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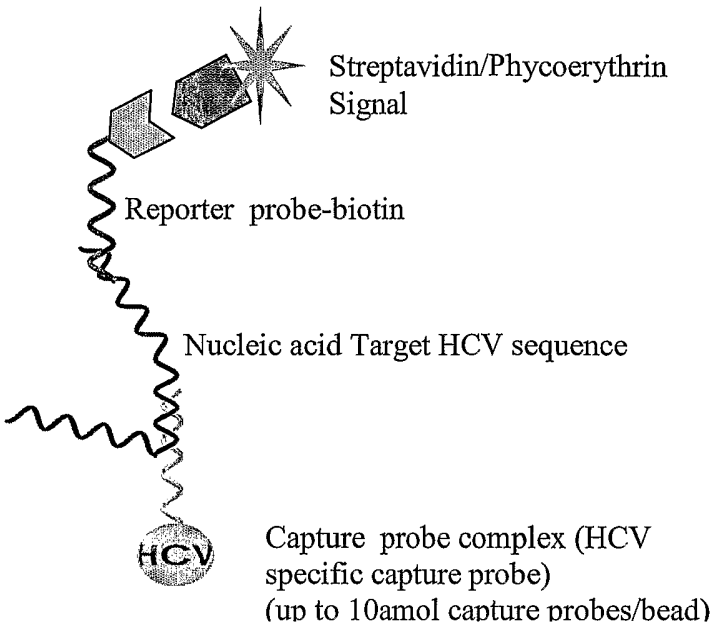

(57) Abstract: The present invention provides a method for the simultaneous analysis, in a homogeneous format, for detection of multiple nucleic acid target sequences. The invention utilizes capture probes conjugated to substrates that are distinguishable based on unique spectral signature in combination with reporter probes having a separately detectable label. In the presence of a target sequence, detectable complexes are formed that are distinguishable for each combination of target, capture probe and reporter probe.

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HOMOGENEOUS MULTIPLEX ASSAY FOR NUCLEIC ACID TARGETS

TECHNICAL FIELD

[0001] The present invention relates to methods for the detection of specific nucleic acid target sequences in biological samples.

BACKGROUND OF THE INVENTION

[0002] Detection and analysis of nucleic acids found in clinical and environmental specimens are important in medicine and scientific study. A wide variety of assays for such nucleic acid detection and/or analysis are known in the art and vary considerably with regard to specificity, sensitivity, ease and speed of use, and capacity for the number of nucleic acids and/or specimens involved in the assay.

[0003] Multiplex analysis is the ability to perform multiple discrete assays in a single reaction vessel with the same sample at the same time. Developments in bioanalytical sciences and bioengineering have lead to multiplexed assays for detection and analysis of nucleic acids. See, for example, U.S. Pat. No. 5,981,180; Fulton et al. (1997) Clinical Chem. 43:1749-1756; PCT Pub. No. WO 01/48244; U.S. Pat. Application Publication Nos. US 2002/0028457 and US 2003/0049620. In some instances, such nucleic acid assays have used multicolor optical coding technology in combination with nucleic acid hybridization technology to specifically identify a particular nucleic acid species within a complex populations of nucleic acids. See, for example, U.S. Pat. Application Publication No. US 2002/0009728; Spiro et al. (2002) *Appl. Environ. Microbiol.* 68:1010-1013; Han et al. (2001) *Nat. Biotechnol.* 19:631-635.

[0004] There continues to be need in the arts for specific, sensitive methods of analyzing samples containing a complex population of nucleic acids for particular nucleic acids. Also needed are such methods which can be performed easily, rapidly, and in a high throughput manner.

[0005] All publications and patent applications cited herein are hereby incorporated by reference in their entirety.

DISCLOSURE OF THE INVENTION

[0006] The present invention provides a homogeneous, multiplexed assay for determination of the presence of nucleic acid target sequences in a sample. The method of the present invention is particularly suitable for the simultaneous detection of multiple nucleic acid target sequences in a single sample with a minimum of physical manipulations. The nucleic acid target sequences can be the products of an amplification reaction ("amplicons").

[0007] The assay utilizes specific capture probe conjugates having distinguishable spectral signatures and reporter probes. The reporter probes are selectively labeled with a detectable reporter moiety having a signal that is distinguishable from that of the capture probe conjugates. The capture probe conjugates and the reporter probe are capable of specific hybridization to the nucleic acid target sequence. In one aspect, a method is provided comprising contacting a sample suspected of containing the nucleic acid target sequence with a capture probe conjugate, a reporter probe and a detectable reporter moiety under conditions that allow hybridization of the capture probe conjugate and the reporter probe to the nucleic acid target sequence, and association of the detectable reporter moiety with the reporter probe. In a preferred embodiment, multiple capture probe conjugates and multiple reporter probes are used allowing the detection of multiple nucleic acid target sequences simultaneously.

[0008] In the method of the present invention, the capture probe conjugate, the reporter probe, the detectable reporter moiety, and the sample suspected of containing the nucleic acid target sequence are combined. A plurality of targets can be detected simultaneously by combining a capture probe conjugate and reporter probe for each desired target polynucleotide. In the presence of nucleic acid target sequence, a complex is formed including the nucleic acid target sequence, capture probe conjugate, reporter probe and detectable reporter moiety. In the absence of nucleic acid target sequence, no complex is formed. Each complex is analyzed individually for the presence of the spectral signature of the capture probe in association with the signal from the detectable reporter moiety.

[0009] The invention described herein is useful for any assay in which a sample can be interrogated regarding an amplification product from a target polynucleotide. Typical assays involve determining the presence of the amplification product in the sample or its relative amount, or the assays may be quantitative or semi-quantitative. Results from such assays can be used to determine the presence or amount of the target polynucleotide originally present in the sample.

[0010] Kits comprising reagents useful for performing the methods of the invention are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] Fig. 1 depicts a single complex formed from capture probe conjugate, nucleic acid target sequence, reporter probe and detectable reporter moiety.

[0012] Fig. 2 shows representative results of a multiplex assay for HAV, HBV, HCV, HIVa, HIVb and Parvovirus B19 nucleic acid target sequences.

[0013] Fig. 3A-3F depicts sequences of capture probe and reporter probe for various viral targets and control.

[0014] Fig. 4A-4B shows the results of multiplex analyses. Beadset 10: HAV capture probe; Beadset 20: HBV capture probe; Beadset 30: HCV capture probe; Beadset 40: HIVa capture probe; Beadset 50: HIVb capture probe; Beadset 60; Parvo B19 capture probe; Beadset 200: control.

MODES OF CARRYING OUT THE INVENTION

[0015] Before the present invention is described in detail, it is to be understood that this invention is not limited to the particular methodology, devices, solutions or apparatuses described, as such methods, devices, solutions or apparatuses can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention.

[0016] Unless defined otherwise or the context clearly dictates otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods and materials are now described.

[0017] All publications mentioned herein are hereby incorporated by reference for the purpose of disclosing and describing the particular materials and methodologies for which the reference was cited. The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

[0018] In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

[0019] Use of the singular forms "a," "an," and "the" include plural references unless the context clearly dictates otherwise. Thus, for example, reference to "a nucleic acid target sequence" includes a plurality of target polynucleotides, reference to "a substrate" includes a plurality of such substrates, reference to "a capture probe" includes a plurality of capture probes, and the like.

[0020] Terms such as "connected," "attached," "linked," and "conjugated" are used interchangeably herein and encompass direct as well as indirect connection, attachment, linkage or conjugation unless the context clearly dictates otherwise. Where a range of values is recited, it is to be understood that each intervening integer value, and each fraction thereof, between the recited upper and lower limits of that range is also specifically disclosed, along with each subrange between such values. The upper and lower limits of any range can independently be included in or excluded from the range, and each range where either, neither or both limits are included is also encompassed within the invention. Where a value being discussed has inherent limits, for example where a component can be present at a concentration of from 0 to 100%, or where the pH of an aqueous solution can range from 1 to 14, those inherent limits are specifically disclosed. Where a value is explicitly recited, it is to be understood that values which are about the same quantity or amount as the recited value are also within the scope of the invention. Where a combination is disclosed, each subcombination of the elements of that combination is also specifically disclosed and is within the scope of the invention. Conversely, where different elements or groups of elements are disclosed, combinations thereof are also disclosed. Where any element of an invention is disclosed as having a plurality of alternatives, examples of that invention in which each alternative is excluded singly or in any combination with the other alternatives are also hereby disclosed; more than one element of an invention can have such exclusions, and all combinations of elements having such exclusions are hereby disclosed.

[0021] The terms "semiconductor nanocrystal," "SCNC," "quantum dot" are used interchangeably herein and refer to an inorganic crystallite of about 1 μm or more and about 1000 nm or less in diameter or any integer or fraction of an integer therebetween, preferably at least about 2 nm and about 50 nm or less in diameter or any integer or fraction of an integer therebetween, more preferably at least about 2 nm and about 20 nm or less in diameter (for example about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nm). SCNCs

are characterized by their uniform nanometer size. An SCNC is capable of emitting electromagnetic radiation upon excitation (i.e., the SCNC is luminescent) and includes a "core" of one or more first semiconductor materials, which may be surrounded by a "shell" of a second semiconductor material. An SCNC core surrounded by a semiconductor shell is referred to as a "core/shell" SCNC. The surrounding "shell" material will preferably have a bandgap energy that is larger than the bandgap energy of the core material and may be chosen to have an atomic spacing close to that of the "core" substrate. The core and/or the shell can be a semiconductor material including, but not limited to, those of the group II-VI (ZnS, ZnSe, ZnTe, CdS, CdSe, CdTe, HgS, HgSe, HgTe, MgS, MgSe, MgTe, CaS, CaSe, CaTe, SrS, SrSe, SrTe, BaS, BaSe, BaTe, and the like) and III-V (GaN, GaP, GaAs, GaSb, InN, InP, InAs, InSb, and the like) and IV (Ge, Si, and the like) materials, Pb, PbS, PbSe, and an alloy or a mixture thereof. Preferred shell materials include CdS and ZnS.

[0022] An SCNC is optionally surrounded by a "coat" of an organic capping agent. The organic capping agent may be any number of materials, but has an affinity for the SCNC surface. In general, the capping agent can be an isolated organic molecule, a polymer (or a monomer for a polymerization reaction), an inorganic complex, or an extended crystalline structure. The coat can be used to convey solubility, e.g., the ability to disperse a coated SCNC homogeneously into a chosen solvent, functionality, binding properties, or the like. In addition, the coat can be used to tailor the optical properties of the SCNC.

[0023] Thus, the terms "semiconductor nanocrystal," "SCNC," "quantum dot" as used herein include a coated SCNC core, as well as a core/shell SCNC. SCNC are described in e.g., U.S. Pat. Nos. 5,990,479, 6,207,229, 6,319,426, 6,322,901, 6,251,303.

[0024] The terms "polynucleotide," "oligonucleotide," "nucleic acid" and "nucleic acid molecule" are used interchangeably herein to refer to a polymeric form of nucleotides of any length, and may comprise ribonucleotides, deoxyribonucleotides, analogs thereof, or mixtures thereof. This term refers only to the primary structure of the molecule. Thus, the term includes triple-, double- and single-stranded deoxyribonucleic acid ("DNA"), as well as triple-, double- and single-stranded ribonucleic acid ("RNA"). It also includes modified, for example by alkylation, and/or by capping, and unmodified forms of the polynucleotide. More particularly, the terms "polynucleotide," "oligonucleotide," "nucleic acid" and "nucleic acid molecule" include polydeoxyribonucleotides (containing 2-deoxy-D-ribose), polyribonucleotides (containing D-ribose), including tRNA, rRNA, hRNA, and mRNA, whether spliced or unspliced, any other type of polynucleotide which is an N- or C-glycoside

of a purine or pyrimidine base, and other polymers containing normucleotidic backbones, for example, polyamide (e.g., peptide nucleic acids ("PNAs")) and polymorpholino (commercially available from the Anti-Virals, Inc., Corvallis, OR., as Neugene) polymers, and other synthetic sequence-specific nucleic acid polymers providing that the polymers contain nucleobases in a configuration which allows for base pairing and base stacking, such as is found in DNA and RNA. There is no intended distinction in length between the terms "polynucleotide," "oligonucleotide," "nucleic acid" and "nucleic acid molecule," and these terms are used interchangeably herein. These terms refer only to the primary structure of the molecule. Thus, these terms include, for example, 3'-deoxy-2',5'-DNA, oligodeoxyribonucleotide N3' P5' phosphoramidates, 2'-O-alkyl-substituted RNA, double- and single-stranded DNA, as well as double- and single-stranded RNA, and hybrids thereof including for example hybrids between DNA and RNA or between PNAs and DNA or RNA, and also include known types of modifications, for example, labels, alkylation, "caps," substitution of one or more of the nucleotides with an analog, intemucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoramidates, carbamates, etc.), with negatively charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), and with positively charged linkages (e.g., aminoalkylphosphoramidates, aminoalkylphosphotriesters), those containing pendant moieties, such as, for example, proteins (including enzymes (e.g. nucleases), toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelates (of, e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide or oligonucleotide.

[0025] It will be appreciated that, as used herein, the terms "nucleoside" and "nucleotide" will include those moieties which contain not only the known purine and pyrimidine bases, but also other heterocyclic bases which have been modified. Such modifications include methylated purines or pyrimidines, acylated purines or pyrimidines, or other heterocycles. Modified nucleosides or nucleotides can also include modifications on the sugar moiety, e.g., wherein one or more of the hydroxyl groups are replaced with halogen, aliphatic groups, or are functionalized as ethers, amines, or the like. The term "nucleotidic unit" is intended to encompass nucleosides and nucleotides.

[0026] Furthermore, modifications to nucleotidic units include rearranging, appending, substituting for or otherwise altering functional groups on the purine or pyrimidine base which

form hydrogen bonds to a respective complementary pyrimidine or purine. The resultant modified nucleotidic unit optionally may form a base pair with other such modified nucleotidic units but not with A, T, C, G or U. Abasic sites may be incorporated which do not prevent the function of the polynucleotide. Some or all of the residues in the polynucleotide can optionally be modified in one or more ways.

[0027] Standard A-T and G-C base pairs form under conditions which allow the formation of hydrogen bonds between the N3-H and C4-oxy of thymidine and the N1 and C6-NH_2, respectively, of adenosine and between the C2-oxy, N3 and C4-NH_2, of cytidine and the C2-NH_2, N'-H and C6-oxy, respectively, of guanosine. Thus, for example, guanosine (2-amino-6-oxy-9-O-D-ribofuran- osyl-purine) may be modified to form isoguanosine (2-oxy-6-amino-9-β-- D-ribofuranosyl-purine). Such modification results in a nucleoside base which will no longer effectively form a standard base pair with cytosine. However, modification of cytosine (1-β-D-ribofuranosyl-2-oxy-4-amino-pyrimidine) to form isocytosine (1-β-D-ribofuranosyl-2-amino-4-oxy-pyrimidine) results in a modified nucleotide which will not effectively base pair with guanosine but will form a base pair with isoguanosine (U.S. Pat. No. 5,681,702). Isocytosine is available from Sigma Chemical Co. (St. Louis, Mo.); isocytidine may be prepared by the method described by Switzer et al. (1993) *Biochemistry* 32:10489-10496 and references cited therein; 2'-deoxy-5-methyl-isocytidine may be prepared by the method of Tor et al. (1993) *J. Am. Chem. Soc.* 115:4461-4467 and references cited therein; and isoguanine nucleotides may be prepared using the method described by Switzer et al. (1993), supra, and Mantsch et al. (1993) *Biochem.* 14:5593-5601, or by the method described in U.S. Pat. No. 5,780,610. Other normatural base pairs may be synthesized by the method described in Piccirilli et al. (1990) *Nature* 343:33-37 for the synthesis of 2,6-diaminopyrimidine and its complement (1-methylpyrazolo-[4,3]pyrimidine-5,7-(4H,6H)-dione). Other such modified nucleotidic units which form unique base pairs are known, such as those described in Leach et al. (1992) *J. Am. Chem. Soc.* 114:3675-3683 and Switzer et al., supra.

[0028] "Nucleic acid probe" and "probe" are used interchangeably and refer to a structure comprising a polynucleotide, as defined above, that contains a nucleic acid sequence that can bind to a corresponding target. The polynucleotide regions of probes may be composed of DNA, and/or RNA, and/or synthetic nucleotide analogs.

[0029] "Complementary" or "substantially complementary" refers to the ability to hybridize or base pair between nucleotides or nucleic acids, such as, for instance, between the two strands of a double stranded DNA molecule or between a polynucleotide primer and a

primer binding site on a single stranded nucleic acid to be sequenced or amplified. Complementary nucleotides are, generally, A and T (or A and U), or C and G. Two single-stranded RNA or DNA molecules are said to be substantially complementary when the nucleotides of one strand, optimally aligned and compared and with appropriate nucleotide insertions or deletions, pair with at least about 80% of the nucleotides of the other strand, usually at least about 90% to 95%, and more preferably from about 98 to 100%.

[0030] Alternatively, substantial complementarity exists when an RNA or DNA strand will hybridize under selective hybridization conditions to its complement. Typically, selective hybridization will occur when there is at least about 65% complementary over a stretch of at least 14 to 25 nucleotides, preferably at least about 75%, more preferably at least about 90% complementary. See, M. Kanehisa *Nucleic Acids Res.* 12:203 (1984).

[0031] "Preferential binding," "preferential hybridization," or "specifically hybridization" refers to the increased propensity of one polynucleotide or capture probe or reporter probe to bind to a complementary target polynucleotide in a sample as compared to noncomplementary polynucleotides in the sample or as compared to the propensity of the one polynucleotide to form an internal secondary structure such as a hairpin or stem-loop structure under at least one set of hybridization conditions.

[0032] Stringent hybridization conditions will typically include salt concentrations of less than about 1M, more usually less than about 500 mM and preferably less than about 200 mM. Hybridization temperatures can be as low as 5°C., but are typically greater than 22°C., more typically greater than about 30°C., and preferably in excess of about 37°C. Longer fragments may require higher hybridization temperatures for specific hybridization. Other factors may affect the stringency of hybridization, including base composition and length of the complementary strands, presence of organic solvents and extent of base mismatching, and the combination of parameters used is more important than the absolute measure of any one alone. Other hybridization conditions which may be controlled include buffer type and concentration, solution pH, presence and concentration of blocking reagents to decrease background binding such as repeat sequences or blocking protein solutions, detergent type(s) and concentrations, molecules such as polymers which increase the relative concentration of the polynucleotides, metal ion(s) and their concentration(s), chelator(s) and their concentrations, and other conditions known in the art. Less stringent, and/or more physiological, hybridization conditions are used where a labeled polynucleotide amplification product cycles on and off a substrate linked to a capture probe during a real-time assay which is monitored during PCR

amplification such as a molecular beacon assay. Such less stringent hybridization conditions can also comprise solution conditions effective for other aspects of the method, for example reverse transcription or PCR.

[0033] The terms "substrate" and "support" are used interchangeably and refer to a material having a rigid or semi-rigid surface.

[0034] As used herein, the term "binding pair" refers to first and second molecules that bind specifically to each other with greater affinity than to other components in the sample. The binding between the members of the binding pair is typically noncovalent. Exemplary binding pairs include immunological binding pairs (e.g., any haptenic or antigenic compound in combination with a corresponding antibody or binding portion or fragment thereof, for example digoxigenin and anti-digoxigenin, fluorescein and anti-fluorescein, dinitrophenol and anti-dinitrophenol, bromodeoxyuridine and anti-bromodeoxyuridine, mouse immunoglobulin and goat anti-mouse immunoglobulin) and nonimmunological binding pairs (e.g., biotin-avidin, biotin-streptavidin, hormone [e.g., thyroxine and cortisol]-hormone binding protein, receptor-receptor agonist or antagonist (e.g., acetylcholine receptor-acetylcholine or an analog thereof) IgG-protein A, lectin-carbohydrate, enzyme-enzyme cofactor, enzyme-enzyme-inhibitor, and complementary polynucleotide pairs capable of forming nucleic acid duplexes) and the like. One or both member of the binding pair can be conjugated to additional molecules.

[0035] The term "antibody" as used herein includes antibodies obtained from both polyclonal and monoclonal preparations, as well as: hybrid (chimeric) antibody molecules (see, for example, Winter et al. (1991) *Nature* 349:293-299; and U.S. Pat. No. 4,816,567); F(ab')2 and F(ab) fragments; Fv molecules (noncovalent heterodimers, see, for example, Inbar et al. (1972) *Proc. Natl. Acad. Sci. USA* 69:2659-2662; and Ehrlich et al. (1980) *Biochem.* 19:4091-4096); single-chain Fv molecules (sFv) (see, for example, Huston et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883); dimeric and trimeric antibody fragment constructs; minibodies (see, e.g., Pack et al. (1992) *Biochem.* 31:1579-1584; Cumber et al. (1992) *J. Immunol.* 149B:120-126); humanized antibody molecules (see, for example, Riechmann et al. (1988) *Nature* 332:323-327; Verhoeyan et al. (1988) *Science* 239:1534-1536; and U.K. Patent Publication No. GB 2,276,169, published Sep. 21, 1994); and, any functional fragments obtained from such molecules, wherein such fragments retain specific-binding properties of the parent antibody molecule.

[0036] A "homogeneous assay" is one that is performed without transfer, separation or washing steps. Thus, for example, a homogeneous high-throughput screening ("HTS") assay involves the initial addition of reagents to a vessel, e.g., a test tube or sample well, followed by the detection of the results from that vessel. A homogeneous HTS assay can be performed anywhere in the vessel, for example in the solution, on the surface of the vessel or on beads or surfaces placed in the vessel. The detection system typically used is a fluorescence, chemiluminescence, or scintillation detection system.

[0037] "Multiplexing" or "multiplex assay" herein refers to an assay or other analytical method in which the presence of multiple nucleic acid target sequences can be assayed simultaneously by using more than one capture probe conjugate, each of which has at least one different detection characteristic, e.g., fluorescence characteristic (for example excitation wavelength, emission wavelength, emission intensity, FWHM (full width at half maximum peak height), or fluorescence lifetime).

[0038] The assays of the present invention may be implemented in a multiplex format. Multiplex methods are provided employing 2, 3, 4, 5, 10, 15, 20, 25, 50, 100, 200, 500, 1000 or more different capture probes which can be used simultaneously to assay for amplification products from corresponding different target polynucleotides. In some embodiments, multiplex methods can also be used to assay for nucleic acid target sequences which have not undergone an amplification procedure. Methods amenable to multiplexing, such as those taught herein, allow acquisition of greater amounts of information from smaller specimens. The need for smaller specimens increases the ability of an investigator to obtain samples from a larger number of individuals in a population to validate a new assay or simply to acquire data, as less invasive techniques are needed.

[0039] Where different substrates are included in a multiplex assay as part of the capture probe conjugates, the different substrates can be encoded so that they can be distinguished. Any encoding scheme can be used; conveniently, the encoding scheme can employ one or more different fluorophores, which can be fluorescent semiconductor nanocrystals. High density spectral coding schemes can be used.

[0040] One or more different populations of spectrally encoded capture probe conjugates can be created, each population comprising one or more different capture probes attached to a substrate comprising a known or determinable spectral code comprising one or more semiconductor nanocrystals or fluorescent nanoparticle. Different populations of the conjugates, and thus different assays, can be blended together, and the assay can be performed

in the presence of the blended populations. The individual conjugates are scanned for their spectral properties, which allows the spectral code to be decoded and thus identifies the substrate, and therefore the capture probe(s) to which it is attached. Because of the large number of different semiconductor nanocrystals and fluorescent nanoparticles and combinations thereof which can be distinguished, large numbers of different capture probes and amplification products can be simultaneously interrogated.

[0041] In one aspect, the invention provides a method for detection of a nucleic acid target sequence in a sample comprising:

(a) contacting said sample with a capture probe conjugate, a reporter probe and a detectable reporter moiety, under conditions allowing the formation of a complex including said a nucleic acid target sequence, if present, said capture probe conjugate, said reporter probe and said detectable reporter moiety,

wherein said capture probe conjugate and said reporter probe each comprise an oligonucleotide that is capable of specifically hybridizing to said nucleic acid target sequence and wherein said capture probe conjugate and said reporter probe do not hybridize to the same or overlapping regions of said nucleic acid target sequence,

wherein said capture probe conjugate comprises a substrate having a distinguishable spectral signature that uniquely identifies the capture probe conjugate,

wherein said reporter probe comprises one member of a binding pair and said detectable reporter moiety comprises the other member of said binding pair,

and wherein said detectable reporter moiety comprises a detectable label; and

(b) detecting the signal individually from each complex so formed.

[0042] Formation of the complex indicative of the presence of the target polynucleotide is measured by the coincidence of the signal from the detectable reporter moiety and the spectral signal from the capture probe conjugate in a single complex.

[0043] In a preferred embodiment of the invention is provided a method for detection of the presence of a plurality of nucleic acid target sequences in a sample, said method comprising

(a) contacting said sample, in a single vessel, with a plurality of capture probe conjugates, a plurality of reporter probes and a detectable reporter moiety, wherein each member of said plurality of capture probe conjugates is specific for one member of said plurality of said target sequences (the specific capture probe conjugate) and each member of said plurality of reporter probes is specific for one member of said plurality of target sequences

(the specific reporter probe), under conditions allowing the formation of a specific complex including said a nucleic acid target sequence, if present, said specific capture probe conjugate, said specific reporter probe and said detectable reporter moiety,

wherein each capture probe conjugate of said plurality of capture probe conjugates and each reporter probe of said plurality of reporter probes each comprise an oligonucleotide that is capable of specifically hybridizing to one member of said plurality of nucleic acid target sequences and wherein said capture probe conjugates and said reporter probes do not hybridize to the same or overlapping regions of said nucleic acid target sequence,

wherein said capture probe conjugates each comprise a substrate having a distinguishable spectral signature that uniquely identifies each capture probe conjugate,

wherein said reporter probes comprise one member of a binding pair and said detectable reporter moiety comprises the other member of said binding pair,

and wherein said detectable reporter moiety comprises a detectable label; and

(b) detecting the signal individually from each specific complex so formed.

[0044] In a further aspect the method provides that the sample is the product of an amplification reaction, wherein the amplification reaction utilizes one or more oligonucleotide primers in a process of enzymatic extension of said primer(s) to produce multiple copies of a nucleic acid target sequence. In this aspect, the capture probe conjugate and the reporter probe are designed so as not to hybridize to the oligonucleotide primers used in the amplification reaction.

[0045] The capture probe conjugate comprises a capture probe and a detectable substrate. A detectable substrate that produces a different distinguishable spectral signal is used for each different capture probe. The capture probe conjugates are provided by binding the capture probe, covalently or non-covalently, to the detectable substrate. A number of such detectable substrates are available (for example, from Luminex Corp, Austin, TX or Quantum Dot Corp., Hayward, CA). These detectable substrates include fluorescent semiconductor nanocrystals, also called quantum dots (for example, as described in U.S. Pat. Nos. 5,990,479, 6,139,426, 6,322,901, 6,207,229, 6,251,303, 6,576,291), quantum beads which are fluorescent microspheres that comprise variable mixtures of quantum dots to provide a large variety of distinguishable emission signals (for example, as described in U.S. Pat. Nos. 6,207,392 and 6,500,622) and fluorescent polymeric microspheres (for example, as described in U.S. Pat. Nos. 6,632,526, 6,649,414, 6,599,331).

[0046] The capture probe is a single stranded oligonucleotide that is capable of hybridizing specifically to a particular nucleic acid target sequence. The sequence of the capture probe will be sufficiently complementary to the sequence of the nucleic acid target sequence to permit specific hybridization under the conditions of the assay described herein. The length of the capture probe can vary and is often from about 5-100 nucleotides in length. Typically, the capture probe is between about 8 and about 60 nucleotides in length. Preferably, the capture probe is between about 20 and about 30 nucleotides in length. Methods of synthesizing oligonucleotides are well known in the art and the capture probe can be synthesized by any convenient method. The capture probe can be attached to the detectable substrate by any appropriate method. Numerous methods for coupling oligonucleotides to such substrates have been described, for example, in U.S. Pat. Nos. 5,990,479, 6,207,392, 6,423,551, 6,251,303, 6,319,426, 6,426,513, 6,444,143, 4,181,636, 6,649,414, Xu et al. (2003) *Nucleic Acids Res.* Vol. 31 e 43, Chan et al. (2002) *Curr. Opin. Biotech.* 13:40, Smith et al. (1998) *Clin. Chem.* 44:2054, Spiro et al. (2000) *Applied Environ. Microbiol.* 66:4258. The capture probe can be linked directly or indirectly to the substrate. The capture probe can be linked to the substrate at any point in the oligonucleotide which allows its use under assay conditions.

[0047] Where the capture probe is a polynucleotide, it can be synthesized directly on the substrate, or can be synthesized separately from the substrate and then coupled to it. Direct synthesis on the substrate may be accomplished by incorporating a monomer that is coupled to a subunit of the capture probe into a polymer that makes up or is deposited on or coupled to the substrate, and then synthesizing the remainder of the capture probe to incorporate that subunit. Alternatively, the substrate or its coating may include or be derivatized to include a functional group which can be coupled to a subunit of the capture probe for synthesis, or may be coupled directly to the complete capture probe. Suitable coupling techniques are known in the art. The length of the capture probe polynucleotide is not critical, but typically is from 5-100 nucleotides in length and is chosen to provide suitably specific hybridization to the target polynucleotide.

[0048] In a preferred embodiment, the substrate can be in the form of a microsphere. Polymeric microspheres or beads can be prepared from a variety of different polymers, including but not limited to polystyrene, cross-linked polystyrene, polyacrylic, polylactic acid, polyglycolic acid, poly(lactide coglycolide), polyanhydrides, poly(methyl methacrylate), poly(ethylene-co-vinyl acetate), polysiloxanes, polymeric silica, latexes, dextran polymers and epoxies. The materials have a variety of different properties with regard to swelling and

porosity, which are well understood in the art. Preferably, the beads are in the size range of approximately 10 nm to 1 mm, and can be manipulated using normal solution techniques when suspended in a solution. The terms "bead," "sphere," "microbead" and "microsphere" are used interchangeably herein.

[0049] The reporter probe is a single stranded oligonucleotide that is capable of hybridizing specifically to a particular nucleic acid target sequence. The sequence of the reporter probe will be sufficiently complementary to the sequence of the nucleic acid target sequence to permit specific hybridization under the conditions of the assay described herein. The length of the reporter probe can vary and is often from about 5-100 nucleotides in length. Typically, the reporter probe is between about 8 and about 60 nucleotides in length. Preferably, the reporter probe is between about 20 and about 30 nucleotides in length. Methods of synthesizing oligonucleotides are well known in the art and the reporter probe can be synthesized by any convenient method.

[0050] In addition, the reporter probe will comprise one member of a binding pair. The other member of the binding pair will be associated with the detectable reporter moiety. A "binding pair" includes any two molecules, macromolecules or other moieties that bind each other with high affinity and specificity. Many such binding pairs are well known, for example antigen and antibody, receptor and ligand, biotin and avidin/streptavidin. Preferably, the reporter probe will comprise a biotin moiety and the detectable reporter moiety will comprise an avidin or streptavidin attached to a detectable label, preferably a fluorescent label. The detectable reporter moiety is preferably a streptavidin-phycoerythrin conjugate. Such detectable reporter moieties are well known and commercially available (e.g., from Molecular Probes, Inc.).

[0051] Typically, the nucleic acid target sequence is an amplified sequence ("amplicon"), although in some instances, unamplified sequences may be suitable, e.g., where the nucleic acid target sequences are present in high concentration without amplification. Amplification of the nucleic acid target sequence can be accomplished using any of the well known nucleic acid amplification methods, including polymerase chain reaction (PCR), RT-PCR, transcription mediated amplification (TMA), Nucleic Acid Sequence Based Amplification (NASBA). The PCR method for amplifying target nucleic acid sequences in a sample is well known in the art and has been described in, e.g., Innis et al. (eds.) *PCR Protocols* (Academic Press, NY 1990); Taylor (1991) *Polymerase chain reaction: basic principles and automation,* in *PCR: A Practical Approach*, McPherson et al. (eds.) IRL Press, Oxford; Saiki et al. (1986) *Nature*

324:163; as well as in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,889,818, all incorporated herein by reference in their entireties. RNAs may be amplified by reverse transcribing the mRNA into cDNA, and then performing PCR (RT-PCR), as described above. Alternatively, a single enzyme may be used for both steps as described in U.S. Pat. No. 5,322,770. mRNA may also be reverse transcribed into cDNA, followed by asymmetric gap ligase chain reaction (RT-AGLCR) as described by Marshall et al. (1994) *PCR Meth. App.* 4:80-84. TMA is described in detail in, e.g., U.S. Pat. No. 5,399,491, the disclosure of which is incorporated herein by reference in its entirety.

[0052] The reporter probe and the capture probe are complementary to and hybridize with non-overlapping regions of the nucleic acid target sequence. Thus, there is no competition between the capture probe and the reporter probe for hybridization to the nucleic acid target sequence and the capture probe and the reporter probes can both hybridize with the nucleic acid target sequence simultaneously. In addition, the reporter probe and the capture probe are designed so as not to hybridize with any amplification primers that may have been used in the amplification reaction to provide the nucleic acid target sequence. This eliminates any competition between the amplicon and the unextended amplification primer for binding to the capture probe or the reporter probe.

[0053] The method of the present invention is most suitable for use in a homogeneous multiplex analysis of multiple nucleic acid target sequences in a sample. Multiple nucleic acid target sequences can be generated by amplification of a sample by multiple amplification oligonucleotide primers or sets of primers, each primer or set of primers specific for amplifying a particular nucleic acid target sequence. For example, a sample can be analyzed for the presence of multiple viral nucleic acid target sequences by amplification with primers specific for amplification of each of multiple viral target sequences, including, e.g., human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis A virus (HAV), parvovirus B19, West Nile Virus, hantavirus, severe acute respiratory syndrome-associated coronavirus (SARS), etc. It will be apparent that only those nucleic acid target sequences that are present in the sample will be amplified to produce amplicons. The amplification reactions of the multiple target sequences can be performed simultaneously in a single reaction vessel. An aliquot of the products of the amplification reaction is transferred to a second reaction vessel for contacting capture probe conjugates and reporter probes specific for each amplified nucleic acid target sequence. The capture probe conjugates, the reporter probes and the detectable reporter moiety are transferred to the second reaction vessel,

separately or in any combination. The capture probe conjugates, the reporter probes and the detectable reporter moiety can be added to the second reaction vessel in any order. The capture probe conjugates, the reporter probes and the detectable reporter moiety can be added to the second reaction vessel before or after the nucleic acid target sequences.

[0054] The capture probe conjugates, the reporter probes, the detectable reporter moiety and the sample containing (or suspected to contain) the nucleic acid target sequences (e.g., an aliquot of the amplification reaction) are incubated under conditions that allow the formation of a complex. When a particular nucleic acid target sequence is present, a complex forms as the capture probe conjugate specific for that target sequence hybridizes to the target, the reporter probe specific for that target sequence also hybridizes to the target and the detectable reporter moiety binds to the reporter probe by the association of the binding pair members. The nucleic acid target sequence forms the link between the capture probe conjugate and the reporter probe (with the detectable reporter moiety).

[0055] Typical conditions for formation of the complex are incubation at 55°C for 30 minutes in 1X tetramethylammonium chloride (TMAC) (3M TMAC, 0.1% Sarkosyl, 50mM TrispH 8, 4mM EDTA) or HEPES buffer (10 mM HEPES(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic]) acid), 0.3M NaCl, pH 7.5), but one of ordinary skill in the art could readily determine other suitable incubation conditions. Where the nucleic acid target is a double stranded polynucleotide, the target is typically subjected to conditions that will denature and separate the strands (e.g., heating at about 98 °C) prior to the annealing reaction.

[0056] After incubation, the sample to be analyzed is subjected to analysis by flow cytometry or other instrumentation that uses microfluidics to align the complexes (as well as the uncomplexed components) into a single file so that the complexes can be analyzed individually. Suitable instrumentation includes the Luminex 100 system or the Becton Dickinson FACScan®. Generally, an array of the complexes can be scanned with a laser scanner having an excitation source and emission filters appropriate for the particular capture probe conjugate substrate (e.g., SCNC(s)) and other fluorophore or reporter label used. Alternatively, the array can be scanned with a wide-field imaging scanner having appropriate excitation and emission filters. Because the capture probe conjugates produce a distinguishable signature signal for each type of capture probe, the coincidence of capture probe signature signal and detectable reporter moiety signal from a single complex is indicative of the presence of nucleic acid target sequence specific for that capture probe.

[0057] Methods of the invention can be implemented in a homogeneous format. This allows for higher assay throughput due to fewer manipulations of the sample, and decreased cross-contamination resulting in more reliable assays and less downtime from cross-contamination. If real time monitoring is used, the entire assay can be disposed of without opening a sealed assay chamber such as a sealed microplate, thus further decreasing the risk of cross-contamination.

[0058] The portion of the sample comprising or suspected of comprising the target polynucleotide can be any source of biological material which comprises polynucleotides that can be obtained from a living organism directly or indirectly, including cells, tissue or fluid, and the deposits left by that organism, including viruses, mycoplasma, and fossils. The sample can also comprise a target polynucleotide prepared through synthetic means, in whole or in part. Typically, the sample is obtained as or dispersed in a predominantly aqueous medium. Nonlimiting examples of the sample include blood, plasma, urine, semen, milk, sputum, mucus, a buccal swab, a vaginal swab, a rectal swab, an aspirate, a needle biopsy, a section of tissue obtained for example by surgery or autopsy, plasma, serum, spinal fluid, lymph fluid, the external secretions of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, tumors, organs, samples of *in vitro* cell culture constituents (including but not limited to conditioned medium resulting from the growth of cells in cell culture medium, putatively virally infected cells, recombinant cells, and cell components), and a recombinant source, e.g., a library, comprising polynucleotide sequences.

[0059] The sample can be a positive control sample which is known to contain the target polynucleotide or a surrogate therefor. A negative control sample can also be used which, although not expected to contain the target polynucleotide, is suspected of containing it, and is tested in order to confirm the lack of contamination by the target polynucleotide of the reagents used in a given assay, as well as to determine whether a given set of assay conditions produces false positives (a positive signal even in the absence of target polynucleotide in the sample).

[0060] The sample can be diluted, dissolved, suspended, extracted or otherwise treated to solubilize and/or purify any target polynucleotide present or to render it accessible to reagents which are used in an amplification scheme or to detection reagents. Where the sample contains cells, the cells can be lysed or permeabilized to release the polynucleotides within the cells. One step permeabilization buffers can be used to lyse cells which allow further steps to be performed directly after lysis, for example a polymerase chain reaction.

[0061] In addition to detecting viral agents or measuring viral load in a sample, methods of the invention can be used to detect the presence of other infectious agents or pathogens in a sample (e.g., bacteria, yeast, parasites). Methods of the invention can also be used to identify and/or study gene variations or mutations. Accordingly, the methods can be used to detect nucleotide sequences associated with increased risk of diseases or disorders, hereditary disorders, drug sensitivity, or drug resistance. Methods of the invention can also be used in forensic applications (e.g., to identify the source of a forensic sample), transplant compatibility testing, paternity testing, in anthropological settings, and so on.

[0062] The nucleic acid target sequence (or "target polynucleotide") can be single-stranded, double-stranded, or higher order, and can be linear or circular. Exemplary single-stranded target polynucleotides include mRNA, rRNA, tRNA, hnRNA, ssRNA or ssDNA viral genomes, although these polynucleotides may contain internally complementary sequences and significant secondary structure. Exemplary double-stranded target polynucleotides include genomic DNA, mitochondrial DNA, chloroplast DNA, dsRNA or dsDNA viral genomes, plasmids, phage, and viroids. The target polynucleotide can be prepared synthetically or purified from a biological source. The target polynucleotide may be purified to remove or diminish one or more undesired components of the sample or to concentrate the target polynucleotide prior to amplification. Conversely, where the target polynucleotide is too concentrated for a particular assay, the target polynucleotide may first be diluted.

[0063] Following sample collection and optional nucleic acid extraction and purification, the nucleic acid portion of the sample comprising the target polynucleotide can be subjected to one or more preparative reactions. These preparative reactions can include in vitro transcription (IVT), labeling, fragmentation, amplification and other reactions. mRNA can first be treated with reverse transcriptase and a primer, which can be the first primer comprising the target noncomplementary region, to create cDNA prior to detection and/or further amplification; this can be done in vitro with purified mRNA or *in situ*, e.g., in cells or tissues affixed to a slide. Nucleic acid amplification increases the copy number of sequences of interest and can be used to incorporate a label into an amplification product produced from the target polynucleotide using a labeled primer or labeled nucleotide. A variety of amplification methods are suitable for use, including the polymerase chain reaction method (PCR), transcription mediated amplification (TMA), the ligase chain reaction (LCR), self sustained sequence replication (3SR), nucleic acid sequence-based amplification (NASBA), the use of Q Beta replicase, reverse transcription, nick translation, and the like, particularly

where a labeled amplification product can be produced and utilized in the methods taught herein.

[0064] In some embodiments, the nucleic acid target sequence does not have a label directly incorporated in the sequence. When the nucleic acid target sequence is made with a directly incorporated label or so that a label can be directly bound to the nucleic acid target sequence, this label is one which does not interfere with detection of the capture probe conjugate substrate and/or the report moiety label.

[0065] Where the target polynucleotide is single-stranded, the first cycle of amplification forms a primer extension product complementary to the target polynucleotide. If the target polynucleotide is single-stranded RNA, a polymerase with reverse transcriptase is used in the first amplification to reverse transcribe the RNA to DNA, and additional amplification cycles can be performed to copy the primer extension products. The primers for a PCR must, of course, be designed to hybridize to regions in their corresponding template that will produce an amplifiable segment; thus, each primer must hybridize so that its 3' nucleotide is paired to a nucleotide in its complementary template strand that is located 3' from the 3' nucleotide of the primer used to prime the synthesis of the complementary template strand.

[0066] The target polynucleotide is typically amplified by contacting one or more strands of the target polynucleotide with a primer and a polymerase having suitable activity to extend the primer and copy the target polynucleotide to produce a full-length complementary polynucleotide or a smaller portion thereof. Any enzyme having a polymerase activity which can copy the target polynucleotide can be used, including DNA polymerases, RNA polymerases, reverse transcriptases, enzymes having more than one type of polymerase activity. The polymerase can be thermolabile or thermostable. Mixtures of enzymes can also be used. Exemplary enzymes include: DNA polymerases such as DNA Polymerase I ("Pol I"), the Klenow fragment of Pol I, T4, T7, Sequenase™ T7, Sequenase™ Version 2.0 T7, Tub, Taq, Tth, Pfx, Pfu, Tsp, Tfl, Tli and Pyrococcus sp GB-D DNA polymerases; RNA polymerases such as E. coli, SP6, T3 and T7 RNA polymerases; and reverse transcriptases such as AMV, M-MuLV, MMLV, RNAse H minus MMLV (SuperScript™), SuperScript™ II, ThermoScript™, HIV-1, and RAV2 reverse transcriptases. All of these enzymes are commercially available. Exemplary polymerases with multiple specificities include RAV2 and Tli (exo-) polymerases. Exemplary thermostable polymerases include Tub, Taq, Tth, Pfx, Pfu, Tsp, Tfl, Tli and Pyrococcus sp. GB-D DNA polymerases.

[0067] Suitable reaction conditions are chosen to permit amplification of the target polynucleotide, including pH, buffer, ionic strength, presence and concentration of one or more salts, presence and concentration of reactants and cofactors such as nucleotides and magnesium and/or other metal ions, optional cosolvents, temperature, thermal cycling profile for amplification schemes comprising a polymerase chain reaction, and may depend in part on the polymerase being used as well as the nature of the sample. Cosolvents include formamide (typically at from about 2 to about 10%), glycerol (typically at from about 5 to about 10%), and DMSO (typically at from about 0.9 to about 10%). Techniques may be used in the amplification scheme in order to minimize the production of false positives or artifacts produced during amplification. These include "touchdown" PCR, hot-start techniques, use of nested primers, or designing PCR primers so that they form stem-loop structures in the event of primer-dimer formation and thus are not amplified. Techniques to accelerate PCR can be used, for example centrifugal PCR, which allows for greater convection within the sample, and comprising infrared heating steps for rapid heating and cooling of the sample. One or more cycles of amplification can be performed. An excess of one primer can be used to produce an excess of one primer extension product during PCR; preferably, the primer extension product produced in excess is the amplification product to be detected. A plurality of different primers may be used to amplify different regions of a particular polynucleotide within the sample. Where the amplification reaction comprises multiple cycles of amplification with a polymerase, as in PCR, it is desirable to dissociate the primer extension product(s) formed in a given cycle from their template(s). The reaction conditions are therefore altered between cycles to favor such dissociation; typically this is done by elevating the temperature of the reaction mixture, but other reaction conditions can be altered to favor dissociation, for example lowering the salt concentration and/or raising the pH of the solution in which the double-stranded polynucleotide is dissolved. Although it is preferable to perform the dissociation in the amplification reaction mixture, the polynucleotides may be first isolated using any effective technique and transferred to a different solution for dissociation, then reintroduced into an amplification reaction mixture for additional amplification cycles.

[0068] This assay can be multiplexed, i.e., multiple distinct assays can be run simultaneously, by using different pairs of primers directed at different targets, which can be unrelated targets, or different alleles or subgroups of alleles from, or chromosomal rearrangements at, the same locus. This allows the quantitation of the presence of multiple target polynucleotides in a sample (e.g., specific genes in a cDNA library). All that is required

is an ability to uniquely identify the different second polynucleotide extension products in such an assay, through either a unique capture sequence or a unique label.

[0069] Amplified target polynucleotides may be subjected to post-amplification treatments. For example, in some cases, it may be desirable to fragment the amplification products prior to hybridization with a polynucleotide array, in order to provide segments which are more readily accessible and which avoid looping and/or hybridization to multiple capture probes. Fragmentation of the nucleic acids can be carried out by any method producing fragments of a size useful in the assay being performed; suitable physical, chemical and enzymatic methods are known in the art.

[0070] The detectable reporter moiety comprises one member of a binding pair and a label. The label comprises an agent selected from a chromophore, a lumiphore, a fluorophore, a chromogen, a hapten, an antigen, a radioactive isotope, a magnetic particle, a metal nanoparticle such as a gold or silver nanoparticle, a quantum dot and combinations thereof. A fluorophore can be any substance which absorbs light of one wavelength and emits light of a different wavelength. Typical fluorophores include fluorescent dyes, semiconductor nanocrystals, lanthanide chelates, and a green fluorescent protein.

[0071] Exemplary fluorescent dyes include fluorescein, 6-FAM, rhodamine, Texas Red®, tetramethylrhodamine, a carboxyrhodamine, carboxyrhodamine 6G, carboxyrhodol, carboxyrhodamine 110, Cascade Blue®, Cascade Yellow®, coumarin, Cy™2, Cy™3, Cy™3.5, Cy™5, Cy™5.5, Cy-Chrome, phycoerythrin, PerCP™ (peridinin chlorophyll-a Protein), PerCP™-Cy™5.5, JOE (6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein), NED, ROX (5-(and-6)-carboxy-X-rhodamine), HEX, lucifer yellow, Marina Blue®, Oregon Green® 488, Oregon Green® 500, Oregon Green® 514, Alexa Fluor® 350, Alexa Fluor® 430, Alexa Fluor® 488, Alexa Fluor® 532, Alexa Fluor® 546, Alexa Fluor® 568, Alexa Fluor® 594, Alexa Fluor® 633, Alexa Fluor® 647, Alexa Fluor® 660, Alexa Fluor® 680, 7-amino-4-methylcoumarin-3-acetic acid, BODIPY® FL, BODIPY® FL-Br_2, BODIPY® 530/550, BODIPY® 558/568, BODIPY® 564/570, BODIPY® 576/589, BODIPY® 581/591, BODIPY® 630/650, BODIPY® 650/665, BODIPY® R6G, BODIPY® TMR, BODIPY® TR, conjugates thereof, and combinations thereof. Exemplary lanthanide chelates include europium chelates, terbium chelates and samarium chelates.

[0072] Kits comprising reagents useful for performing the methods of the invention are also provided. In one embodiment, a kit comprises a detectable substrate attached to a capture probe and first and second primers for amplifying the target polynucleotide. The substrate can

be an encoded bead comprising a first spectral code comprising a first semiconductor nanocrystal and first fluorescence characteristics. The capture probe can bind to the amplification product produced from the target polynucleotide, and a sample may be assayed for the presence of such a target polynucleotide or amplification product produced therefrom using the components of the kit. The kit may also contain a reporter probe that can bind to the amplification product produced from the target polynucleotide and a detectable reporter moiety.

[0073] The components of the kit are retained by a housing. Instructions for using the kit to perform a method of the invention are provided with the housing, and may be located inside the housing or outside the housing, and may be printed on the interior or exterior of any surface forming the housing which renders the instructions legible. The kit may be in multiplex form, containing pluralities of one or more different capture probe conjugates, reporter probes and/ or pairs of amplification primers. The substrate may comprise a plurality of polynucleotides of different sequence for performing a plurality of individual assays thereon such as a microarray, or a plurality of different beads can be provided for a multiplexed assay wherein each of the different beads comprises a different capture probe for binding to a corresponding different amplification product.

EXAMPLES

[0074] The following examples are set forth so as to provide those of ordinary skill in the art with a complete description of how to make and use the present invention, and are not intended to limit the scope of what is regarded as the invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental error and deviation should be accounted for. Unless otherwise indicated, parts are parts by weight, temperature is degree centigrade and pressure is at or near atmospheric, and all materials are commercially available.

Example 1

Homogeneous Multiplex Assay for viral nucleic acid targets using Luminex beads.

[0075] The Luminex xMap® System (Luminex Corp, Austin, TX) uses polystyrene microspheres of 5.5 microns in diameter to measure multiple analytes simultaneously in a single reaction vessel. One hundred subsets of microspheres are available, which have been created by impregnating a combination of two fluorescent dyes in different proportions to

produce a unique spectral signature for each subset. One of two lasers is used to identify the bead subset. A second laser is used to identify a fluorescent label. This allows the use of a single fluorescent label (detectable reporter moiety) associated with different reporter probes in conjunction with defined subsets of capture probe conjugates for different targets for multiplexing. By using streptavidin-coupled fluorescent label for the detectable reporter moiety, and biotinylated oligonucleotide for reporter probe, the technology can be easily applied to a wide range of targets.

[0076] For each target that one desires to detect, thousands of oligonucleotides (capture probes) are conjugated to the surface of a subset of the microspheres having a particular spectral signature. As shown in Fig. 1, the capture probes and reporter probes hybridize to different regions of the target sequence. The fluorescent label of the detectable reporter moiety in turn binds to the reporter probe to signal the extent of the reaction.

[0077] To perform an assay, the capture probe conjugate, reporter probe, fluorescent reporter moiety and the sample containing the target sequences are combined. This mixture is then injected into an instrument that uses microfluidics to align the microspheres in a single file where lasers illuminate the colors inside and on the surface of each microsphere. Since the lasers score only for the dual feature of capture probe bead fluorescence and associated reporter moiety fluorescent marker this has the advantage of being homogeneous assay. Advanced optics capture the color signals and digitally process them into real-time, quantitative data for each reaction. Utilizing the 100 subsets of microspheres, each subset conjugated to a different capture probe, and target specific reporter probes, this approach has the advantage of being a mutiplexed homogeneous assay.

[0078] Transcription Mediated Amplification (TMA) of HCV and HIV RNA produces RNA amplicons. Capture probes complementary to the RNA amplicons for HCV and HIV are carbodiimide coupled, each to a distinct subset of Luminex carboxylated microspheres according to instructions provided by the supplier (Luminex Corp.).

[0079] Six different subtypes of microspheres are used to couple to capture probes specific for each of the targets in similar fashion to that described above. HAV capture probe was coupled to Luminex beads L100-C110-04 to produce Bead set 10; HBV capture probe was coupled to Luminex beads L100-C120-04 to produce Bead set 20; HCV capture probe was coupled to Luminex beads L100-C130-04 to produce Bead set 30; HIVa capture probe was coupled to Luminex beads L100-C140-04 to produce Bead set 40; HIVb capture probe was coupled to Luminex beads L100-C150-04 to produce Bead set 50; ParvoB19 capture probe

was coupled to Luminex beads L100-C160-04 to produce Bead set 60; Control capture probe was coupled to Luminex beads L100-C200-04 to produce Bead set 20.

[0080] Target sequences for multiplexing studies were generated using the Invitrogen (Carlsbad, CA) RNA amplifier kit or synthetic oligonucleotides corresponding to the regions of interest. The nucleic acid targets corresponded to sequences from HCV, HIV, HBV, Parvovirus B19 and HAV. RNA amplicons to the following corresponding regions of HAV(nt 61-300, GenBank Accession M14707), HBV(nt 2881-3121, GenBank Accession X02763), HCV(nt 121-420, GenBank Accession M62321), HIV-1a (nt 4561-4800, GenBank Accession K034551), Parvo B19(nt 3241-3480, GenBank Accession M13178), and internal control (pBR322- nt 600-820, GenBank Accession J01749) derivative sequence were prepared using the RNA amplifier kit. The sequences of the capture probes, reporter probes, and targets used are shown in Figures 3A-3F.

[0081] The target amplicons, capture probe-microsphere conjugates, biotinylated reporter probes and Streptavidin-phycoerythrin (i.e., detectable reporter moiety) were combined and incubated for 30 minutes at 55°C for hybridization of target, reporter, and capture oligonucleotides. Equimolar amounts of the capture probe-microsphere conjugates, biotinylated reporter probes and Streptavidin-phycoerythrin were combined in one reaction. The reactions were read from a 96 well plate on a Luminex flow cytometric plate reader for high throughput screening and analyzed with special software.

[0082] Fig. 2 depicts representative graphic results of a multiplex assay using amplified synthetic target. The synthetic target is a synthetic single stranded DNA sequence having sequences from all six viral targets (HAV, HBV, HCV, HIVa , HIVb, ParvoB19). "IC" in Fig. 2 represents results with an internal control. When six different capture probe conjugates hybridized to six different nucleic acid target sequences all combined together in one reaction, the signal from respective target-capture probe-reporter probe complexes was distinguishable, and simultaneously detectable.

[0083] Fig. 4 shows the data from one multiplex assay using the synthetic targets.

[0084] Performance of such assays has demonstrated that one microliter of amplicon generated by a TMA reaction, equivalent to amplicons generated from 1.5 IU of HCV, is sufficient for detection. Also, 1x TMAC buffer (3M TMAC, 0.1% Sarkosyl, 50mM TrispH 8, 4mM EDTA) provided optimal conditions for homogenous nucleic acid assay, while HEPES buffer (10 mM HEPES acid), 0.3M NaCl, pH 7.5) was also adequate. 55°C for 30 minutes is an optimal condition for the reaction in TMAC buffer. Slightly shorter incubation times were

adequate, but 30 minutes gave the most reproducible results in TMAC buffer. The titration of the streptavidin-phycoerythrin detectable reporter moiety indicated that one to three ug/ul provided the best overall signal and signal to noise ratio.

[0085] Although the invention has been described in some detail with reference to the preferred embodiments, those of skill in the art will realize, in light of the teachings herein, that certain changes and modifications can be made without departing from the spirit and scope of the invention.

Claims

What is claimed is:

1. A method for detecting a nucleic acid target sequence in a sample, the method comprising:

(a) contacting said sample with a capture probe conjugate, a reporter probe and a detectable reporter moiety, under conditions allowing formation of a complex including said nucleic acid target sequence, if present, said capture probe conjugate, said reporter probe and said detectable reporter moiety,

wherein said capture probe conjugate and said reporter probe each comprise an oligonucleotide that is capable of specifically hybridizing to said nucleic acid target sequence and wherein said capture probe conjugate and said reporter probe do not hybridize to the same or overlapping regions of said nucleic acid target sequence,

wherein said capture probe conjugate comprises a substrate having a distinguishable spectral signal signature that uniquely identifies the capture probe conjugate,

wherein said reporter probe comprises one member of a binding pair and said detectable reporter moiety comprises the other member of said binding pair,

and wherein said detectable reporter moiety comprises a detectable label; and

(b) detecting a signal from said capture probe conjugate substrate and said detectable reporter moiety label individually from each complex so formed.

2. A multiplex assay method for detecting the presence of a plurality of nucleic acid target sequences in a sample, the method comprising:

(a) contacting said sample, in a single vessel, with a plurality of capture probe conjugates, a plurality of reporter probes, and a detectable reporter moiety, wherein each member of said plurality of capture probe conjugates is specific for one member of said plurality of said target sequences (the specific capture probe conjugate) and each member of said plurality of reporter probes is specific for one member of said plurality of target sequences (the specific reporter probe), under conditions allowing the formation of a specific

complex including said nucleic acid target sequence, if present, said specific capture probe conjugate, said specific reporter probe and said detectable reporter moiety,

wherein each capture probe conjugate of said plurality of capture probe conjugates and each reporter probe of said plurality of reporter probes comprise an oligonucleotide that is capable of specifically hybridizing to one member of said plurality of nucleic acid target sequences and wherein said capture probe conjugates and said reporter probes do not hybridize to the same or overlapping regions of said nucleic acid target sequence,

wherein said capture probe conjugates each comprise a substrate having a distinguishable spectral signal signature that uniquely identifies each capture probe conjugate,

wherein said reporter probes comprise one member of a binding pair and said detectable reporter moiety comprises the other member of said binding pair,

and wherein said detectable reporter moiety comprises a detectable label; and

(b) detecting a signal from said capture probe conjugate substrate and said detectable reporter moiety label individually from each specific complex so formed.

3. The method of claim 1 or claim 2, wherein said sample is a biological sample.

4. The method of claim 1 or claim 2, wherein said nucleic acid target sequence is a sequence from a pathogen nucleic acid.

5. The method of claim 4, wherein said pathogen is a virus.

6. The method of claim 5, wherein said virus is selected from the group consisting of HIV, HBV, HCV, HAV, parvovirus B19, West Nile Virus, hantavirus and SARS.

7. The method of claim 1 or claim 2, wherein the nucleic acid target sequence in the sample is a product of an amplification procedure.

8. The method of claim 7, wherein the amplification procedure is selected from the group consisting of polymerase chain reaction method (PCR), transcription mediated amplification (TMA), ligase chain reaction (LCR), self sustained sequence replication (3SR),

nucleic acid sequence-based amplification (NASBA), Q Beta replicase method, reverse transcription, and nick translation.

9. The method of claim 1 or claim 2, wherein said capture probe is about 8 to about 60 nucleotides in length.

10. The method of claim 1 or claim 2, wherein the substrate comprises fluorescent semiconductor nanocrystals, quantum beads or fluorescent polymeric microspheres.

11. The method of claim 1 or claim 2, wherein the reporter probe is about 8 to about 60 nucleotides in length.

12. The method of claim 1 or claim 2, wherein the one member of the binding pair is biotin and the other member of the binding pair is avidin or streptavidin.

13. The method of claim 1 or claim 2, wherein the detectable reporter moiety label comprises an agent selected from the group consisting of a chromophore, a lumiphore, a fluorophore, a chromogen, a hapten, an antigen, a radioactive isotope, a magnetic particle, a metal nanoparticle, and a quantum dot.

14. The method of claim 13, wherein the detectable reporter moiety label is a fluorophore.

15. A kit for a multiplex assay for detecting the presence of a plurality of nucleic acid target sequences in a sample, the kit comprising:

a plurality of capture probe conjugates, a plurality of reporter probes and a detectable reporter moiety, wherein each member of said plurality of capture probe conjugates is specific for one member of said plurality of said target sequences (the specific capture probe conjugate) and each member of said plurality of reporter probes is specific for one member of said plurality of target sequences (the specific reporter probe),

wherein each capture probe conjugate of said plurality of capture probe conjugates and each reporter probe of said plurality of reporter probes comprise an oligonucleotide that is capable of specifically hybridizing to one member of said plurality of nucleic acid target

sequences and wherein said capture probe conjugates and said reporter probes do not hybridize to the same or overlapping regions of said nucleic acid target sequence,

wherein said capture probe conjugates each comprise a substrate having a distinguishable spectral signal signature that uniquely identifies each capture probe conjugate,

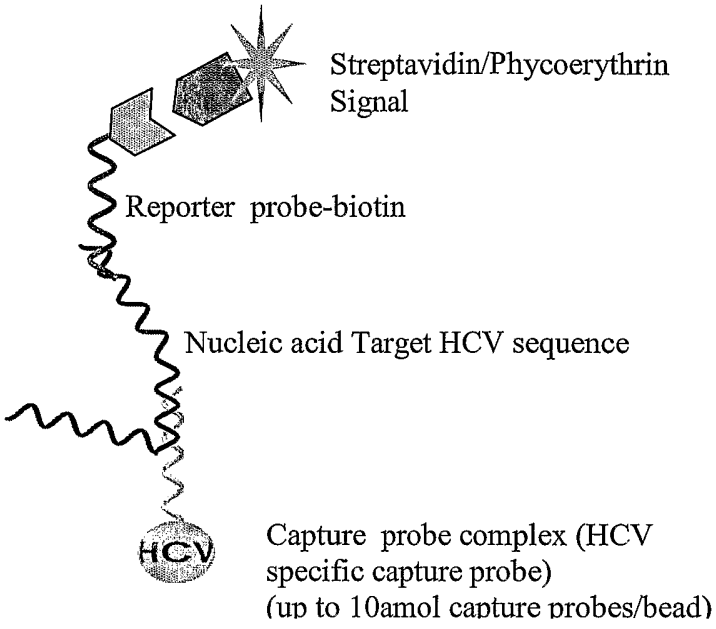
wherein said reporter probes comprise one member of a binding pair and said detectable reporter moiety comprises the other member of said binding pair,

wherein said detectable reporter moiety comprises a detectable label;

a housing for retaining said capture probe conjugates, reporter probes and reporter probe moiety; and

instructions provided with said housing that describe how to use the components of the kit to assay a sample by contacting said sample, in a single vessel, with said plurality of capture probe conjugates, said plurality of reporter probes and said detectable reporter moiety under conditions allowing formation of a specific complex including said nucleic acid target sequence, if present, said specific capture probe conjugate, said specific reporter probe, and said detectable reporter moiety, and detecting a signal from said capture probe conjugate substrate and said detectable reporter moiety label individually from each specific complex so formed.

Fig. 1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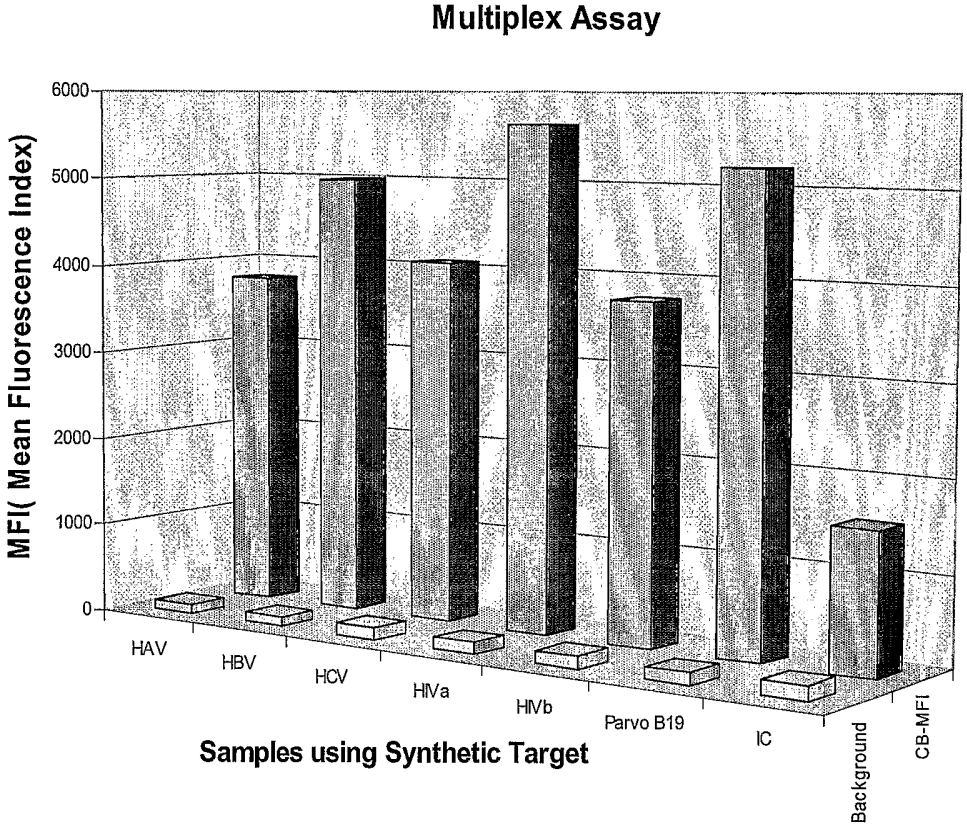
Reporter probe-biotin
Nucleic acid Target HCV sequence
HCV
Capture probe complex (HCV
specific capture probe)
(up to 10amol capture probes/bead)

Fig. 2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5000
4000
3000
2000
1000
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HAV
HBV
HCV
HIVa
HIVb
Parvo B19
IC
Background
CB-MFI
Samples using Synthetic Target

Fig. 3

Primer Design

Capture Probe (LCap)	5' [Amino Modifier 12] + 25bp 3'
Reporter Probe (LRep)	5' 20bp + [Biotin] 3'
Target (LTar)	5' 75bp 3'

Fig. 3A Control

LCap-Con (SEQ ID NO:1)
5'-ZTGCTAATCGATGGCCTAGGCTCATT-3' (Z=amino modifier 12)

LRep-Con (SEQ ID NO:2)
5'-XAATGAGCCTAGGCCATCGATTAGCA (X=Biotin)

Fig. 3B Parvovirus B19 (PVBAUA, Shade)

(SEQ ID NOS:3-4)

3241 ATTTTCCAGACAGTTTTTAAT**TCCATATGACCCAGAGCACCATTATAAGGTGTTTTCTCC**
TAAAAGGTCTGTCAAAAATTA**AGGTATACTGGGTCTCGTGG**TAATATTCCACAAAAGAGG

3301 **CGCAGCGAGTAGCTGCCACAATGCCAGTGGAAAGGA**GGCAAAGGTTTGCACCATCAGTCC
GCGTCGCTCAT**CGACGGTGTTACGGTCACCTTTCCT**CCGTTTCCAAACGTGGTAGTCAGG

LCap-B19 (SEQ ID NO:5)
5'-ZTCCTTTCCACTGGCATTGTGGCAGC-3' (Z=amino modifier 12)

LRep-B19 (SEQ ID NO:6)
5'-GGTGCTCTGGGTCATATGGAX-3' (X=Biotin)

LTar-B19 (SEQ ID NO:7)
5'-TCCATATGACCCAGAGCACCATTATAAGGTGTTTTCTCCCGCAGCGAGTAGCTGC
CACAATGCCAGTGGAAAGGA-3'

Fig. 3C HAV (HPA, Cohen)

(SEQ ID NOS:8-9)

481 TAAGACAAAAACCATTCAACGCCGGAGGACTGAC**TCTCATCCAGTGGATGCATTGAGTGG**
ATTCTGTTTTTGGTAAGTTGCGGCCTCCTGACTG**AGAGTAGGTCACCTACGTAA**CTCACC

541 **ATTGACTGTCAGGGCTGTCTTTAGGCTTAATTCCAGACCTCTCTGTGCT**TAGGGCAAACA
TAACTGACAGTCCCGACAGAAATC**CGAATTAAGGTCTGGAGAGACACGA**ATCCCGTTTGT

LCap-HAV (SEQ ID NO:10)
5'-ZAGCACAGAGAGGTCTGGAATTAAGC-3' (Z=amino modifier 12)

LRep-HAV (SEQ ID NO:11)
5'-AATGCATCCACTGGATGAGAX-3' (X=Biotin)

LTar-HAV (SEQ ID NO:12)
5'-TCTCATCCAGTGGATGCATTGAGTGGATTGACTGTCAGGGCTGTCTTTAGGCTTA
ATTTCAGACCTCTCTGTGCT-3'

Fig. 3 (continued)

Fig. 3D HBV (HBVADW2, Valenzuela)

(SEQ ID NOS:13-14)

1741 CACTCACCAACCTCCTGTCCTCCAATTTG**TCCTGGTTATCGCTGGATGTGTCTGCGGCGT**
GTGAGTGGTTGGAGGACAGGAGGTTAAAC**AGGACCAATAGCGACCTACA**CAGACGCCGCA

1801 **TTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTT**ATTGGTTCTTCTGGAT
AAATAGTATAAGGAGAAGT**AGGACGACGATACGGAGTAGAAGAA**TAACCAAGAAGACCTA

LCap-HBV (SEQ ID NO:15)
5'-ZAAGAAGATGAGGCATAGCAGCAGGA-3' (Z=amino modifier 12)

LRep-HBV (SEQ ID NO:16)
5'-ACATCCAGCGATAACCAGGAX-3' (X=Biotin)

LTar-HBV (SEQ ID NO:17)
5'-TCCTGGTTATCGCTGGATGTGTCTGCGGCGTTTTATCATATTCCTCTTCATCCTGC
TGCTATGCCTCATCTTCTT-3'

Fig. 3E HCV (HCV1a, Choo)

(SEQ ID NOS:18-19)

61 TCTTCACGCAGAAAGCGTCT**AGCCATGGCGTTAGTATGAGTGTCG**TGCAGCCTCCAGGAC
AGAAGTGCGTCTTTCGCAGA**TCGGTACCGCAATCATACTCACAGCACGTCGGAGGTCCTG**

121 CCCCCCTCCCGGGAG**AGCCATAGTGGTCTGCGGAA**CCGGTGAGTACACCGGAATTGCCAG
GGGGGGAGGGCCCTCTCGGTATCACCAGACGCCTTGGCCACTCATGTGGCCTTAACGGTC

LCap-HCV (SEQ ID NO:20)
5'-ZAGCCATGGCGTTAGTATGAGTGTCG-3' (Z=amino modifier 12)

LRep-HCV (SEQ ID NO:21)
5'-AGCCATAGTGGTCTGCGGAAX-3' (X=Biotin)

LTar-HCV (SEQ ID NO:22)
5'-TTCCGCAGACCACTATGGCTCTCCCGGGAGGGGGGGTCCTGGAGGCTGCACGAC
ACTCATACTAACGCCATGGCT-3'

Fig. 3F HIV (HIVHXB2CG, Ratner)

(SEQ ID NOS:23-24)

4741 ATCTTAAGACAGCAGTACAAATGGCAGTATTCAT**CCACAATTTTAAAAGAAAAGGGGGG**A
TAGAATTCTGTCGTCATGTTTACCGTCATAAGTA**GGTGTTAAAATTTTCTTTTCCCCCCT**

4801 TTGGGGGGTACAGTGCAGGGGAAAGAATA**GTAGACATAATAGCAACAGA**CATACAAACTA
AACCCCCCATGTCACGTCCCCTTTCTTATCATCTGTATTATCGTTGTCTGTATGTTTGAT

(SEQ ID NOS:25-26)

9661 TGACT**CTGGTAACTAGAGATCCCTCAGACC**CTTTTAGTCAGTGTGGAAAATCTCTAGCA
ACTGA**GACCATTGATCTCTAGGGAGTCTGGGAAAATCAGTCACACCTTTTAGAGATCGT**

9721* G**TGGCGCCCGAACAGGGACCT**GAAAGCGAAAGGGAAACCAGAGGAGCTCTCTCGACGCA
CACCGCGGGCTTGTCCCTGGACTTTCGCTTTCCCTTTGGTCTCCTCGAGAGAGCTGCGT

Fig. 3 (continued)

Fig. 3F HIV (HIVHXB2CG, Ratner) (continued)

LCap-HIVa (SEQ ID NO:27)
5'-ZCCACAATTTTAAAAGAAAAGGGGGG-3' (Z=amino modifier 12)

LRep-HIVa (SEQ ID NO:28)
5'-GTAGACATAATAGCAACAGAX-3' (X=Biotin)

LTar-HIVa (SEQ ID NO:29)
5'-TCTGTTGCTATTATGTCTACTATTCTTTCCCCTGCACTGTACCCCCCAATCCCCCCT
TTTCTTTTAAAATTGTGG-3'

LCap-HIVb (SEQ ID NO:30)
5'-ZCTGGTAACTAGAGATCCCTCAGACC-3' (Z=amino modifier 12)

LRep-HIVb (SEQ ID NO:31)
5'-TGGCGCCCGAACAGGGACCTX-3' (X=Biotin)

LTar-HIVb (SEQ ID NO:32)
5'-AGGTCCCTGTTCGGGCGCCACTGCTAGAGATTTTCCACACTGACTAAAAGGGTCT
GAGGGATCTCTAGTTACCAG-3'

Fig. 4A

Location	Sample	Sample Contents	Beadset 10	Beadset 20	Beadset 30	Beadset 40	Beadset 50	Beadset 60	Notes	Total Events
A1	1	TMAC Buffer Only	0	0	0	0	0	0	Sample Empty	0
B1	2	TMAC Buffer Only	0	0	0	0	0	0	Sample Empty	0
C1	3	10-60 Beads 0 ul sa/pe	105	1.75	1.944444444	4.419161677	7.75	12.58415842		1020
D1	4	10-60 Beads 0 ul sa/pe	1.44444444	1.726415094	2.317460317	4.451882845	7.751295337	11.84699454		1293
E1	5	10-60 Beads 0 ul sa/pe	1.5	1.535211268	2.066666667	4.826086957	7.840277778	11.90551181		1016
F1	6	TMAC Buffer Only	1.8	2	2.333333333	4.466666667	6.727272727	13.47058824	Sample Empty	73
G1	7	TMAC Buffer Only	0	0	1	4	3	14.5	Sample Empty	5
H1	8	10-60 Beads 1 ul sa/pe	2767.53333	3369.105769	2300.658892	4642.869919	2936.853881	3550.34434		1460
A2	9	10-60 Beads 1 ul sa/pe	3012.28889	3659.405714	2540.17619	4896.572687	3187.468085	4157.528571		1085
B2	10	10-60 Beads 1 ul sa/pe	2825.68889	3117.722222	2089.89071	4181.153652	2590.518828	3289.974468		1729
C2	11	TMAC Buffer Only	2816.66667	2848.333333	1949.75	4524.384615	2606.666667	3459.666667	Sample Empty	36
D2	12	TMAC Buffer Only	0	0	1815	0	2244	0	Sample Empty	3
E2	13	10-60 Beads 2 ul sa/pe	3484.31111	3943.569106	2759.68018	5484.815217	3689.776786	4560.395604		910
F2	14	10-60 Beads 2 ul sa/pe	3532.97778	4085.22381	2710.003195	5371.207358	3365.861224	4279.218935		1468
G2	15	10-60 Beads 2 ul sa/pe	2811.71111	35953888158	2382.971154	4695.477352	3114.984772	3558.060773		1347
H2	16	TMAC Buffer Only	2584	3938	2423.045455	4527.157895	3012.5625	3546.333333	Sample Empty	86
A3	17	TMAC Buffer Only	3111	4279	3468	4272.75	2848.5	0	Sample Empty	12
B3	18	10-60 Beads 3 ul sa/pe	2884.33333	3337.088	2268.787402	4601.80203	2934.350877	3603.139241		1099
C3	19	10-60 Beads 3 ul sa/pe	3112.01111	3211.275862	2565.679856	5356.787645	3131.165094	3915.982558		1311
D3	20	10-60 Beads 3 ul sa/pe	3597.16667	3645.493243	2948.547619	5983.593458	3580.257143	3977.026316		1178
E3	21	TMAC Buffer Only	3093.75	3585.909091	2881.190476	5529.136364	3430.444444	3919.333333	Sample Empty	93

Fig. 4B

Location	Sample	Sample Contents	Beadset 10	Beadset 20	Beadset 30	Beadset 40	Beadset 50	Beadset 60	Notes	Total Events
F3	22	TMAC Buffer Only	3362	0	3040.5	4989.5	2826	4392.666667	Sample Empty	9
G3	23	10-60 Beads 4 ul sa/pe	3042.01111	3346.329545	2571.724576	4727.833948	2990.568702	3631.793103		1372
H3	24	10-60 Beads 4 ul sa/pe	3176.81111	3579.419162	2530.36255	5253.322835	3288.407609	3838.972727		1029
A4	25	10-60 Beads 4 ul sa/pe	3250.58889	3926.447674	2722.829861	5505.709016	3489.547619	4505.514706		1260
B4	26	TMAC Buffer Only	2972.57143	3615.090909	2509.888889	5357.133333	3204.470588	4145.090909	Sample Empty	81
C4	27	TMAC Buffer Only	0	4335	2800	5048	4007	0	Sample Empty	4
D4	28	10-60 Beads 5 ul sa/pe	3641.34444	4250.723404	3242.615132	6354.752632	3746.831818	4982.958333		1256
E4	29	10-60 Beads 5 ul sa/pe	2696.12222	3163.833795	2401.973244	4326.634921	2853.435407	3646.536842		1551
F4	30	10-60 Beads 5 ul sa/pe	3331.6	3640.195455	2787.891986	5394.425414	3272.978632	4145.8		1318
G4	31	TMAC Buffer Only	3156.6	2984.125	2665.176471	4897.571429	2945.3125	3883.714286	Sample Empty	67
H4	32	TMAC Buffer Only	0	0	0	3929	3008	0	Sample Empty	2
A5	33	10-60 Beads 6 ul sa/pe	3636.21111	3885.863366	2412.055901	5406.101504	3337.90458	4493.171717		1817
B5	34	10-60 Beads 6 ul sa/pe	3169.67778	3610.651982	2598.744939	5171.807882	2545.643902	4100.642045		1270
C5	35	10-60 Beads 6 ul sa/pe	3121.04444	3529.565217	2590.96875	4866.455319	3181.154867	3958.868421		1454
D5	36	TMAC Buffer Only	2773.5	3584.375	2400.4	4670.454545	3442.307692	3979.5	Sample Empty	61
E5	37	TMAC Buffer Only	0	3064	0	4575	2564	3348	Sample Empty	4
F5	38	10-60 Beads 7 ul sa/pe	3233.23333	3517.65285	2927.535088	5029.122363	3199.345528	4137.320988		1280
G5	39	10-60 Beads 7 ul sa/pe	2674.05556	3185.848485	2563.692042	4592.608696	2535.403756	3688.982659		1348
H5	40	10-60 Beads 7 ul sa/pe	2500.61111	2920.986577	2426.772429	4160.665644	2674.314286	3392.003521		2040
A6	41	TMAC Buffer Only	2525.33333	2908.6	2380.7	4240.1	2516.3	3184.909091	Sample Empty	61
B6	42	TMAC Buffer Only	0	0	1916	0	0	0	Sample Empty	1
C6	43	10-60 Beads 8 ul sa/pe	2947.55556	3258.928571	2502.289231	4376.504132	545.3617747	3597.026042		1574
D6	44	10-60 Beads 8 ul sa/pe	2842.57778	3089.876923	2486.388889	4451.689189	5553.220532	3866.959302		1462
E6	45	10-60 Beads 8 ul sa/pe	2736.71111	2962.730942	2356.564246	4454.503846	602.7521739	3189.033333		1485

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SHYMALA, Venkatakrishna
NGUYEN, Steve H.

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