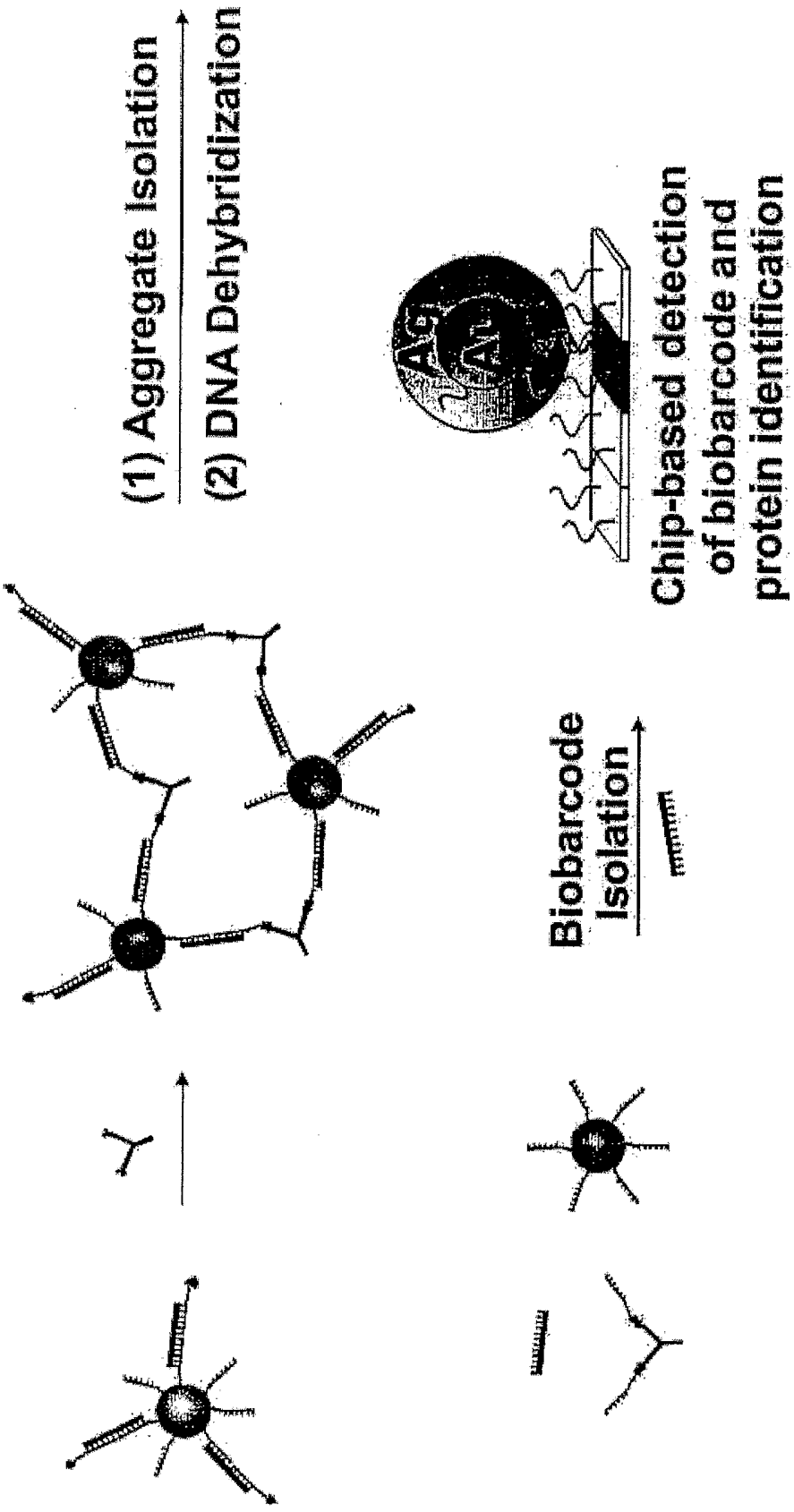

IgG1
IgE
Only IgG1 present
Only IgE present
Both IgE and IgG1 present

Figure 4


(1) Aggregate Isolation
(2) DNA Dehybridization
Chip-based detection of biobarcode and protein identification
Biobarcode Isolation
Ag
Au

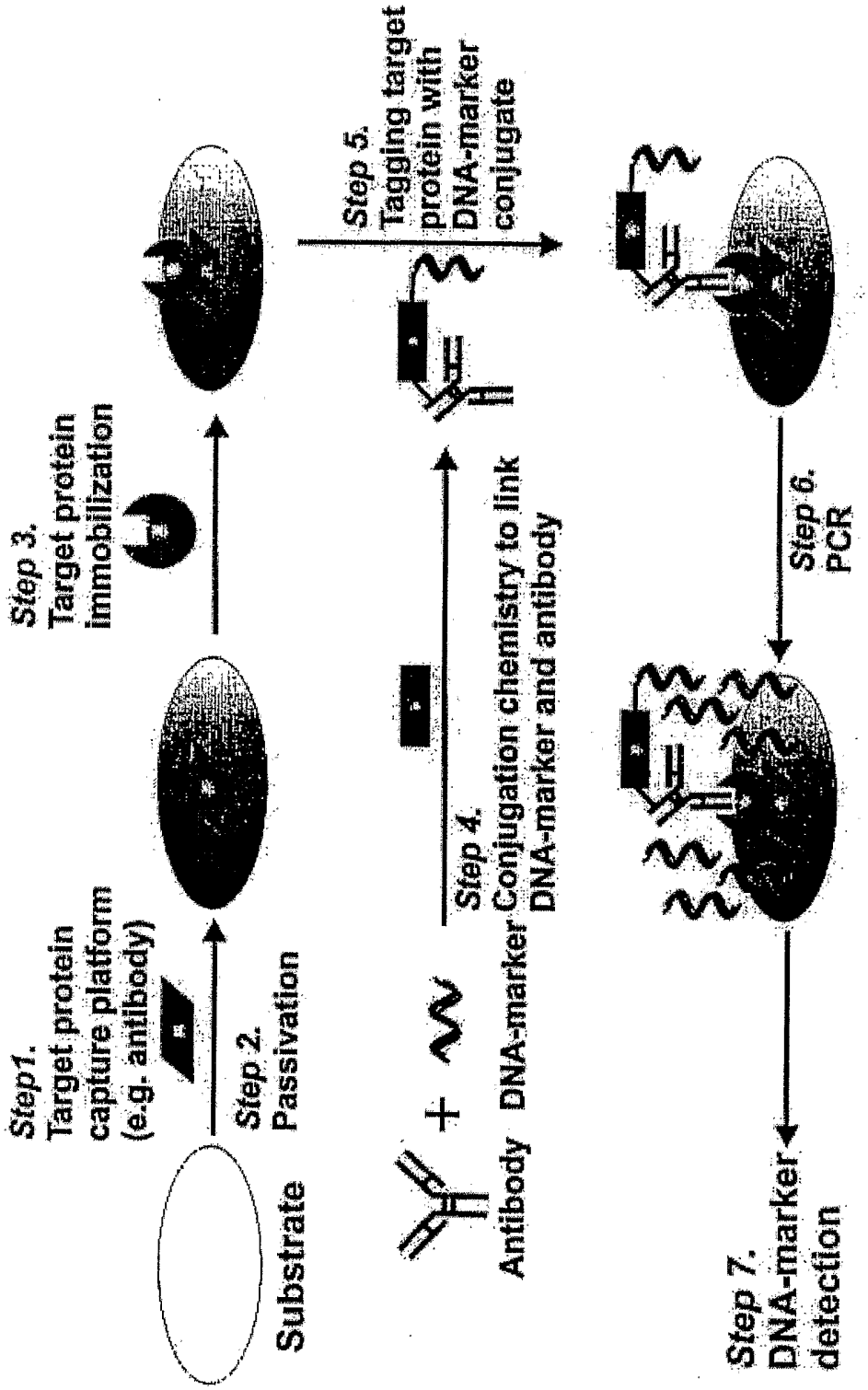

Step1.
Target protein
capture platform
(e.g. antibody)
Step 2.
Passivation
Substrate
Step 3.
Target protein
immobilization
Step 4.
Conjugation chemistry to link
DNA-marker and antibody
Antibody
DNA-marker
Step 5.
Tagging target
protein with
DNA-marker
conjugate
Step 6.
PCR
Step 7.
DNA-marker
detection

Figure 5

Figure 6A

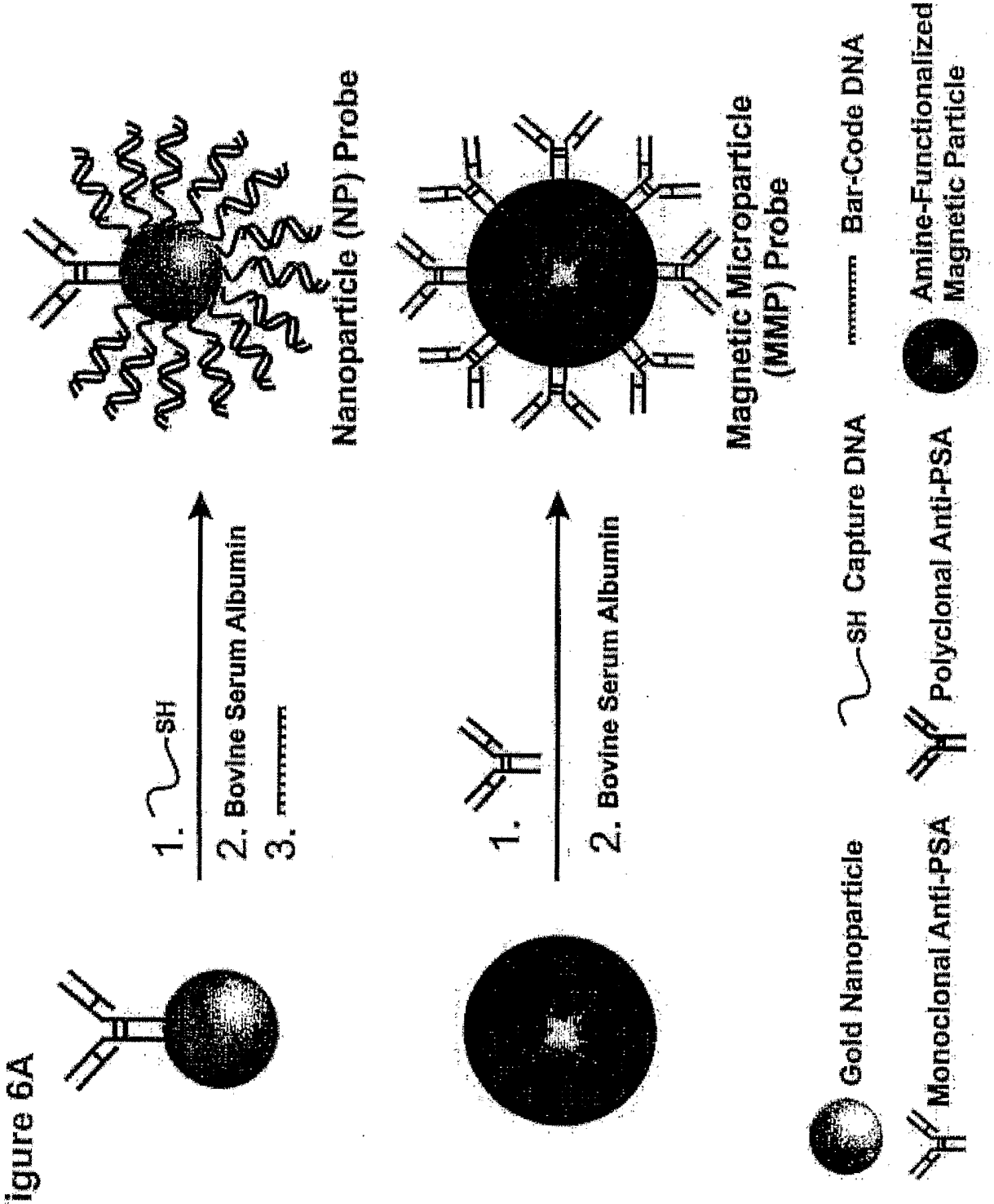
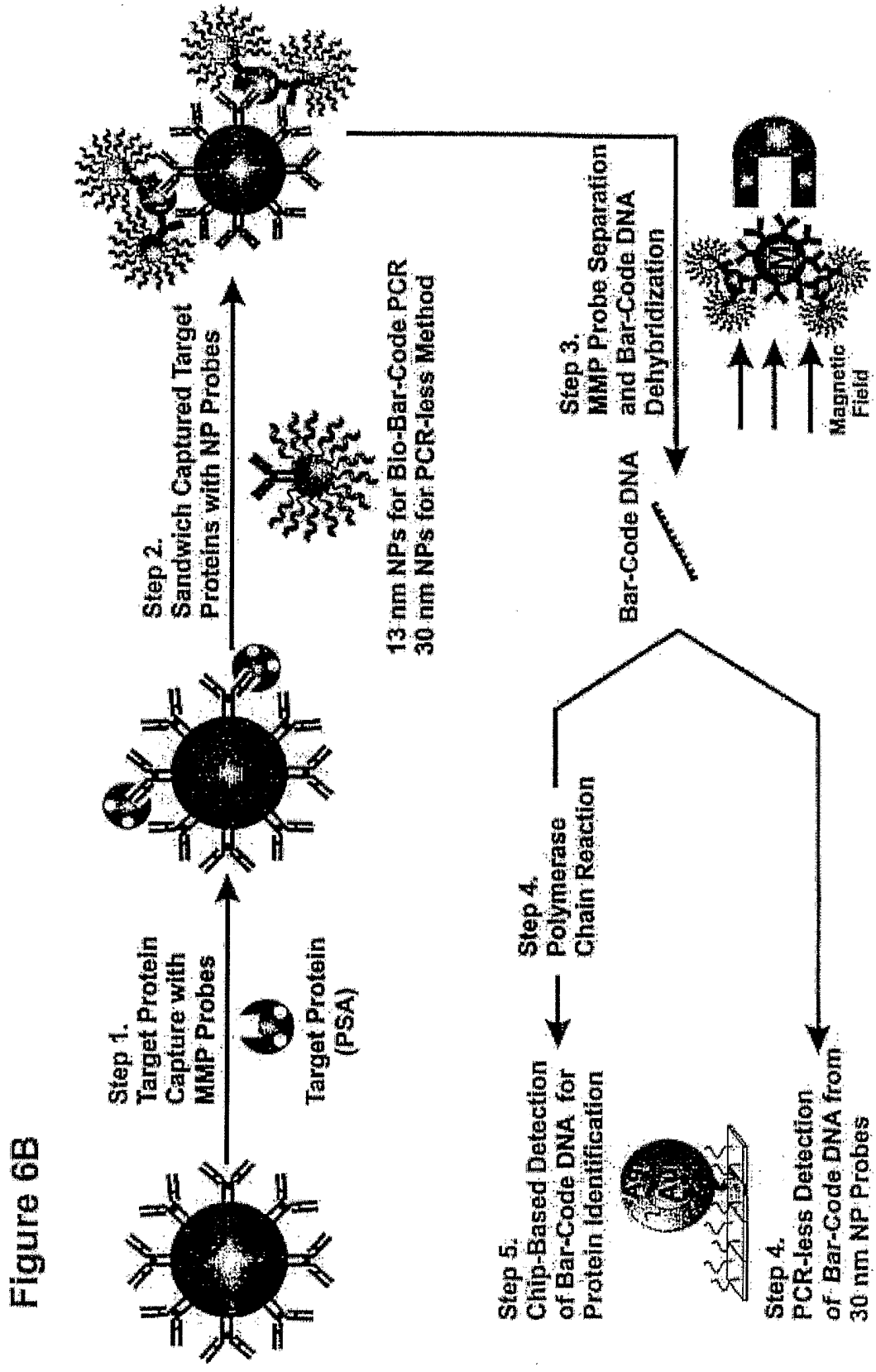

1.
SH
2. Bovine Serum Albumin
3.
Nanoparticle (NP) Probe
1.
2. Bovine Serum Albumin
Magnetic Microparticle (MMP) Probe
Gold Nanoparticle
SH Capture DNA
Bar-Code DNA
Monoclonal Anti-PSA
Polyclonal Anti-PSA
Amine-Functionalized Magnetic Particle

Figure 6B


Step 1.
Target Protein
Capture with
MMP Probes
Target Protein
(PSA)
Step 2.
Sandwich Captured Target
Proteins with NP Probes
13 nm NPs for Bio-Bar-Code PCR
30 nm NPs for PCR-less Method
Step 3.
MMP Probe Separation
and Bar-Code DNA
Dehybridization
Magnetic
Field
Bar-Code DNA
Step 4.
Polymerase
Chain Reaction
Step 5.
Chip-Based Detection
of Bar-Code DNA for
Protein Identification
Step 4.
PCR-less Detection
of Bar-Code DNA from
30 nm NP Probes

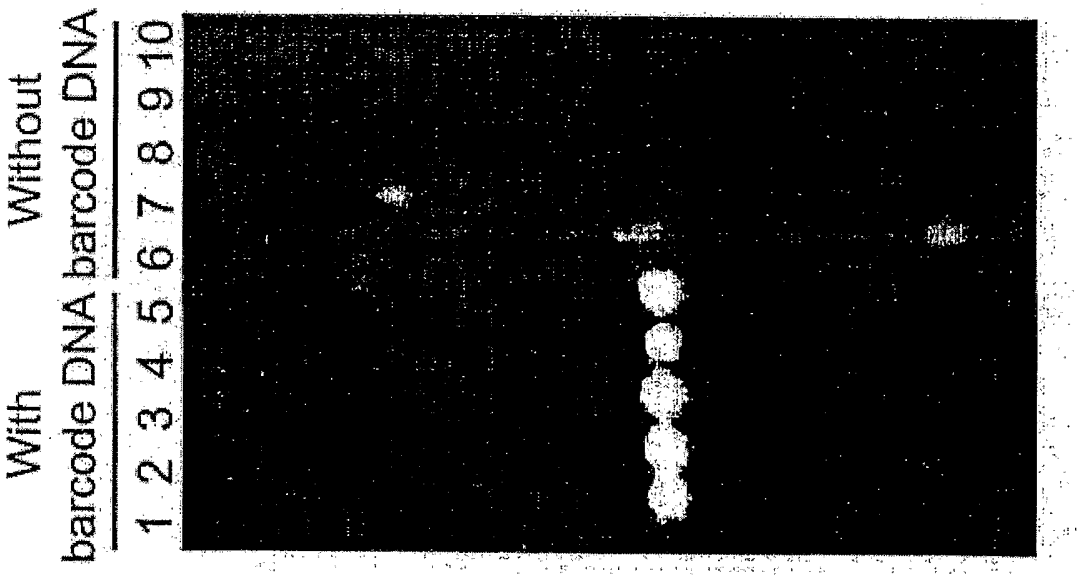


Figure 7

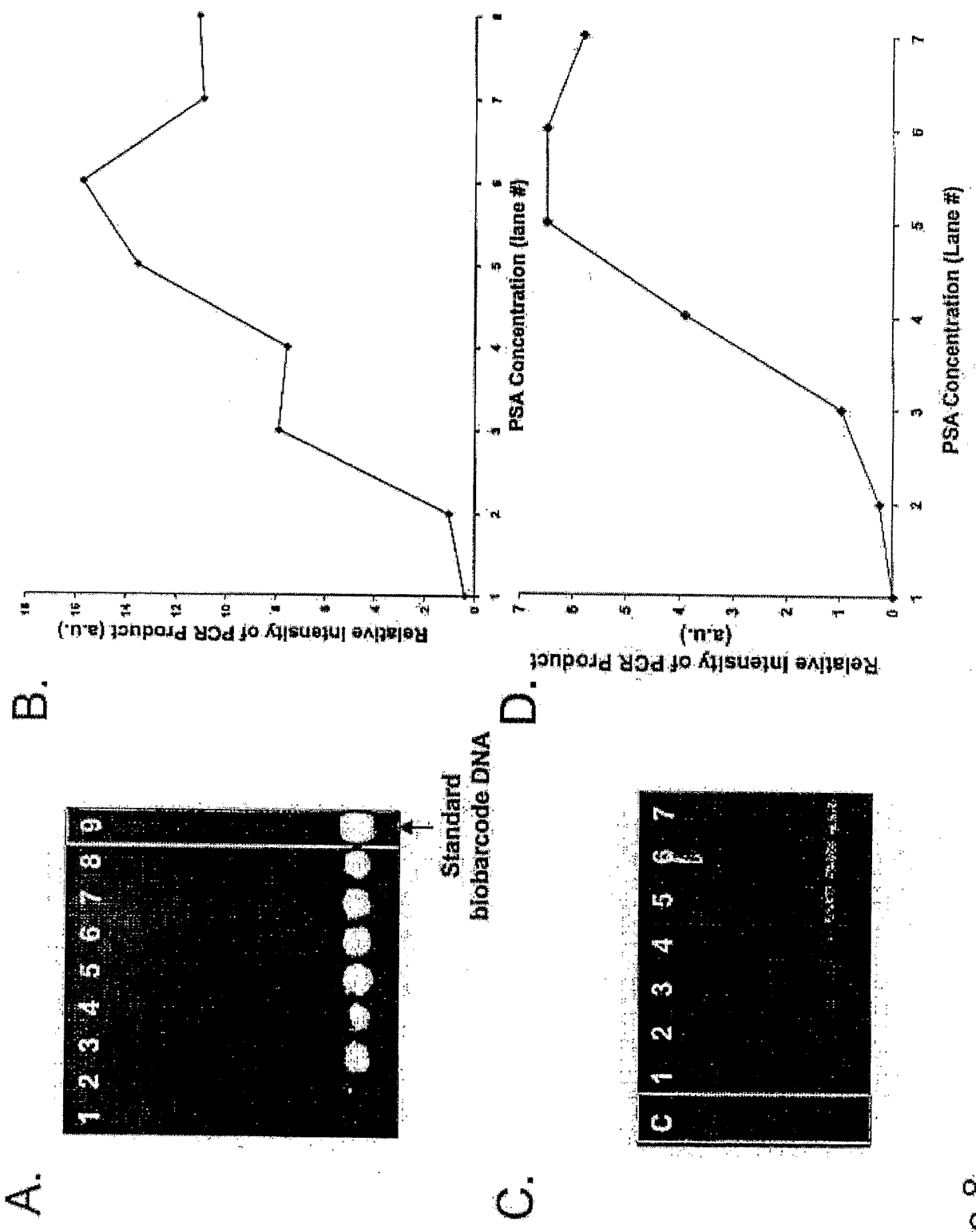

A.
1 2 3 4 5 6 7 8 9
Standard biobarcode DNA
B.
Relative Intensity of PCR Product (a.u.)
PSA Concentration (lane #)
C.
C 1 2 3 4 5 6 7
D.
Relative Intensity of PCR Product (a.u.)
PSA Concentration (Lane #)

Figure 8

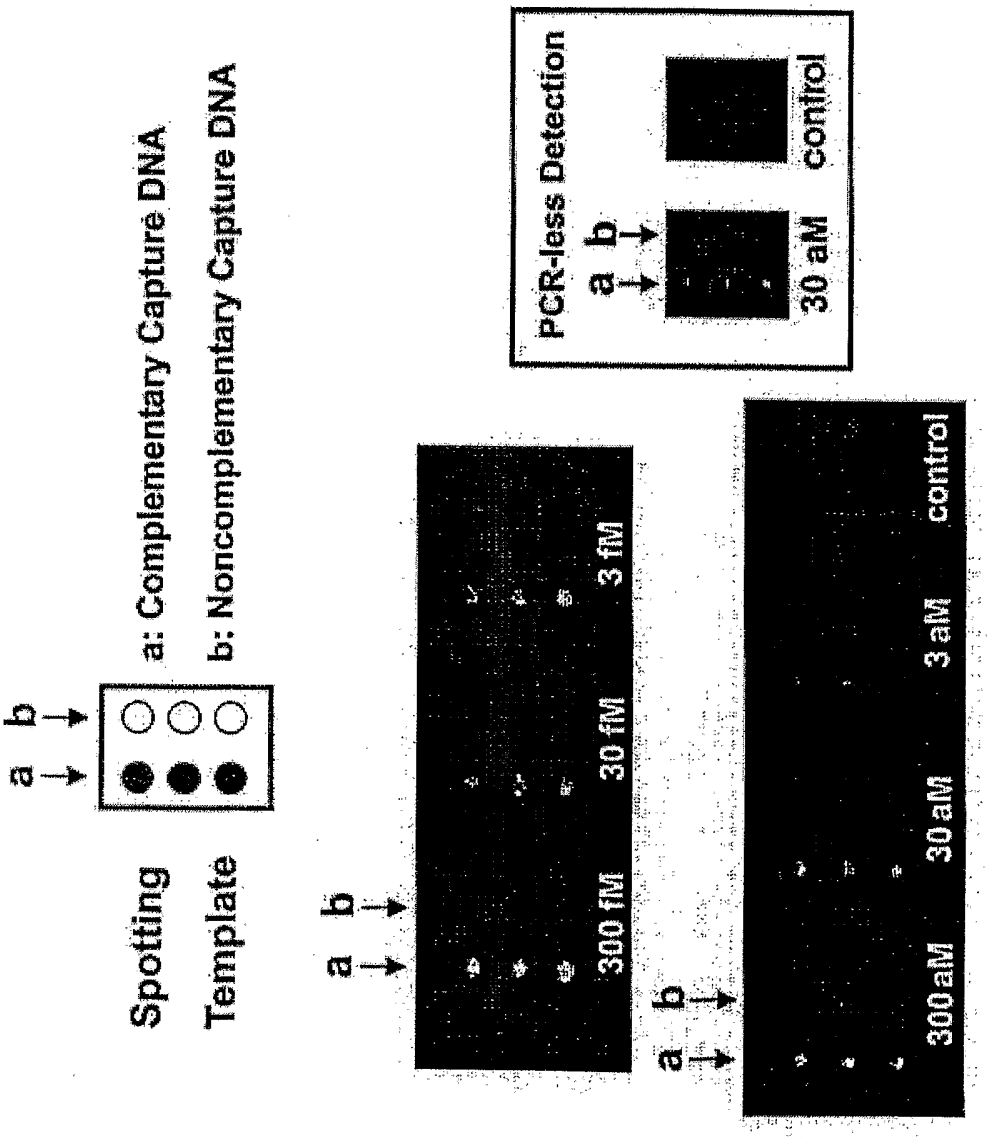
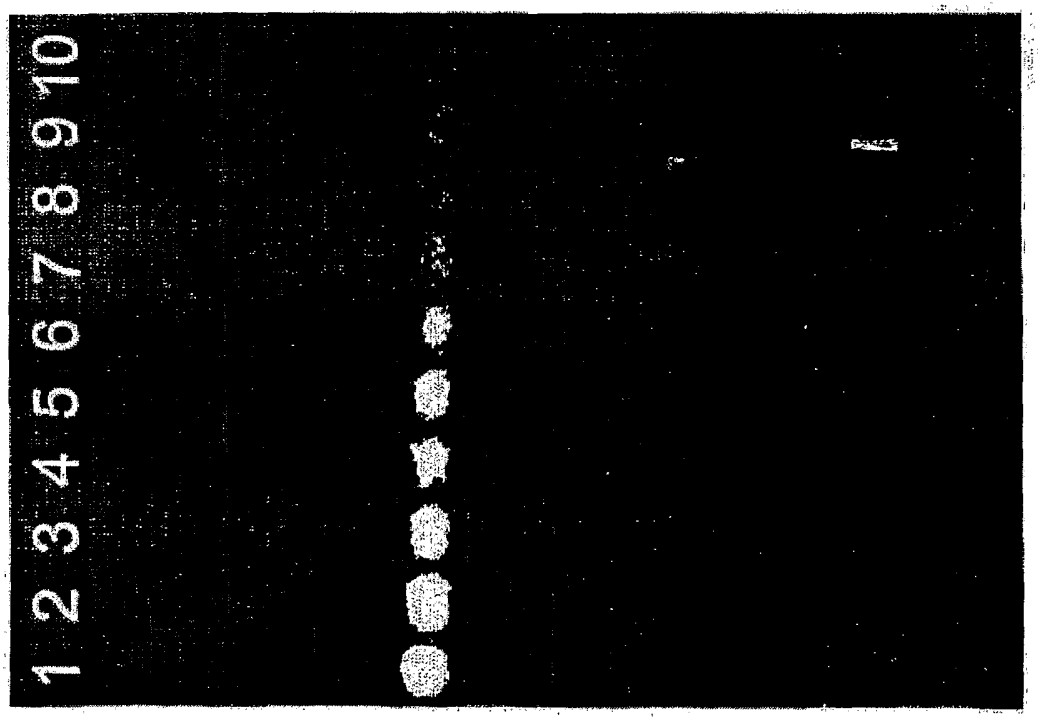

a: Complementary Capture DNA
b: Noncomplementary Capture DNA
Spotting
Template
a b
a b
300 fM
30 fM
3 fM
a b
300 aM
30 aM
3 aM
control
PCR-less Detection
a b
30 aM
control

Figure 9


1 2 3 4 5 6 7 8 9 10

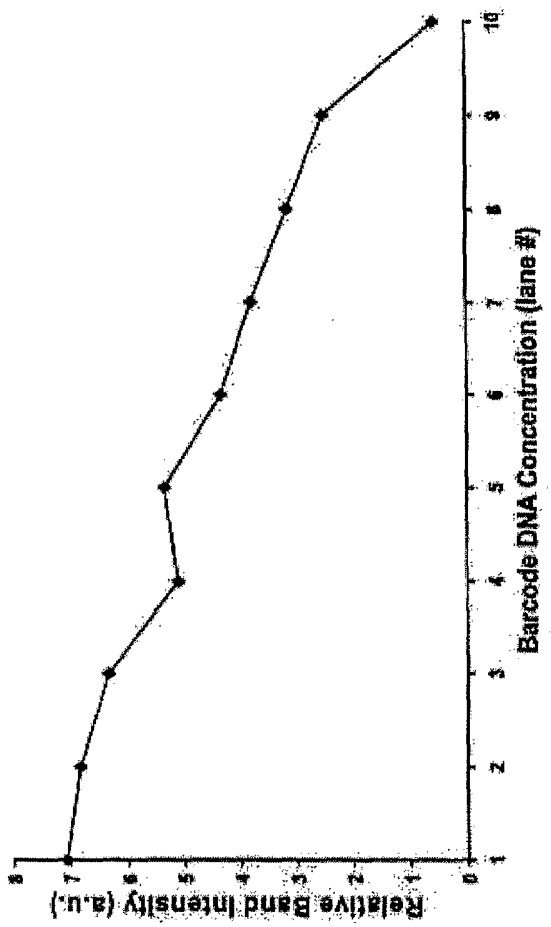

Relative Band Intensity (a.u.)
Barcode DNA Concentration (lane #)

Figure 10

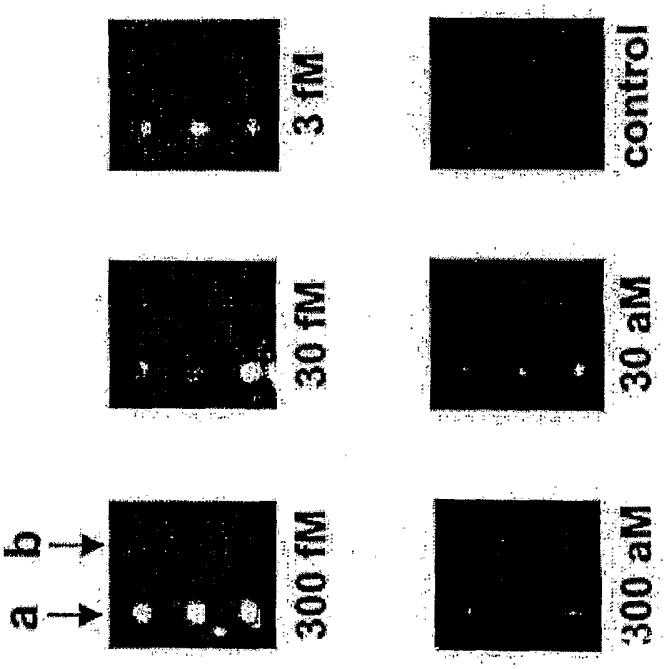


Figure 11

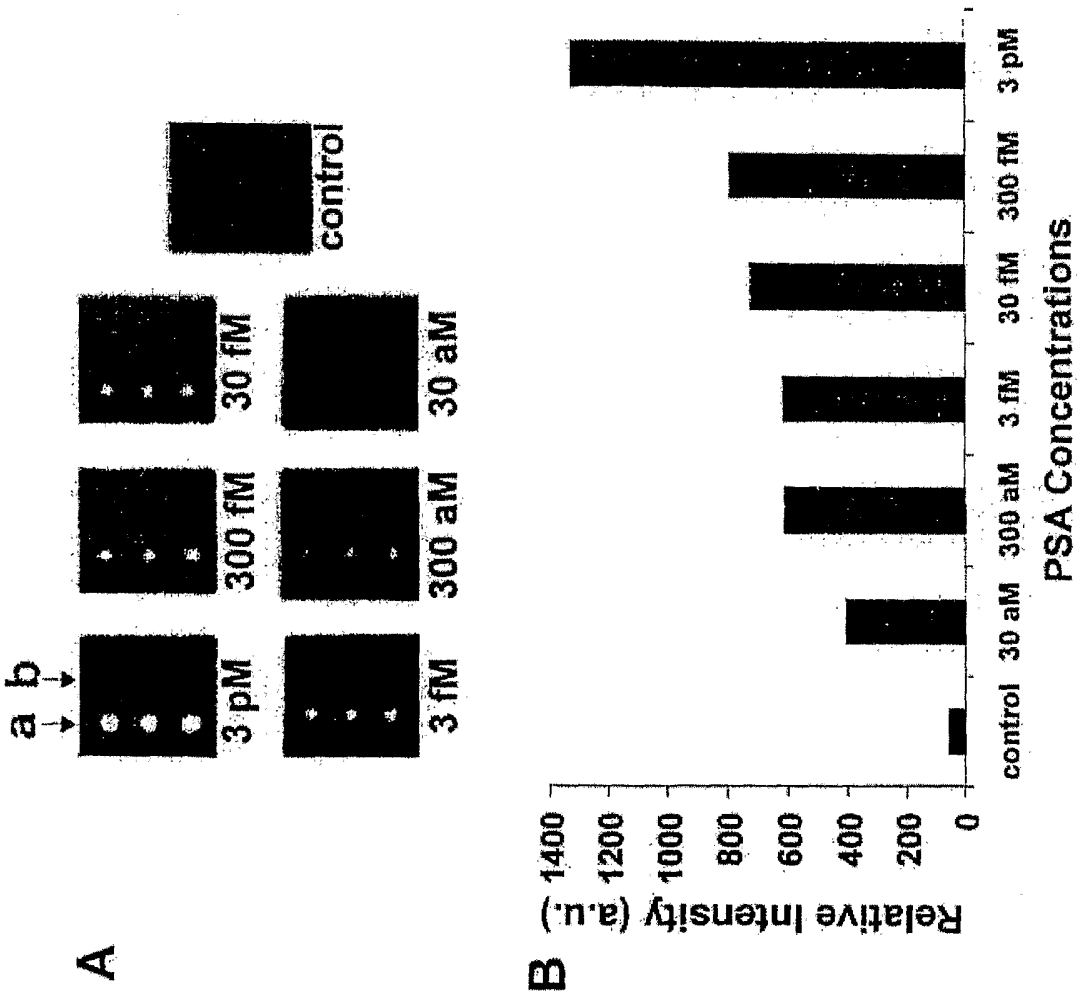

A
a b
3 pM
3 fM
300 fM
300 aM
30 fM
30 aM
control
B
Relative Intensity (a.u.)
0
200
400
600
800
1000
1200
1400
control
30 aM
300 aM
3 fM
30 fM
300 fM
3 pM
PSA Concentrations

Figure 12

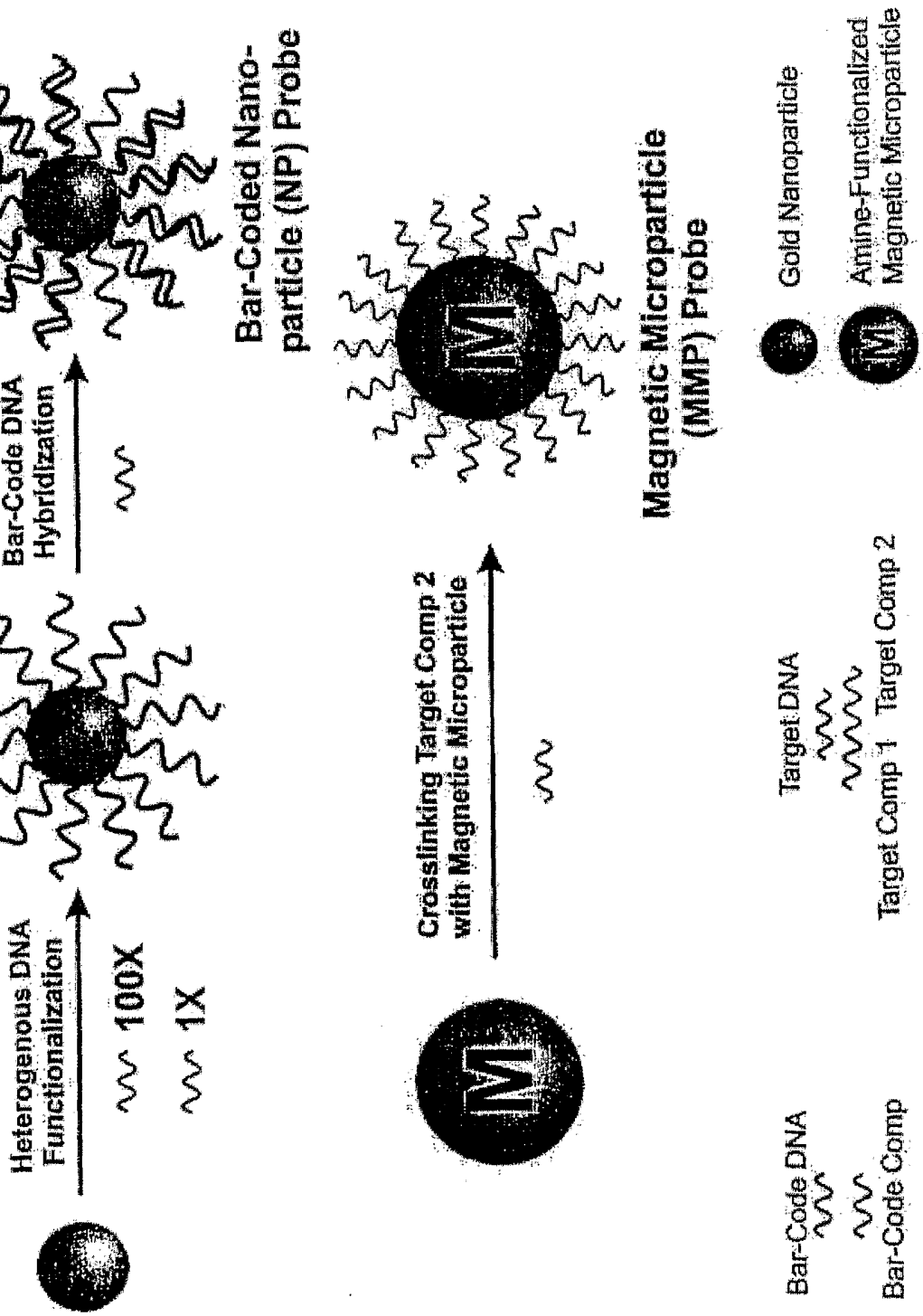

Heterogenous DNA Functionalization
100X
1X
Bar-Code DNA Hybridization
Bar-Coded Nano-particle (NP) Probe
Crosslinking Target Comp 2 with Magnetic Microparticle
Magnetic Microparticle (MMP) Probe
Gold Nanoparticle
Amine-Functionalized Magnetic Microparticle
Target DNA
Bar-Code DNA
Bar-Code Comp
Target Comp 1
Target Comp 2

Figure 13A.

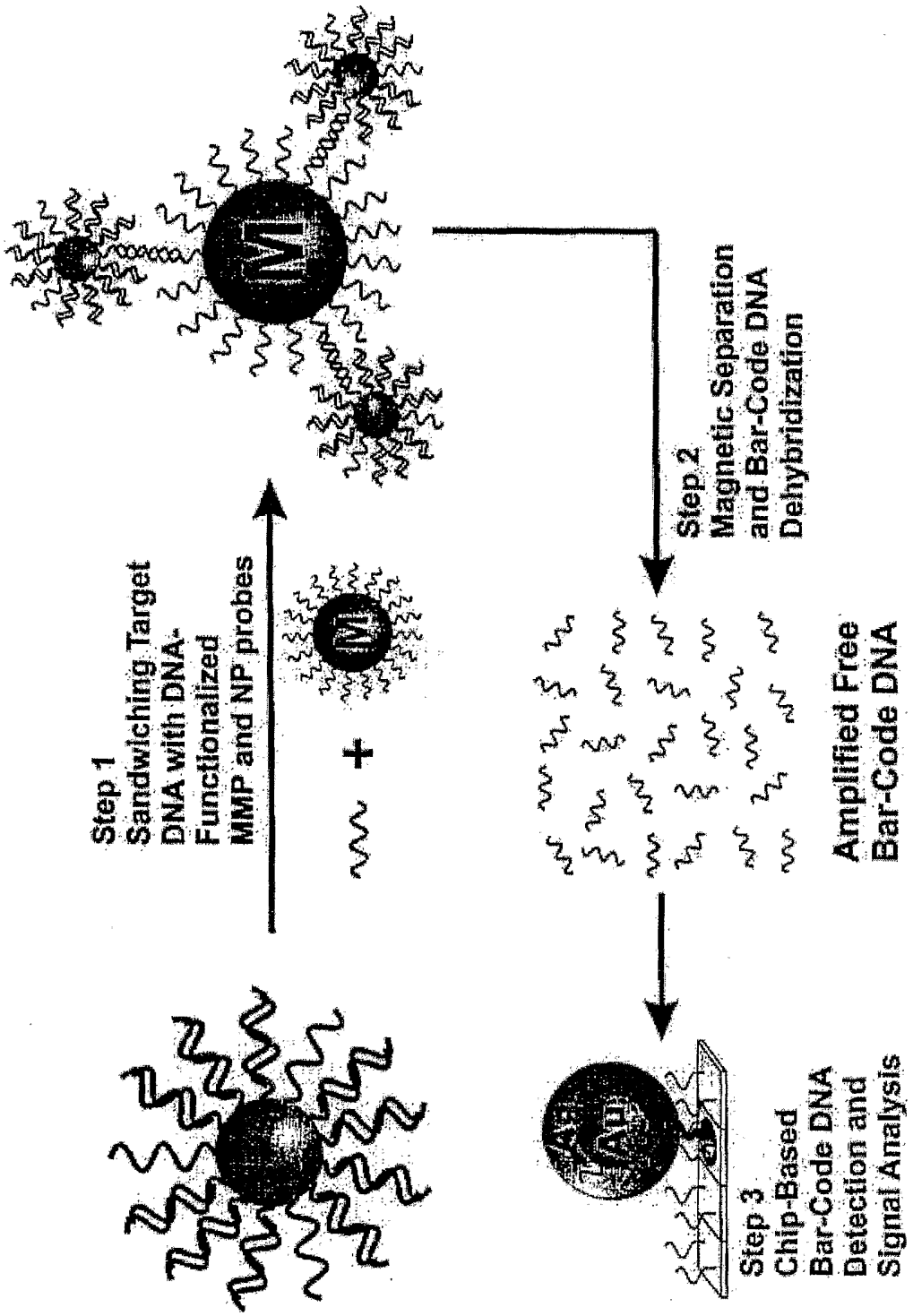

Step 1
Sandwiching Target
DNA with DNA-
Functionalized
MMP and NP probes
Step 2
Magnetic Separation
and Bar-Code DNA
Dehybridization
Amplified Free
Bar-Code DNA
Step 3
Chip-Based
Bar-Code DNA
Detection and
Signal Analysis

Figure 13B.

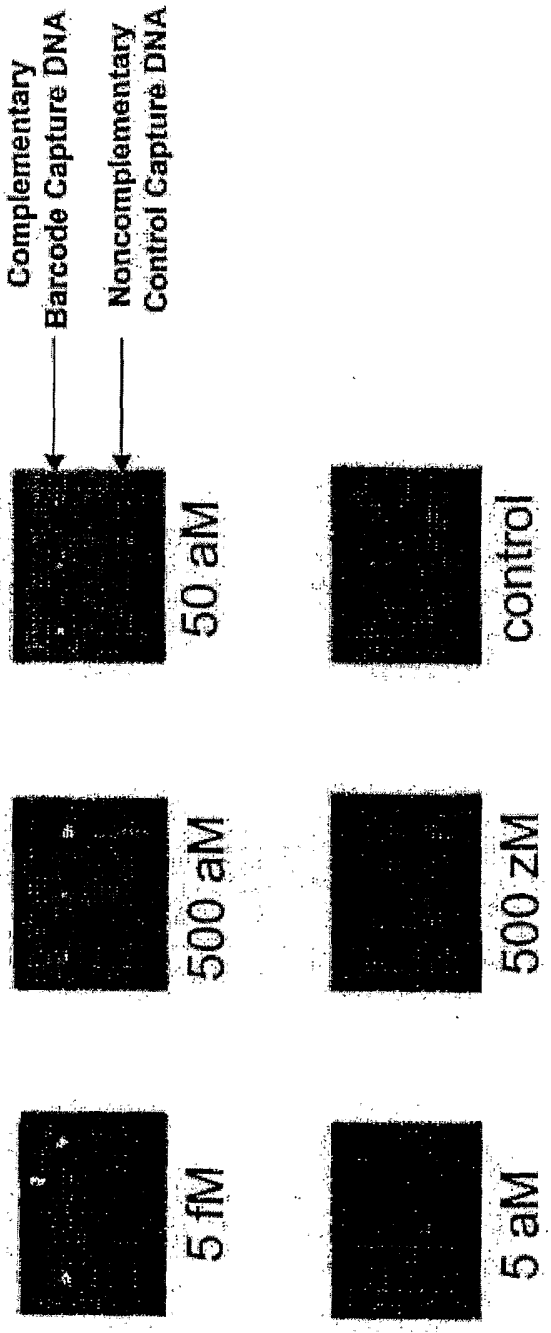

Complementary
Barcode Capture DNA
Noncomplementary
Control Capture DNA
50 aM
control
500 aM
500 zM
5 fM
5 aM

Figure 14A.

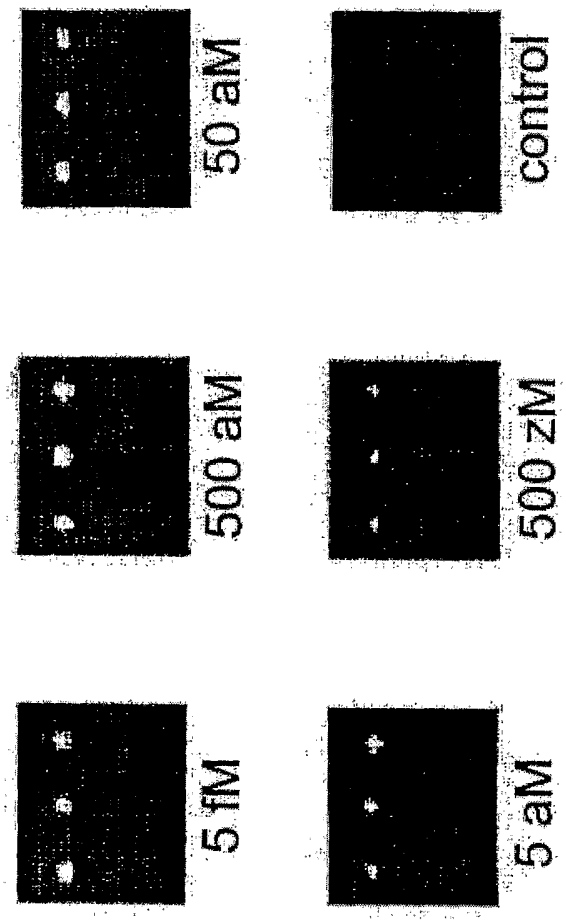


Figure 14B.

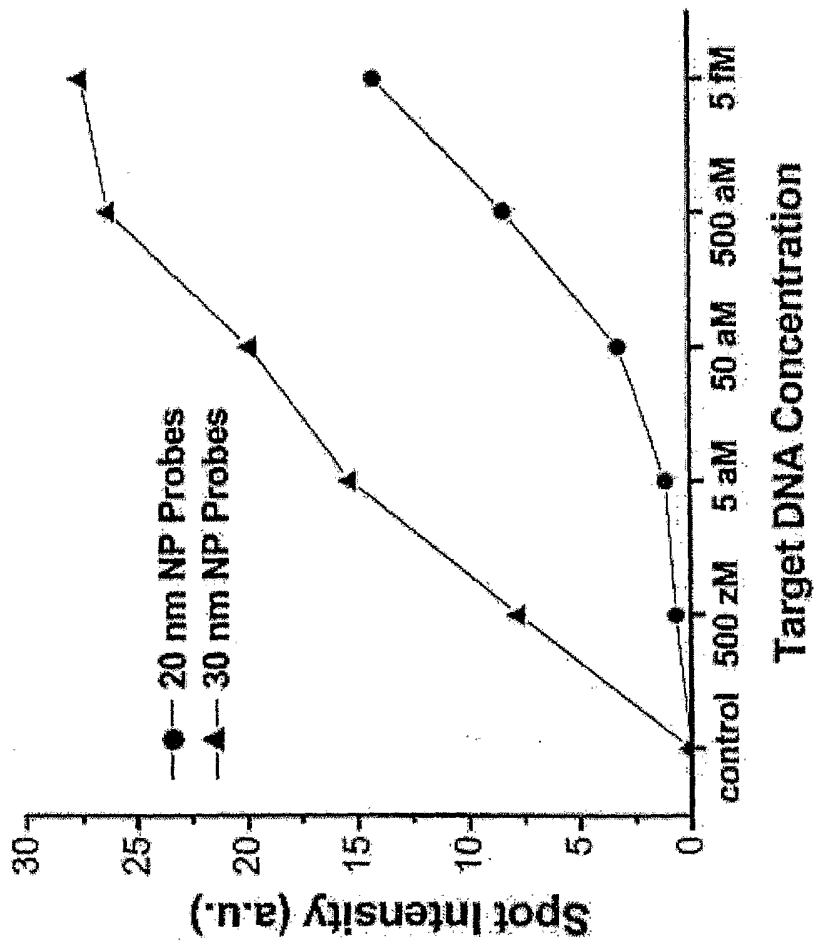
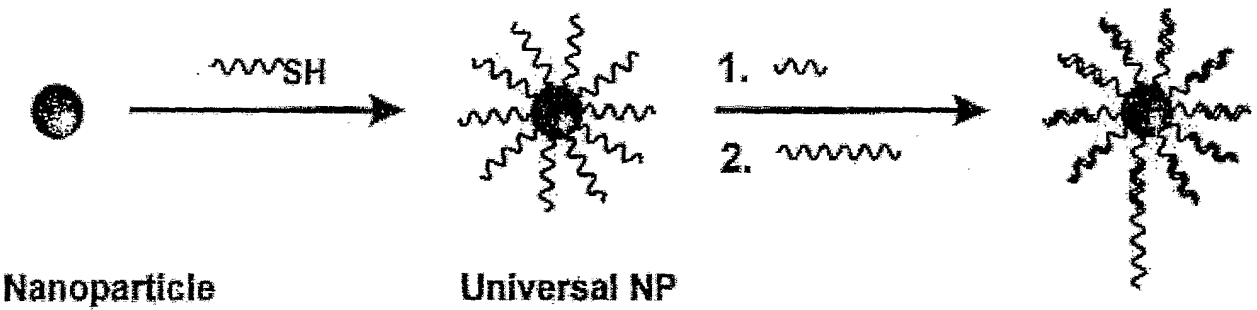

Spot Intensity (a.u.)
30
25
20
15
10
5
0
20 nm NP Probes
30 nm NP Probes
control
500 zM
5 aM
50 aM
500 aM
5 fM
Target DNA Concentration

Figure 15

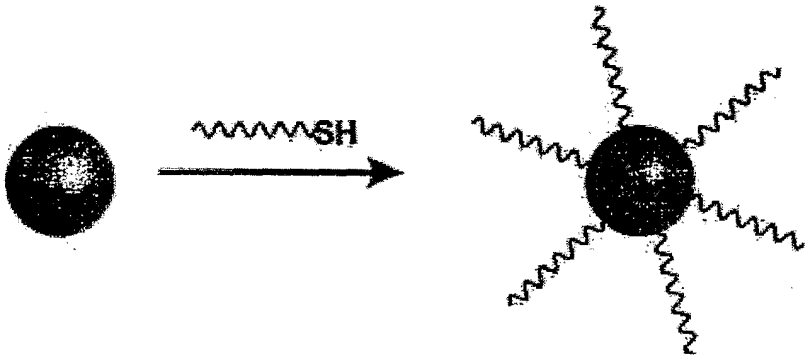
Figure 16A **Universal Nanoparticle Probe**



Gold Nanoparticle (NP)

Universal NP Probe

Universal NP Probe can be loaded with a high ratio of unique barcode DNA to target recognition DNA.



Amine-Functionalized Magnetic Microparticle (MMP)

MMP Probe

SH Bar-Code Capture DNA

Bar-Code DNA

Target Recognition DNA on NP

SH Target Capture DNA on MMP

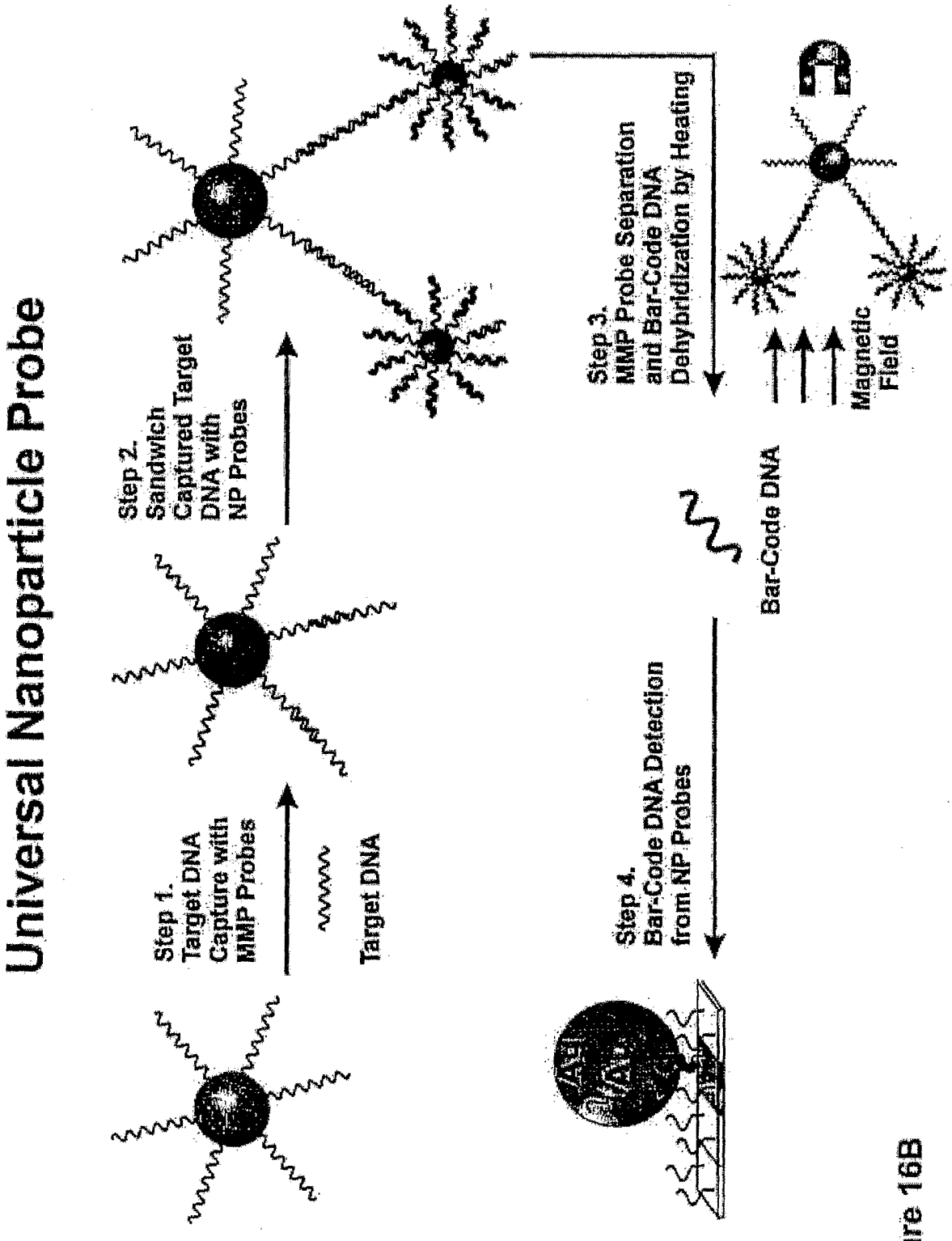

Universal Nanoparticle Probe
Step 1.
Target DNA
Capture with
MMP Probes
Target DNA
Step 2.
Sandwich
Captured Target
DNA with
NP Probes
Step 3.
MMP Probe Separation
and Bar-Code DNA
Dehybridization by Heating
Magnetic
Field
Bar-Code DNA
Step 4.
Bar-Code DNA Detection
from NP Probes

Figure 16B

Dendrimer and Amplified Bio-Bar-Code Probes
Amplified Nanoparticle Probe
Dendrimer-Nanoparticle Hybrid Probe
Dendrimer Probe
Amplified Dendrimer Probe
Dendrimer
Nanoparticle
Bar-Code DNA

Figure 17