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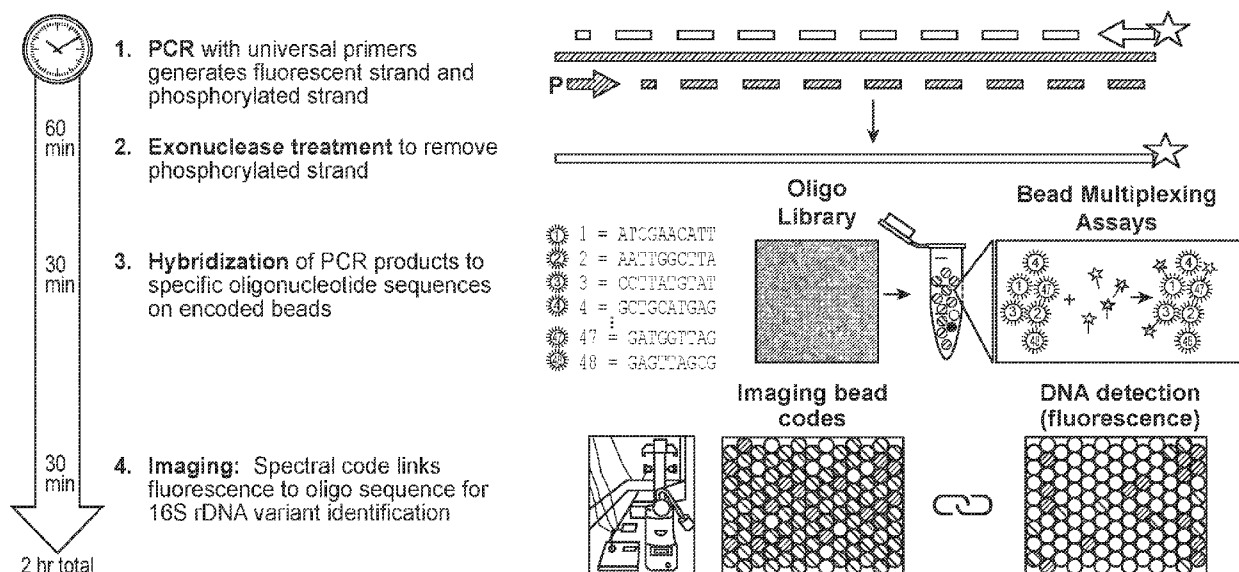


FIG. 1

(57) **Abstract:** Methods for characterizing a population of microbes in a sample are described. The methods include: amplifying microbial polynucleotides obtained from the sample to form a plurality of amplicons and combining the amplicons with a plurality of microbeads, wherein each of the microbeads has a lanthanide spectral signature paired with the sequence of capture polynucleotides immobilized on the microbead. At least some of the capture polynucleotides comprise a sequence substantially complementary to a microbe-identifying sequence in one or more amplicons, such that at least some amplicons are captured onto beads by the capture polynucleotides and the microbe can be identified based on the lanthanide spectral signature of with which the capture polynucleotide is paired. Further described are methods for the identification of pathogens present in a sample by evaluating patterns of hybridization of a capture oligonucleotide to amplicons. Microbead compositions and methods for the preparation thereof are also described.

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METHOD FOR MULTIPLEXED DETECTION OF NUCLEIC ACIDS USING SPECTRALLY ENCODED BEADS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority benefit of U.S. Provisional Patent Application No. 62/853,494, filed May 28, 2019, which is incorporated by reference in its entirety for all purposes.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[0002] This invention was made with government support under Grant No. 1DP2GM123641 awarded by the National Institutes of Health. The government has certain rights in the invention

BACKGROUND OF THE INVENTION

[0003] Sepsis causes millions of deaths globally each year and is the leading cause of death in people who have been hospitalized. In sepsis, the time to antibiotic treatment is critical for patient survival, as the difference of an hour can mean life or death. However, as antibiotics are effective against only certain classes of microbial infections, the choice of appropriate treatment requires knowledge of the pathogen responsible. Such knowledge limits the use of inappropriate antibiotics that can have significant side effects and drive hospital-acquired resistance. The current gold-standard method in clinical use for identifying pathogens in blood infections relies on cultures that can take *days*, too slow to affect the initial antibiotic selection. To address this, several recent nucleic acid-based technologies have emerged, but none completely meet this urgent need. PCR-based nucleic acid tests can identify bacterial sequences in clinical samples in hours, but are limited to detecting ~1–4 pathogens simultaneously. In order to test for multiple pathogens, these methods typically require sample-splitting and compartmentalization into multiple different reactions, and this statistical sampling of blood volumes lowers the overall sensitivity of these assays and therefore requires pre-amplification steps that can complicate

detection and lead to false-positive detections. Recently developed approaches that rely on next-generation sequencing enable unbiased (although still targeted) pathogen discovery and can detect a large number of potential pathogens, but take many hours to days.

BRIEF SUMMARY OF THE INVENTION

5 [0004] Provided herein are methods for characterizing a population of microbial strains in a sample. The different microbial strains contain polynucleotides with different microbe-identifying sequences, and the methods include:

(i) amplifying polynucleotides obtained from the sample to form a plurality of amplicons, wherein the amplicons comprise microbe-identifying sequences;

10 (ii) combining the amplicons with a plurality of microbeads, wherein each of the microbeads has a lanthanide spectral signature and a plurality of copies of a capture polynucleotide immobilized on the microbead,

wherein each of the capture polynucleotides comprises a predetermined sequence,

15 wherein each predetermined sequence is paired with a specific lanthanide spectral signature, such that the capture polynucleotide immobilized on a microbead can be identified based on the lanthanide spectral signature of the microbead,

wherein at least some of the capture polynucleotides comprise a sequence substantially complementary to a microbe-identifying sequence of one or more amplicons,

20 wherein the combining is conducted under conditions in which at least some amplicons are captured onto the beads by the capture polynucleotides, thereby producing captured amplicons, and

wherein the amplicons are labeled with one or more signal-generating moieties prior to, simultaneously with, or after being captured onto the beads, such that microbeads comprising immobilized signal-generating moieties are produced;

25 (iii) detecting signal from immobilized signal-generating moieties of individual microbeads from step (ii); and

(iv) determining the lanthanide spectral signatures of the individual microbeads from step (ii), thereby determining the microbe-identifying sequences of the captured amplicons and characterizing the population of microbial strains in the sample.

[0005] In one approach, step (iii) occurs prior to step (iv). In another approach, step (iii) occurs simultaneously with step (iv). In yet another approach, step (iii) occurs after step (iv).

[0006] In some instances, the amplicons are captured by hybridization of microbe-identifying sequences in the amplicons to the substantially complementary sequences in the capture polynucleotides.

[0007] In one aspect, the polynucleotides in step (i) are DNA. In another aspect, the polynucleotides in step (i) are RNA.

[0008] In some instances, the plurality of microbeads comprises at least 50 different spectral signatures and immobilized capture polynucleotides comprising at least 50 different predetermined sequences. In some instances, the combining in step (ii) comprises hybridizing amplicons to at least 50 microbeads having different spectral signatures and different capture polynucleotides.

[0009] In one approach, the signal-generating moiety produces a fluorescent or chemiluminescent signal. In one approach, the amplicons are labeled during the amplifying in step (i). In one approach, the magnitude of signal detected from an individual microbead in step (iii) corresponds to the amount of the amplicons captured on the microbead.

[0010] In some instances, the population of microbial strains comprise bacterial strains. In some instance, at least one of the microbe identifying sequences in the amplicons comprises a bacterial 16S ribosomal RNA (rRNA) gene sequence. In some instances, the amplification is conducted using primer pairs that hybridize to conserved regions flanking one or more variable regions in a bacterial 16S rRNA gene sequence. In some instances, the amplification in step (i) comprises amplifying a V3 variable region, a V4 variable region, or a V6 variable region in a bacterial 16S rRNA gene sequence. In one approach, at least one of the microbe identifying sequences in the amplicons comprises an antibiotic resistance gene sequence. In some instances, the microbial strains comprise one or more species selected from the group consisting of *Pseudomonas*, *Streptococcus*, *Staphylococcus*, *Neisseria*, *Acinetobacter*, *Escherichia*, *Enterobacter*, and *Klebsiella*.

[0011] In some instances, each of the lanthanide spectral signatures comprises a europium (Eu) signal, a dysprosium (Dy) signal, a samarium (Sm) signal, a cerium (Ce) signal, a terbium (Tb)

signal, a lanthanum (La) signal, a praseodymium (Pr) signal, a neodymium (Nd) signal, a gadolinium (Gd) signal, a holmium (Ho) signal, an erbium (Er) signal, a thulium (Tm) signal, an ytterbium (Yb) signal, or a combination thereof. In some instances, each of the microbeads comprises a plurality of lanthanide nanoparticles. In some instances, the lanthanide nanoparticle
5 comprises a lanthanide-doped host lattice. In some instances, the host lattice is yttrium orthovanadate, lanthanum phosphate, or a combination thereof.

[0012] In some instances, each of the microbeads further comprises a crosslinked polymer, and the capture polynucleotides are covalently bonded to the crosslinked polymer.

10 [0013] In some instances, the plurality of microbeads are dispersed on a microscope slide prior to step (iii) or step (iv).

[0014] In some instances, the method according to the present disclosure further comprises separating uncaptured amplicons from microbeads having captured amplicons. In some instances, the method further comprises separating microbeads having immobilized signal-generating moieties from microbeads not having immobilized signal-generating moieties. In one
15 aspect, the immobilized signal-generating moieties are fluorescent labels. In one approach, the immobilized signal-generating moieties are detected in step (iii) via fluorescence microscopy.

[0015] In some instances, the sample is a biological sample from an animal, a food sample, a beverage sample, or an environmental sample. In some aspects, the biological sample is a blood sample, an intestinal sample, a stool sample, a urine sample, a saliva sample, or a sputum sample.

20 [0016] Also provided herein are microbeads comprising a lanthanide spectral signature and a plurality of copies of a capture polynucleotide immobilized on the microbeads, wherein each capture polynucleotide comprises a predetermined sequence and each capture polynucleotide is paired with a lanthanide spectral signature, such that the predetermined sequence can be identified based on the lanthanide spectral signature.

25 [0017] In some instances, each capture polynucleotide comprises a nucleic acid sequence that hybridizes to a bacterial 16S rRNA gene sequence. In some instances, the capture polynucleotides comprise a nucleic acid sequence that hybridizes to a V3 variable region, a V4 variable region, or a V6 variable regions in a bacterial 16S rRNA gene sequence. In some instances, each of the capture polynucleotides comprises a nucleic acid sequence able to

hybridize a *Pseudomonas* gene sequence, a *Streptococcus* gene sequence, *Staphylococcus* gene sequence, a *Neisseria* gene sequence, an *Acinetobacter* gene sequence, an *Escherichia* gene sequence, an *Enterobacter* gene sequence, or a *Klebsiella* gene sequence.

[0018] In some instances, each of the lanthanide spectral signatures comprises one or more

5 signals selected from a europium (Eu) signal, a dysprosium (Dy) signal, a samarium (Sm) signal, a cerium (Ce) signal, a terbium (Tb) signal, a lanthanum (La) signal, a praseodymium (Pr) signal, a neodymium (Nd) signal, a gadolinium (Gd) signal, a holmium (Ho) signal, an erbium (Er) signal, a thulium (Tm) signal, and an ytterbium (Yb) signal. In some instances, each of the microbeads comprises a plurality of lanthanide nanoparticles. In some instances, the lanthanide
10 nanoparticles comprise a lanthanide-doped host lattice. In some instances, the host lattice is yttrium orthovanadate, lanthanum phosphate, or a combination thereof. In some instances, each of the microbeads further comprise a crosslinked polymer, and the capture polynucleotides are covalently bonded to the crosslinked polymer. In some instances, the microbeads comprise at least 20 lanthanide spectral signatures. In some instances, the microbeads comprise at least 1000
15 lanthanide spectral signatures.

[0019] In another aspect, provided is a method for characterizing a population of microbial strains in a sample. The different microbial strains contain polynucleotides with different microbe-identifying sequences, and the methods include:

(i) amplifying polynucleotides obtained from the sample to form a plurality of
20 amplicons, wherein the amplicons comprise microbe-identifying sequences;

(ii) combining the amplicons with a panel of microbeads,
wherein each of the microbeads has a lanthanide spectral signature and a plurality of copies of a capture polynucleotide immobilized on the microbead,

wherein each of the capture polynucleotides comprises a predetermined sequence,
25 wherein each predetermined sequence is paired with a specific lanthanide spectral signature, such that the capture polynucleotide immobilized on a microbead can be identified based on the lanthanide spectral signature of the microbead,

wherein at least some of the capture polynucleotides comprise a sequence substantially complementary to a microbe-identifying sequence of one or more amplicons,

wherein the combining is conducted under conditions in which at least some amplicons hybridize to the capture polynucleotides, thereby producing captured amplicons, and

wherein the amplicons are labeled with one or more signal-generating moieties prior to, simultaneously with, or after being captured onto the beads, such that microbeads comprising immobilized signal-generating moieties are produced; wherein the magnitude of signal detected from an individual microbead corresponds to the amount of the amplicons captured on the microbead.

(iii) measuring the magnitude of the signal from immobilized signal-generating moieties of individual microbeads from step (ii);

(iv) determining the lanthanide spectral signatures of the individual microbeads from step (ii), thereby determining the microbe-identifying sequences of the captured amplicons;

(v) generating a pattern of measured amount of hybridization of the captured amplicons to individual capture polynucleotides;

(vi) comparing the pattern of hybridization measured in (v) to a predicted pattern of hybridization of amplicons to each capture oligonucleotide, wherein prediction of the pattern of hybridization of the amplicons to a capture oligonucleotide comprises calculating the level of hybridization of the capture oligonucleotide to the amplicons, *e.g.*, by calculating the Gibbs free energy of hybridization; and

(vii) correlating the pattern of hybridization measured in (v) to the predicted pattern of hybridization for each species to identify the species that has a predicted pattern of hybridization that has the strongest correlation with the pattern of hybridization measured in (v).

[0020] In some instances, step (iii) occurs prior to step (iv). In some instances, step (iii) occurs simultaneously with step (iv). In some instances, step (iii) occurs after step (iv).

[0021] In some instances, the polynucleotides in step (i) are DNA.

[0022] In some instances, the plurality of microbeads comprises at least 50 different spectral signatures and immobilized capture polynucleotides comprising at least 50 different predetermined sequences. In some instances, the combining in step (ii) comprises hybridizing amplicons to at least 50 microbeads having different spectral signatures and different capture polynucleotides.

[0023] In some instances, the signal-generating moiety produces a fluorescent or chemiluminescent signal. In some instances, the amplicons are labeled during the amplifying in step (i).

[0024] In some instances, the population of microbial strains comprises bacterial strains. In one aspect, at least one of the microbe identifying sequences in the amplicons comprises a bacterial 16S ribosomal RNA (rRNA) gene sequence. In some instances, the amplification is conducted using primer pairs that hybridize to conserved regions flanking one or more variable regions in a bacterial 16S rRNA gene sequence. In some instances, the amplification in step (i) comprises amplifying one or more of a V3 variable region, a V4 variable region, or a V6 variable region in a bacterial 16S rRNA gene sequence. In some instances, the amplification step (i) comprises amplifying the V3 variable region and the V6 variable region. In some instances, the microbial strains comprise one or more species selected from the group consisting of *Pseudomonas*, *Streptococcus*, *Staphylococcus*, *Neisseria*, *Acinetobacter*, *Escherichia*, *Enterobacter*, *Klebsiella*, *Haemophilus*, *Proteus*, *Serratia*, *Enterococcus*, and *Listeria*.

[0025] In some instances, each of the lanthanide spectral signatures comprises a europium (Eu) signal, a dysprosium (Dy) signal, a samarium (Sm) signal, a cerium (Ce) signal, a terbium (Tb) signal, a lanthanum (La) signal, a praseodymium (Pr) signal, a neodymium (Nd) signal, a gadolinium (Gd) signal, a holmium (Ho) signal, an erbium (Er) signal, a thulium (Tm) signal, an ytterbium (Yb) signal, or a combination thereof. In some instances, each of the microbeads comprises a plurality of lanthanide nanoparticles. In some instances, the lanthanide nanoparticle comprises a lanthanide-doped host lattice. In some instances, the host lattice is yttrium orthovanadate, lanthanum phosphate, or a combination thereof. In one aspect, each of the microbeads further comprises a crosslinked polymer, and the capture polynucleotides are covalently bonded to the crosslinked polymer. In some instances, the plurality of microbeads are dispersed on a microscope slide prior to step (iii) or step (iv).

[0026] In some instances, the method further comprises separating uncaptured amplicons from microbeads having captured amplicons. In some instances, the method further comprises separating microbeads having immobilized signal-generating moieties from microbeads not having immobilized signal-generating moieties. In one aspect, the immobilized signal-generating

moieties are fluorescent labels. In one aspect, the immobilized signal-generating moieties are measured in step (iii) via fluorescence microscopy.

[0027] In some instances, the sample is from blood, cerebrospinal fluid, lymph, or urine.

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BRIEF DESCRIPTION OF THE DRAWINGS

[0028] FIG. 1 shows a schematic representation of assays according to the present disclosure.

[0029] FIG. 2A shows algorithmic steps used for the design of capture polynucleotides.

[0030] FIG. 2B shows a heat-map depicting the specificity of candidate capture polynucleotides.

10 [0031] FIG. 2C shows an alignment of 16S variable region V6 for several species of bacteria.

[0032] FIG. 3A shows a scheme for microbead functionalization.

[0033] FIG. 3B shows a fluorescence microgram of microbeads following hybridization of Cy5-labeled oligonucleotides.

15 [0034] FIG. 3C shows fluorescence signal intensity of microbeads following hybridization of Cy5-labeled oligonucleotides.

[0035] FIG. 3D shows that the fluorescence signal from the beads correlates to the amount of input oligonucleotide.

[0036] FIG. 4 shows the testing sensitivity of a whole blood assay according to the present disclosure.

20 [0037] FIG. 5 shows the multiplexed detection of different pathogens.

[0038] FIG. 6 shows that hybridization affinity is correlated with the free energy of hybridization.

[0039] FIG. 7 shows a schematic depiction of 16S rRNA gene targeting.

[0040] FIG. 8 shows the simultaneous measurement of multiple variable regions.

[0041] FIG. 9A shows a heatmap illustrating three species that bind with different affinities to three capture polynucleotides.

[0042] FIG. 9B shows illustrative examples of Pearson correlation coefficients (r) for the measured hybridization signal and predicted hybridization energies.

5 [0043] FIG. 9C shows a heatmap illustrating the Pearson correlation coefficients shown in FIG. 9B.

[0044] FIG. 9D shows a heatmap illustrating four species that bind with different affinities to four capture polynucleotides of the V3 (top) and V4 (bottom) region.

10 [0045] FIG. 9E shows illustrative examples of Pearson correlation coefficients (r) for the measured hybridization signal and predicted hybridization energies for both the V3 and V6 region.

[0046] FIG. 9F shows a heatmap illustrating the Pearson correlation coefficients shown in FIG. 9E.

15 [0047] FIG. 10A shows the fluorescence signal intensity of microbeads reflecting the hybridization of *Acinetobacter baumannii* amplicons to different capture polynucleotides.

[0048] FIG. 10B shows Pearson correlations (R) of measured hybridization signal intensities with the predicted hybridization energies for V3 (top row) and V6 (bottom row) *Acinetobacter baumannii* amplicons against a panel of capture polynucleotides.

20 [0049] FIG. 11. shows a heatmap displaying the Gibbs free energy of hybridization for distinguishing groups of bacteria.

[0050] FIG. 12A and FIG. 12C show summary heatmaps of correlations between measured hybridization signal intensity and predicted hybridization affinity.

[0051] FIG. 12B and FIG. 12D show prediction matrices for the probes used for experiments shown in FIG. 12A and FIG. 12C, respectively.

25 [0052] FIG. 13A shows the area under a receiver operating characteristic curve (AUROC) analysis for the combined V3 and V6 correlations.

[0053] FIG. 13B shows the AUROC analysis for the V3 amplicons.

[0054] FIG. 13C shows the AUROC analysis for the V6 amplicons.

[0055] FIG. 14 shows clustering of probe candidates (columns) and bacterial species (rows) based on probe-to-amplicon hybridization affinities.

DETAILED DESCRIPTION OF THE INVENTION

5 [0056] Provided herein are highly multiplexed, sensitive, specific, and fast methods for the detection of pathogens without blood compartmentalization for use in clinical scenarios such as suspected cases of sepsis. These new methods, useful in rapid screening for the presence of multiple bacteria species in a single reaction, employ microfluidically synthesized beads embedded with distinct spectral codes. The methods can be used to detect different bacterial
10 species in a variety of samples (*e.g.*, clinical samples such as blood or stool, and environmental samples such as water or food sources) in multiplexed fashion, allowing for screen the parallel screening of large numbers samples (*e.g.*, 96 samples or more) against a pathogen panels in short timeframes (*e.g.*, 2 hours or less). This is already beyond the current throughput and multiplexing provided by conventional methods. The methods can also be used for identifying further
15 characteristics such as the presence of antibiotic-resistant bacteria.

[0057] Beads according to the present disclosure are chemically compatible with wide variety of solvents and reagents, and bead sets can be prepared with over 1100 distinct spectral codes. Assays according to the present disclosure are economical, easy to use, compatible with standard laboratory equipment, and adaptable to a variety of assays. The assays are less expensive than
20 DNA sequencing, and a higher number of DNA sequences may be targeted than by conventional PCR assays.

I. Definitions

[0058] As used herein, the term “sample” refers to a specimen that is suspected of containing microbial nucleic acids. Samples include, but are not limited to, clinical samples, food samples,
25 and water samples, as described in more detail below.

[0059] As used herein, the term “microbial strain” refers to a strain of bacterium, virus, fungus, protist, or other microscopic organism.

[0060] As used herein, the term “microbead” refers to a particle having one or more dimensions (*e.g.*, length, width, diameter, or circumference) of about 1000 μm or less, *e.g.*, less than about 500 μm , 100 μm , or 10 μm . Microbeads may have a generally spherical shape or a non-spherical shape. Microbeads used in the methods of the present disclosure are characterized by a detectable spectral signature as described in more detail below.

[0061] As used herein, the term “nanoparticle” refers to a particle having at least one dimension (*e.g.*, length, width, diameter, or circumference) ranging from 1 to 1,000 nm. Nanoparticles may have a generally spherical shape or a non-spherical shape. A “plurality” of nanoparticles refers to a population of microbeads ranging in size from a few nanoparticles to thousands of nanoparticles, or more.

[0062] As used herein, the term “host lattice” refers to a material having constituent atoms packed in a regularly ordered, repeating pattern which can accommodate the incorporation of lanthanide atoms or ions. By “lanthanide-doped,” it is meant that the host lattice material contains lanthanide atoms or ions.

[0063] As used herein, the terms “polynucleotide” and “nucleic acid” refer to DNA or RNA, including, for example, genomic DNA; complementary DNA (cDNA); and DNA molecules produced synthetically or by amplification. Polynucleotides may be single-stranded or double-stranded. The term “microbial polynucleotide” refers to a polynucleotide originating from a strain of bacterium, virus, fungus, protist, or other microscopic organism.

[0064] As used herein, the term “microbe-identifying sequence” refers to a polynucleotide sequence that is characteristic of the microbial strain from which the polynucleotide originated. For example, a microbe-identifying sequence may be unique to a particular bacterial strain, allowing the strain to be positively identified upon finding the microbe-identifying sequence in a sample obtained from a source suspected to contain the strain. The microbe-identifying sequences may range from around five bases in length to hundreds of bases in length.

[0065] As used herein, the term “capture polynucleotide” refers to an oligonucleotide containing a predetermined sequence selected to hybridize to a microbe-identifying sequence in an amplicon. The terms “hybridize,” “anneal,” and “bind,” in reference to two polynucleotide sequences, are used interchangeably and have the usual meaning in the art. Two complementary

sequences (*e.g.*, DNA and/or RNA) anneal or hybridize by forming hydrogen bonds with complementary bases to produce a double-stranded polynucleotide or a double-stranded region of a polynucleotide.

[0066] The predetermined sequence of a capture polynucleotide is selected such that is
5 complementary or substantially complementary to a microbe-identifying sequence suspected to be present in a sample under study. The term “substantially complementary,” in the context of a capture oligonucleotide, refers to a sequence that is not perfectly complementary to its target sequence, but can hybridize selectively to the desired target sequences. Such a sequence may have one or more “mismatches”, *i.e.*, one or more positions in which the nucleotide in the
10 capture polynucleotide and the nucleotide in the target amplicon with which it is aligned are not complementary. Selectivity of hybridization exists when hybridization occurs that is more selective than total lack of specificity. Typically, selective hybridization will occur when there is at least about 55% identity over a stretch of at least 14-25 nucleotides, *e.g.*, at least about 65%, at least about 75%, or at least about 90% identity. *See*, M. Kanehisa, *Nucleic Acids Res.* 12:203
15 (1984).

[0067] As used herein, the term “immobilized” refers to a substance (*e.g.*, a capture polynucleotide or signal-generating moiety) which is covalently bonded to microbeads, or non-covalently bound such that it is not unintentionally removed during characterization of the sample under study (*e.g.*, during amplification, combination, labeling, and/or detection steps as
20 described herein).

[0068] As used herein, the terms “amplifying” and “amplification reaction” refer to a process whereby a portion of a target polynucleotide is replicated using a primer extension reaction such as a polymerase chain reaction (PCR). Amplifying the polynucleotide can include a single replication, as well as arithmetic, logarithmic, or exponential amplification. The term
25 “amplicon” refers to the portion of a target polynucleotide that is replicated in an amplification reaction. Amplicons generated in the methods of the present disclosure typically range from tens of bases in length to hundreds of bases in length, or more.

[0069] As used herein, the term “predetermined” refers to a nucleotide sequence that has been specifically selected for hybridization to an amplicon sequence suspected to arise from an
30 amplification reaction conducted with a particular sample. For example, predetermined

sequences may be selected for hybridization to known bacterial gene sequences as described herein.

[0070] As used herein, the term “lanthanide spectral signature” refers to the combined luminescent signals in the range of 350–850 nm emitted from lanthanide nanoparticles contained in a single microbead upon excitation with an appropriate wavelength of light, *e.g.*, UV light (*e.g.*, 292 nm for excitation of downconverting lanthanides) or IR light (*e.g.*, 980 nm for excitation of upconverting lanthanides). The luminescence intensity at a characteristic wavelength or wavelengths (*e.g.*, 620 nm, 630 nm, or 650 nm) for a particular lanthanide (*e.g.*, Eu) indicates the presence and quantity of the particular lanthanide in the source (*e.g.*, a microbead) from which the spectral signature originates. As used herein, the term “lanthanide” refers to elements 57-71 of the periodic table, namely lanthanum (La), cerium (Ce), praseodymium (Pr), neodymium (Nd), promethium (Pm), samarium (Sm), europium (Eu), gadolinium (Gd), terbium (Tb), dysprosium (Dy), holmium (Ho), erbium (Er), thulium (Tm), ytterbium (Yb), and lutetium (Lu).

[0071] The term “paired,” in the context of a spectral signature and the capture oligonucleotides for a particular microbead, refers to a microbead having a known spectral signature and copies of a capture oligonucleotide having a known predetermined sequence immobilized thereto. Because both the spectral signature and the predetermined sequence are known, the identity of the predetermined sequence can be determined based on the features of the spectral signature (*e.g.*, the luminescence intensity at one or more characteristic wavelengths for one or more particular lanthanides).

[0072] The term “label,” as used herein, refers to any atom or molecule that can be used to provide a detectable and/or quantifiable signal. In some embodiments, the label can be attached, directly or indirectly, to a biomolecule. Labels include, but are not limited to, radioisotopes, fluorophores, chromophores, mass labels, electron dense particles, magnetic particles, spin labels, molecules that emit chemiluminescence, electrochemically active molecules, enzymes, cofactors, and enzyme substrates.

II. Embodiments of the Invention

[0073] Provided herein are methods for characterizing a population of microbial strains in a sample. The different microbial strains contain polynucleotides with different microbe-identifying sequences, and the methods include:

- 5 (i) amplifying polynucleotides obtained from the sample to form a plurality of amplicons, wherein the amplicons comprise microbe-identifying sequences;
- (ii) combining the amplicons with a plurality of microbeads, wherein each of the microbeads has a lanthanide spectral signature and a plurality of copies of a capture polynucleotide immobilized on the microbead,
- 10 wherein each of the capture polynucleotides comprises a predetermined sequence, wherein each predetermined sequence is paired with a specific lanthanide spectral signature, such that the capture polynucleotide immobilized on a microbead can be identified based on the lanthanide spectral signature of the microbead,
- wherein at least some of the capture polynucleotides comprise a sequence
- 15 substantially complementary to a microbe-identifying sequence of one or more amplicons, wherein the combining is conducted under conditions in which at least some amplicons are captured onto the beads by the capture polynucleotides, thereby producing captured amplicons, and
- wherein the amplicons are labeled with one or more signal-generating moieties
- 20 prior to, simultaneously with, or after being captured onto the beads, such that microbeads comprising immobilized signal-generating moieties are produced;
- (iii) detecting signal from immobilized signal-generating moieties of individual microbeads from step (ii); and
- (iv) determining the lanthanide spectral signatures of the individual microbeads
- 25 from step (ii), thereby determining the microbe-identifying sequences of the captured amplicons and characterizing the population of microbial strains in the sample.

[0074] The plurality of microbeads may range in population size. For example, a plurality of microbeads may include 10 or more, 100 or more, 500 or more, 10^3 or more, 10^4 or more, 10^5 or more, 10^6 or more, or 10^7 or more microbeads. The microbeads of the present disclosure

30 generally have one or more dimensions (*e.g.*, diameter) of about 1000 μm or less, *e.g.*, about 500

μm or less, about 100 μm or less, about 50 μm or less, about 10 μm or less, or about 5 μm or less. For example, a microbead may have one or more dimensions (*e.g.*, diameter) of from about 1000 μm to about 1 μm, from about 500 μm to about 1 μm, from about 100 μm to about 1 μm, from about 50 μm to about 1 μm, from about 10 μm to about 1 μm, or from about 5 μm to about 1 μm.

- 5 The microbeads may have a generally spherical shape or a non-spherical shape. Each microbead in a plurality of microbeads may have approximately the same one or more dimensions. In some embodiments, individual microbeads have a diameter such that the diameter variation among all the members of the plurality is no greater than about 10 percent, *e.g.*, no greater than about 5 percent, or no greater than about 1 percent.

10 **A. Amplification of Microbial Nucleic Acids**

- [0075] A number of nucleic acid amplification methods can be used in the methods described herein, *e.g.*, PCR and variations thereof (*e.g.*, TaqMan, real time PCR, quantitative PCR), reverse transcription, strand displacement reaction (SDR), ligase chain reaction (LCR), transcription mediated amplification (TMA), Qbeta replication, hybridization chain reaction, recombinase
15 polymerase amplification, or rolling circle amplification.

- [0076] Samples from a variety of sources can be processed for use in the amplification reactions. In some embodiments, the sample is a blood sample, a stool sample, a urine sample, a saliva sample, a sputum sample, a food sample, a beverage sample, or an environmental sample (*e.g.*, a soil sample, a water sample, an oil sample, or an agricultural sample). Bacterial DNA,
20 for example, can be amplified from a clinical sample obtained from a septic patient. In some embodiments, a small portion of a clinical sample obtained from a subject can be used directly in the PCR mixture for amplification and detection of microbial nucleic acids. Alternatively, one or more nucleic acid purification steps can be conducted with body fluid sample prior to the amplification step in the method. In such cases, the sample may be a mixture containing purified
25 microbial nucleic acids (if present) along with other nucleic acids associated with the sample (*e.g.*, genomic DNA or a portion thereof from the subject). The term “purified,” as used herein to refer to microbial nucleic acids purified from a sample, means that at least a portion of other components (*e.g.*, polysaccharides, polypeptides, and the like) have been removed. In some
30 embodiments, for example, the amount of protein in a purified DNA mixture will be less than 50% of the protein present in the initial sample from which the purified mixture is obtained.

Purifying DNA from a sample can therefore include removing at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of the protein present in the sample. Protein concentrations can be determined by assay methods such as a Bradford assay, a Lowry assay, a BCA assay, or like similar techniques. Similarly, other carbohydrate, amino acid, and lipid components can be removed from body fluid samples during purification of nucleic acids.

[0077] For example, proteins and other contaminants can be removed from samples by extraction with phenol-chloroform or similar organic solvents, and the remaining DNA can be precipitated with an alcohol such as ethanol. Solid-phase adsorbent materials can also be employed for purification of nucleic acids from samples prior to PCR. Examples of such materials include, but are not limited to, silica or glass fibers/particles that are used in conjunction with chaotropic agents such as sodium iodide, guanidinium isothiocyanate, and the like. Commercially available materials designed for such protocols include ILLUSTRA GFX products (GE Life Sciences) and MONARCH nucleic acid purification products (New England BioLabs).

[0078] Different variable region sequences within the amplified portions of the 16S rRNA gene can be detected using microbeads having capture polynucleotides with predetermined sequences that are selected to specifically identify various bacterial species, *e.g.*, *Pseudomonas* species, *Streptococcus* species, *Staphylococcus* species, *Neisseria* species, *Acinetobacter* species, *Escherichia* species, *Enterobacter* species, *Klebsiella* species, and combinations thereof.

Polynucleotides from Gram positive and Gram negative bacteria can be assessed using the methods of the present disclosure. The bead compositions and methods disclosed herein can also be adapted to detect other infectious agents such as viruses, or to validate the presence of DNA sequences that are predicted from de novo metagenomic sequencing.

[0079] In some embodiments, the capture polynucleotides contain predetermined sequences that are present in any one or more variable regions V1-V9 of a bacterial 16S rRNA gene. By selecting a predetermined sequence (*e.g.*, a sequence present within the V3 variable region, V4 variable region, or V6 variable region) that is unique for a particular species of bacteria, *e.g.*, *Staphylococcus aureus*, that species can be identified as the cause of, or a contributor to, infection in a subject from whom a clinical sample under study has been obtained. Using the same capture polynucleotide sequence on beads having different spectral signatures can provide

can provide technical replicates, as shown in FIG. 7. In addition, as shown in FIG. 8, simultaneous assessment of sequences in two or more variable regions (*e.g.*, the V3 region using capture polynucleotides having one or more predetermined sequences set forth in Table 2 and the V6 region using capture polynucleotides having one or more predetermined sequences set forth in Table 3) can further increase confidence in pathogen detection, reduce false positives, and allow for discrimination between species that may be identical or extremely similar in one variable region but not another.

[0080] In some embodiments, at least one of the microbe identifying sequences in the amplicons comprises a bacterial 16S ribosomal RNA (rRNA) gene sequence. In some embodiments, the amplification is conducted using primer pairs that hybridize to conserved regions in a bacterial 16S rRNA gene sequence. As used herein, the term “conserved” refers to any sequence of bases in comparable segments of different nucleotides that tend to show similarity greater than that due to chance alone. In some embodiments, a conserved sequence flanks a known variable region in a bacterial gene such as a 16S rRNA gene. The amplification can be conducted using primer pairs that hybridize, for example, to conserved regions flanking: a V1 variable region corresponding to nucleotides 69-99 (numbering by the *E. coli* system of nomenclature; *see*, Brosius et al., 1978); a V2 variable region corresponding to nucleotides 137-242; a V3 variable region corresponding to nucleotides 433-497); a V4 variable region corresponding to nucleotides 576-682); a V5 variable region corresponding to nucleotides 822-879); a V6 variable region corresponding to nucleotides 986-1043); a V7 variable region corresponding to nucleotides 1117-1173); a V8 variable region corresponding to nucleotides 1243-1294); a V9 variable region corresponding to nucleotides 1435-1465); or two or more of these variable regions. For example, degenerate primers (“515F” having the sequence GTGCCAGCMGCCGCGGTAA, SEQ ID NO:1; and “806R” having the sequence GGACTACHVGGGTWTCTAAT, SEQ ID NO:2; *see*, Table 1) can anneal to common regions in the 16S gene to specifically amplify the V4 variable region. In some embodiments, the amplification in step (i) comprises amplifying a V3 variable region, a V4 variable region, or a V6 variable region in a bacterial 16S rRNA gene sequence. Alternatively, the entire 16S rRNA gene can be amplified and microbe-identifying sequences within the resulting amplicons can be identified by annealing to predetermined sequences in immobilized capture polynucleotides and analysis of the corresponding lanthanide spectral signatures. Non-limiting examples of primers

for use in such embodiments include “27F” having the sequence AGRGTTYGATYMTGGCTCAG (SEQ ID NO:164) and “1492R” having the sequence RGYTACCTIGTTACGACTT (SEQ ID NO:165).

Table 1. Primer Sequences for Amplification of 16S rRNA Variable Regions.

Description	Sequence	SEQ ID NO.
515F(V4), labeled	/A647/GTGCCAGCMGCCGCGGTAA	1
806R(V4), phosphorylated	/Phos/GGACTACHVGGGTWTCTAAT	2
515F(V4)	GTGCCAGCMGCCGCGGTAA	3
806R(V4),	GGACTACHVGGGTWTCTAAT	4
V6-Forward, labeled	/A647/TGGAGCATGTGGTTTAATTCGA	5
V6-Forward	TGGAGCATGTGGTTTAATTCGA	6
V6-Reverse, phosphorylated	/Phos/AGCTGACGACANCCATGCA	7
V6-Reverse	AGCTGACGACANCCATGCA	8
V3-Forward, labeled	/A647/CCAGACTCCTACGGGAGGCAG	9
V3-Forward	CCAGACTCCTACGGGAGGCAG	10
V3-Reverse, phosphorylated	/Phos/CGTATTACCGCGGCTGCTG	11
V3-Reverse	CGTATTACCGCGGCTGCTG	12

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[0081] As set forth above, the methods of the present disclosure generally include labeling amplicons with a signal-generating moiety, *e.g.*, a moiety that produces a fluorescent or chemiluminescent signal which can then be detected on individual microbeads. Well-known fluorophores including, but not limited to, fluoresceins, rhodamines, eosins, cyanines, boron-dipyrromethenes, and coumarins can be used as signal generating moieties for labeling. Affinity tags such as biotin or haptens such as digoxigenin can be used for detection with labeled detection reagents such as streptavidin or antibodies. Labeling of the amplicons can occur before, during, or after combination with the microbeads. In some embodiments, the labeling in step (iii) is conducted during the amplifying in step (i). For example, the amplification can be conducted using a forward primer containing a 5'-label (*e.g.*, a cyanine dye such as Cy5 or Alexa Fluor 647) as in SEQ ID NO: 1, SEQ ID NO: 5, and SEQ ID NO: 9. Oligonucleotides for preparation of labeled amplicons are available from commercial sources such as Integrated DNA

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Technologies, Inc. (Coralville, Iowa, USA). In some embodiments, labeled amplicons are prepared using forward primers containing a 5'-label and reverse primers containing a 5'-phosphate group, as in as in SEQ ID NO: 2, SEQ ID NO: 7, and SEQ ID NO: 11. Following amplification, remaining phosphorylated primers and corresponding amplicon strands containing 5'-phosphate groups can be digested by treatment with commercially-available bacteriophage lambda exonuclease. The exonuclease treatment can increase the amount of single stranded, fluorescently labeled amplicon available for highly efficient hybridization to capture polynucleotides on the microbeads.

[0082] Alternatively, labeling of the amplicons can occur after combination with the microbeads. For example, a DNA intercalator such as ethidium bromide or SYBR dye (e.g., SYBR Green I) can be used to identify amplicons that have formed double-stranded complexes with capture oligonucleotides on the microbeads.

Table 2. Capture Polynucleotide Sequences for Hybridization to V6 Amplicons.

Description	Species	Sequence	SEQ ID NO
v6Sau_1_1806 26, amine modified	<i>S. aureus</i>	/5AmMC12/TTTTTTTCTAGAGTT GTCAAAGGATGTCAAGATTGGT AAG	13
v6Sau_1_1806 26	<i>S. aureus</i>	CTAGAGTTGTCAAAGGATGTCAA GATTGGTAAG	14
v6Pae_1_1806 26, amine modified	<i>P. aeruginosa</i>	/5AmMC12/TTTTTTTACCTGTGTC TGAGTTCCCGAAGG	15
v6Pae_1_1806 26	<i>P. aeruginosa</i>	ACCTGTGTCTGAGTTCCCGAAGG	16
v6Pae_2_1806 26, amine modified	<i>P. aeruginosa</i>	/5AmMC12/TTTTTTTGGAAAGTT CTCAGCATGTCAAGGC	17
v6Pae_2_1806 26	<i>P. aeruginosa</i>	GGAAAGTTCTCAGCATGTCAAGG C	18
v3Abau_3_18, amine modified 0626	<i>A. baumannii</i>	/5AmMC12/TTTTTTTGCCGTATTA ACTAAATCCTCCTCGCTTAAAG	19
v3Abau_3_18	<i>A. baumannii</i>	GCCGTATTA ACTAAATCCTCCTC GCTTAAAG	20
v6Abau_1_18 0626, amine modified	<i>A. baumannii</i>	/5AmMC12/TTTTTTTGGAAAGTT TCTAGTATGTCAAGGCCAGG	21

Description	Species	Sequence	SEQ ID NO
v6Abau_1_180626	<i>A. baumannii</i>	GGAAAGTTTCTAGTATGTCAAGGCCAGG	22
v6Abau_2_180626, amine modified	<i>A. baumannii</i>	/5AmMC12/TTTTTTTGCACCTGTATCTAGATTCCCGAAGG	23
v6Abau_2_180626	<i>A. baumannii</i>	GCACCTGTATCTAGATTCCCGAAGG	24
v6Ecl_2_180626, amine modified	<i>E. cloacae</i>	/5AmMC12/TTTTTTTGTCTAAGTTCTCTGGATGTCAAGAGTAGG	25
v6Ecl_2_180626	<i>E. cloacae</i>	GCTAAGTTCTCTGGATGTCAAGAGTAGG	26
v6Efa_2_180626, amine modified	<i>E. faecium</i>	/5AmMC12/TTTTTTTGACCTGGTAAGGTTCTTTCGCGTTG	27
v6Efa_2_180626	<i>E. faecium</i>	GACCTGGTAAGGTTCTTTCGCGTTG	28
v6Efa_3_180626, amine modified	<i>E. faecium</i>	/5AmMC12/TTTTTTTAGAGTGGTCAAAGGATGTCAAGA	29
v6Efa_3_180626	<i>E. faecium</i>	AGAGTGGTCAAAGGATGTCAAGAA	30
v6Kpn_1_180626, amine modified	<i>K. pneumoniae</i>	/5AmMC12/TTTTTTTGTCTCAGAGTTCCCGAAGGCA	31
v6Kpn_1_180626	<i>K. pneumoniae</i>	GTCTCACAGTTCCCGAAGGCA	32
v6Kpn_2_180626, amine modified	<i>K. pneumoniae</i>	/5AmMC12/TTTTTTTGGAAAGTTCTGTGGATGTCAAGA	33
v6Kpn_2_180626	<i>K. pneumoniae</i>	GGAAAGTTCTGTGGATGTCAAGA	34
v6Sep_1_180626, amine modified	<i>S. epidermis</i>	/5AmMC12/TTTTTTTGTCACCTCTGTCCCCCGAAGG	35
v6Sep_1_180626	<i>S. epidermis</i>	GTCACCTCTGTCCCCCGAAGG	36
v6Sep_2_180626, amine modified	<i>S. epidermis</i>	/5AmMC12/TTTTTTTGAGGGGTCAAGAGATGTCAAGATTG	37
v6Sep_2_180626	<i>S. epidermis</i>	GAGGGGTCAAGAGATGTCAAGATTG	38
v6Spn_1_180626, amine modified	<i>S. pneumoniae</i>	/5AmMC12/TTTTTTTGTACCTCTGTCCCGAAGGAAA	39

Description	Species	Sequence	SEQ ID NO
v6Spn_1_1806 26	<i>S. pneumoniae</i>	GTCACCTCTGTCCCGAAGGAAA	40
v6Spn_3_1806 26, amine modified	<i>S. pneumoniae</i>	/5AmMC12/TTTTTTTGTAGAGCGGT CAGAGGGATGTCAAG	41
v6Spn_3_1806 26	<i>S. pneumoniae</i>	AGAGCGGTCAGAGGGATGTCAA G	42

Table 3. Capture Polynucleotide Sequences for Hybridization to V3 Amplicons.

Description	Species	Sequence	SEQ ID NO.
v3Eco_n, amine modified	<i>E. coli</i>	/5AmMC12/TTTTTTTGAGCAAAGG TATTAACCTTACTCCCTTCC	43
v3Eco_n	<i>E. coli</i>	GAGCAAAGGTATTAACCTTACTC CCTTCC	44
v3Sau_n, amine modified	<i>S. aureus</i>	/5AmMC12/TTTTTTTGATGTGCAC AGTTACTTACACATATGT	45
v3Sau_n	<i>S. aureus</i>	GATGTGCACAGTTACTTACACAT ATGT	46
v3Pae_n, amine modified	<i>P. aeruginosa</i>	/5AmMC12/TTTTTTTGTATTAACCT TACTGCCCTTCCTCCCAAC	47
v3Pae_n	<i>P. aeruginosa</i>	GTATTAACCTTACTGCCCTTCCTCC CAAC	48
v3Abau_n, amine modified	<i>A. baumannii</i>	/5AmMC12/TTTTTTTCTATCTCTA GGTAGCCGTATTAACCTAAA	49
v3Abau_n	<i>A. baumannii</i>	CTATCTCTAGGTAGCCGTATTAA CTAAA	50
v3Ecl_n, amine modified	<i>E. cloacae</i>	/5AmMC12/TTTTTTTAGGGATGAA CAGTTACTCTCATCCT	51
v3Ecl_n	<i>E. cloacae</i>	AGGGATGAACAGTTACTCTCATC CT	52
v3Efa_n, amine modified	<i>E. faecium</i>	/5AmMC12/TTTTTTTCGGCAGGGT TATTAACCCTGTCGC	53
v3Efa_n	<i>E. faecium</i>	CGGCAGGGTTATTAACCCTGTCG C	54
v3Kpn_n, amine modified	<i>K. pneumoniae</i>	/5AmMC12/TTTTTTTTCGACAAGG TTATTAACCTTATCG	55
v3Kpn_n	<i>K. pneumoniae</i>	CGACAAGGTATTAACCTTATCG	56

Description	Species	Sequence	SEQ ID NO.
v3Ngo_n, amine modified	<i>N. gonorrhoeae</i>	/5AmMC12/TTTTTTTCGGCCGCCG ATATTGGCAACGGCCTT	57
v3Ngo_n	<i>N. gonorrhoeae</i>	CGGCCGCCGATATTGGCAACGGC CTT	58
v3Sep_n, amine modified	<i>S. epidermis</i>	/5AmMC12/TTTTTTTGACGTGCAT AGTTACTTACACATTG	59
v3Sep_n	<i>S. epidermis</i>	GACGTGCATAGTTACTTACACAT TTG	60
v3Spn_n, amine modified	<i>S. epidermis</i>	/5AmMC12/TTTTTTTCAGTGTGAA CTTCCACTCTCACACTC	61
v3Spn_n	<i>S. pneumoniae</i>	CAGTGTGAACTTTCCACTCTCAC ACTC	62

Table 4. Capture Polynucleotides for Hybridization to Amplicons Generated from Environmental Samples.

Description	Sequence	Sample Type	SEQ ID NO.
#Ga0181610_1 0375322_v1	GGACTACAGGGGTATCTAATCCCTTTTG	SS (Salton Sea water)	63
#Ga0181610_1 0375322_v2	GGTGACAGGGGTTTACAATCCGAG	SS	64
#Ga0181610_1 0375322_v3	GCGTTGCACCATCAGGGTTT	SS	65
#Ga0181610_1 0397925_v1	CGGCTTCATGCAGTCGAGTTG	SS	66
#Ga0181610_1 0397925_v2	CAGTGTACTCCGCTCCGAAGAG	SS	67
#Ga0181610_1 0397925_v3	AGTAACTCCGAACAACGCTTGC	SS	68
#Ga0181610_1 0398315_v1	GAACTGTGGCTGGGTTTGATGAGATT	SS	69
#Ga0181610_1 0398315_v2	CATGCAGGCGAGTTTCAGCC	SS	70
#Ga0181610_1 0398315_v3	GTTTCGCTCCCCTTTGTCCCAA	SS	71
#Ga0181610_1 052119_v1	GTCAATTCATTTGAGTTTAACTTGCG	SS	72
#Ga0181610_1 052119_v2	AGCTCGCCAGTTTTGGATGCAG	SS	73
#Ga0181610_1	AGTCGACATCGTTTACGGCGTG	SS	74

Description	Sequence	Sample Type	SEQ ID NO.
052119 v3			
#Ga0181610_1 054535 v1	GAAGTGAAGATGGCTTTTGGAGATTTCG	SS	75
#Ga0181610_1 054535 v2	GAGCCAGGATCAAAGTCTCAGGTTT	SS	76
#Ga0181610_1 054535 v3	GTCTGGCCGACATCGTTTACGG	SS	77
#Ga0181610_1 054817 v1	TTAGCTTTTGTAGCCTTTTCCCTGCTG	SS	78
#Ga0181610_1 054817 v2	CGAAGTGAAGTTTTCAGTATCTAAAG C	SS	79
#Ga0181610_1 054817 v3	AGGAATTCCGCTTGCTTTTCCCG	SS	80
#Ga0181610_1 060662 v1	CAGTTACTAGTTTACCTAGGCAGC	SS	81
#Ga0181610_1 060662 v2	GAAGTGAAGATAGTGTTAAGGGATTTCG C	SS	82
#Ga0181610_1 060662 v3	ACGTAGAAGGGTTTATCCCAAACAAA A	SS	83
#Ga0181610_1 064264 v1	TACGACCGGTTTTTCGGGATTGG	SS	84
#Ga0181610_1 064264 v2	ATGTCAAAGGTGGGTAAAGTTTTTCG	SS	85
#Ga0181610_1 064264 v3	GACTACGACCGGTTTTTCGGGATTG	SS	86
#Ga0181610_1 076632 v1	GATCGCTCCCTTTTACCTCTCGG	SS	87
#Ga0181610_1 076632 v2	GGTAAGGTTCTTCGGTTTGCATCGAATT	SS	88
#Ga0181610_1 076632 v3	GTACTCCAGTCCGACGGTTTCG	SS	89
#Ga0181610_1 079111 v1	TGCAGCACCTGTGTTTAGGTT	SS	90
#Ga0181610_1 079111 v2	GTTCTAGCAAGCTAGCACTCTCATATTT	SS	91
#Ga0181610_1 079334 V1	CGGACTACGACGAGTTTTTTGGGATT	SS	92
#Ga0181610_1 081755 v1	ATAGGGCTCGGCTTCATGCG	SS	93
#Ga0181610_1 081755 v2	ATATCACTATAGGGCTCGGCTTCATGC	SS	94
#Ga0181610_1 081755 v3	TATAGGGCTCGGCTTCATGCG	SS	95
#Ga0181610_1	GAGACCGGCTTTTTTGGGATTTCG	SS	96

Description	Sequence	Sample Type	SEQ ID NO.
082181_v1			
#Ga0181610_1 082181_v2	AACTGAGACCGGCTTTTTGGGATTG	SS	97
#Ga0181610_1 082181_v3	tCtCCTTTCAGGAAGAGGCCCCCTTT	SS	98
#Ga0181610_1 087371_v1	CATCAGTCCTTTTTCCCCGACAAAAGG	SS	99
#Ga0181610_1 087371_v2	CCATCAGTCCTTTTTCCCCGACAAAAG	SS	100
#Ga0181610_1 087371_v3	tCtCGGTTGAGCCGTGGTATTTTACGC	SS	101
#Ga0181610_1 090741_v1	tCGCTACTGATCGTCGCCATGGTAAG	SS	102
#Ga0181610_1 09080141_v1	TTTCCTTACTCACCATGCAGTAAGTAAT	SS	103
#Ga0181610_1 09080141_v2	tCtCGTGCGCCAGTGTACTCTGCT	SS	104
#Ga0181610_1 0916949_v1	GTGCAGCACCTGTCTTTAGGTTCTTG	SS	105
#Ga0181610_1 0916949_v2	CGTGGACGGTAGCCTATTTAGCATT	SS	106
#Ga0181610_1 0916949_v3	ATGTTTTAGAGATTTGCTCCACCTCGC	SS	107
#Ga0181610_1 0937620_v1	GCGGTATTGCATCTTTTTGTCCTT	SS	108
#Ga0181610_1 0937620_v2	AGCCGTGCAGCACCTGTTTT	SS	109
#Ga0181610_1 0937620_v3	tCCGAAGTGAAGAGAAAGGTTTTGAGATTAGC	SS	110
#Ga0181610_1 0945623_v1	ACGGCCGGCTTTTTGCGA	SS	111
#Ga0181610_1 0945623_v2	GAGTTAAGCTCCAGGTTTTACGC	SS	112
#Ga0181610_1 0945623_v3	TACGGCCGGCTTTTTGCGATT	SS	113
#Ga0181610_1 0955219_v1	TACATTTAGTTTTTCTCCCTGCACCATG	SS	114
#Ga0181610_1 0955219_v2	GTACATTTAGTTTTTCTCCCTGCACCAT	SS	115
#Ga0181610_1 0955219_v3	tCAAAGTTGAGCTTCGGCTTTTCAC	SS	116
#Ga0181610_1 097186_v1	ACTGACAGAGTTTTACACCCCAAGGG	SS	117
#Ga0181610_1	CGATCCGAAGTGAAGATAGGTTTAAGA	SS	118

Description	Sequence	Sample Type	SEQ ID NO.
097186_v2	G		
#Ga0181610_1 097186_v3	CAGCGTGCTGATCTGCGTTTACTAG	SS	119
#Ga0181610_1 100471_v1	GCAACTCCCATTITTTGGGTTGGATG	SS	120
#Ga0181610_1 100471_v2	TGAACTGAGGACGGTTTTATGGATTTGC	SS	121
#Ga0181610_1 101123_v1	GAGCGTATGCGGTATTAGCGTAAGTTT	SS	122
#Ga0181610_1 101123_v2	ACGGTATTAGCGCAACCCCTTT	SS	123
#Ga0181610_1 101123_v3	GTTTGGCAACCCTTTGTACCGA	SS	124
#Ga0181610_1 108361_v1	ATGTCAAGGGTAGGTAAGGTTTTTCG	SS	125
#Ga0181610_1 114317_v1	GAACCTACGAACAGCTTTTTGAGATTCG C	SS	126
#Ga0181610_1 114317_v2	ATAATATCCGGTTTTAGCACTCCTTTCG	SS	127
#Ga0181610_1 114317_v3	AAGCCCTCCTTTAGCACCTTAGTTTT	SS	128
#Ga0181610_1 116582_v1	ATAGAGCTCGGCTTCATGCGGTATTAG	SS	129
#Ga0181610_1 116582_v2	TATAGAGCTCGGCTTCATGCGGTATTAG	SS	130
#Ga0181610_1 116582_v3	ATATCACTATAGAGCTCGGCTTCATGCG	SS	131
#Ga0181610_1 117382_v1	GAACCTGAGACGCACTTTTAGAGGTTGG	SS	132
#Ga0181610_1 117382_v2	TGCAGCACCTTGTTTCAGGTCATTG	SS	133
#Ga0181610_1 117382_v3	tCGCGTTTTCTTCCCAGATAAAAGCAGT TT	SS	134
Evi_Hyb1	CatAAATTCCAtgTaCCcCatCGGc	Purified <i>E. vietnamensis</i> DNA	135
Evi_Hyb2	CCGGAAACTTTCACCGCTGAC	Purified <i>E. vietnamensis</i> DNA	136
Phe_Hyb1	tgTcAcATTCCgCCTaCCTCTAgtGTAt	Purified <i>P. heparinus</i> DNA	137
Phe_Hyb2	cGTCAGTATCAAaggGCAcTgCgata	Purified <i>P. heparinus</i>	138

Description	Sequence	Sample Type	SEQ ID NO.
		DNA	
Mru_Hyb1	CTcCGaccgTCTAGCcTca	Purified <i>M. ruber</i> DNA	139
Mru_hyb2	TCGGTCTTTAGCATCGGACTTGAGAG	Purified <i>M. ruber</i> DNA	140
Eco_Hyb1	GgAATTcTACCcCCCTCTACGagAC	Purified <i>E. coli</i> DNA	141
#Ga0181608_1 001153_v1	GGGTATTATCCCCAGTTTTCGGGG	Oil (tar sands tailing pond, Alberta Canada)	142
#Ga0181608_1 0018514_v1	GCCTTGGTGGGCTTTTACCCC	Oil	143
#Ga0181608_1 0024138_v1	GAAGTGAAGAACGGTTTTTTCGATTAGC	Oil	144
#Ga0181608_1 0053911_v1	ATTCGACAGGGGTTTACGATCCGAA	Oil	145
#Ga0181608_1 009102_v1	CGGTATTAGCGGTCGTTTCCAACC	Oil	146
#Ga0181608_1 0091215_v1	CGAAGGAACGGACTATCTCTAGTCTTTT	Oil	147
#Ga0181608_1 013222_v1	GTAAAGCTGTGAGTTTTACGAACAAC G	Oil	148
#Ga0181608_1 015781_v1	GCCATGCAGCACCTTGTTTCATG	Oil	149
#Ga0181608_1 0192727_v1	GCTACTTGTGGAATTCCATTTTCTCCGT	Oil	150
#Ga0181608_1 0241723_v1	GGTACCGCCGCTTTTGAGAG	Oil	151
#Ga0181608_1 037542_v1	GAAGTGGGGCGCGCTTTTTG	Oil	152
#Ga0181608_1 062404_v1	TGAGCCAGGATCAAACCTCTCCAGTTTTT	Oil	153
#Ga0181608_1 073854_v1	TTGCATGTCAAGCCTTGGTAAGGTTTTT	Oil	154
#Ga0181608_1 115897_v1	GAAGTGGCGCCTCTTTTAAGGATTG	Oil	155
#Ga0181608_1 119449_v1	GGCTTCGGCACCGATAGGTTTT	Oil	156
#Ga0181608_1 140976_v1	TGAGGACGGCTTTTGGGATTGG	Oil	157
#Ga0181608_1 160854_v1	TGAGACTGGTTTTTTGAGATTAGCTTGG	Oil	158

[0083] Predetermined sequences in capture oligonucleotides can be selected by manual inspection of genetic data for bacteria of interest, or with the assistance of algorithmic tools. Algorithms for selected sequences to discriminate between two or more species of bacteria may include one or more steps of: (1) determining K-mer length (*e.g.*, a 5-, 6-, or 7-base length) to uniquely distinguish targets; (2) finding locations of uniquely identifying K-mers; (3) identifying maximally separated K-mers; (4) performing sliding window probe design (similar to primer design); and (5) re-running candidate sequences for further pruning and selection. A graphical representation of an exemplary algorithmic processes is depicted in FIG. 2A. Identifying sequences can be selected in this manner for detection of pathogens associated with hospital-acquired infections, including but not limited the pathogens listed in Table 2, Table 3, and Table 6 as well as for the detection of microbes relevant to the safety and/or quality of water, soil, foods, agricultural products, and natural resources, including but not limited to those listed in Table 4.

[0084] The assay according to the present disclosure can also be used for gram typing of bacteria. In one embodiment, the V6 variable region may be used for the detection of Gram positive or negative bacteria. Exemplary sequences that can be used in such embodiments are described, for example, in Yang, S., *et al.* "Rapid PCR-Based Diagnosis of Septic Arthritis by Early Gram-Type Classification and Pathogen Identification" J CLIN MICROBIOL, Apr. 2008, Vol 47(4), p. 1386–1390. A non-limiting example of a sequence that can be used as a capture polynucleotide for identifying Gram positive organisms includes: AGGTGGTGCATGGTTGTCGTCAGC. A non-limiting example of a sequence that can be used as a capture polynucleotide for identifying Gram negative organisms includes: ACAGGTGCTGCATGGCTGTCGTCAGCT. In one aspect, these probes are modified at the 5' end to have an amine (and spacer) for conjugation reactions. Exemplary V6 primers suited for use in such embodiments are described in Table 1.

[0085] In addition to determining the species and/or strain of microbes present in a particular sample, the methods of the present disclosure can also be used for identifying further characteristics relevant to pathogenicity and/or virulence, *e.g.*, the presence of antibiotic-resistance bacteria. Many strains of methicillin-resistant *S. aureus* (MRS), for example, are known to possess the *mecA* gene located in a staphylococcal cassette chromosome *mec*

(SCCmec). See, e.g., Song, et al. (FEBS Lett. 1987, 221(1): 167–171); Baba et al. (The Lancet, 2002, 359:1819–1827). MRSA strains can be identified by using microbeads that contain a capture polynucleotide having a sequence or sequences present in *mecA* and/or SCCmec. In some embodiments, at least one of the microbe identifying sequences in the amplicons comprises an antibiotic resistance gene sequence. Capture polynucleotides employed in the present disclosure can contain identifying sequences present in a variety of acquired antibiotic resistance genes including, but not limited to, aminoglycoside resistance genes, quinolone resistance genes, and tetracycline resistance genes. Examples of such acquired antibiotic resistance genes are described, e.g., by van Hoek (Front. Microbiol. 2011, 2: 203) and Böckelmann (Applied and Environmental Microbiology 2008, 75: 154-63), and sequences from such genes can be used in capture polynucleotides on microbeads of the present disclosure. This can include directly targeting extended-spectrum beta-lactamase genes, such as TEM-1. For example, in *Staphylococcus aureus* (“Sau”), primers and hybridization oligonucleotides may be designed to target fragments of *mecA* (encoding methicillin resistance), *aacA-aphD* (aminoglycoside resistance), *tetK*, *tetM* (tetracycline resistance), *erm(A)*, *erm(C)* (macrolide-lincosamide-streptogramin B resistance), *vat(A)*, *vat(B)*, and *vat(C)* (streptogramin A resistance). A non-limiting example of a primer and hybridization set for *mecA* is:

forward primer: CATTGATCGCAACGTTCAATTTAAT (SEQ ID NO:161);

reverse primer: TGGTCTTTCTGCATTCCTGGA (SEQ ID NO:162); and

hybridization oligonucleotide: CTATGATCCCAATCTAACTTCCACATACC (SEQ ID NO:163).

[0086] Methods of the present disclosure may also be applied to detect viruses. In some embodiments, the virus is influenza A virus including subtype H1N1, influenza B virus, influenza C virus, rotavirus A, rotavirus B, rotavirus C, rotavirus D, rotavirus E, SARS coronavirus, including SARS-CoV-1 and SARS-CoV-2, human adenovirus types (HAdV-1 to 55), human papillomavirus (HPV) Types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, parvovirus B19, molluscum contagiosum virus, JC virus (JCV), BK virus, Merkel cell polyomavirus, coxsackie A virus, norovirus, Rubella virus, lymphocytic choriomeningitis virus (LCMV), yellow fever virus, measles virus, mumps virus, respiratory syncytial virus, rinderpest virus, California encephalitis virus, hantavirus, rabies virus, ebola virus, dengue virus (DENV), zika

virus (ZIKV), marburg virus, herpes simplex virus- 1 (HSV-1), herpes simplex virus-2 (HSV-2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), herpes lymphotropic virus, roseolovirus, Kaposi's sarcoma-associated herpesvirus, hepatitis A (HAV), hepatitis B (HBV), hepatitis C (HCV), hepatitis D (HDV), hepatitis E (HEV), human immunodeficiency virus (HIV), Human T-lymphotropic virus Type I (HTLV-1), Friend spleen focus-forming virus (SFFV) or Xenotropic MuLV-Related Virus (XMRV).

[0087] For example, methods of the present disclosure can be used to detect the SARS-CoV-2 virus. Exemplary primer and capture polynucleotide sequences for use in such embodiments can be obtained from the United States Department of Health and Human Services Centers for Disease Control and Prevention. In one aspect, suitable sequences are modified at the 5' end with an amino-terminated 12-carbon spacer (the "5AmMC12" modification) and an oligo(T) spacer for use in the assay according to the present disclosure.

[0088] In one embodiment, the method of the present disclosure utilizes all of these capture polynucleotide in parallel using beads of different spectral codes to target the different viral sequences. In another embodiment, these capture polynucleotide are combined with capture polynucleotides targeting other viruses, such as the SARS-CoV-1, influenza A, influenza B, dengue virus or zika virus. For example, a flu panel may include capture polynucleotides for targeting influenza A, influenza B, SARS-CoV-1, and SARS-CoV-2.

[0089] In another embodiment, a panel could include viral and bacterial capture polynucleotides to simultaneously test for viruses and bacterial co-infections. For example, the panel may include capture polynucleotides for influenza A, influenza B, SARS-CoV-1, and SARS-CoV-2 and capture polynucleotides for bacterial species, such as the bacterial capture polynucleotides described above.

[0090] Similarly, it is possible to extend the method to the analysis of host response genes, such as a procalcitonin gene, a c-reactive protein gene, a tumor necrosis factor alpha gene, or an interleukin gene. Amplicons may be prepared using mRNA from cells, or cell-free RNA. In some such embodiments, reverse transcription may be performed followed by PCR or quantitative PCR (*e.g.*, RT-qPCR). The PCR product can then be hybridized to the beads, optionally after a treatment step with lambda exonuclease. This may be done in parallel with

PCR for amplification of 16S variable regions and detection of pathogens. A non-limiting example of a primer and hybridization set for procalcitonin is:

forward primer: 5'-TCTAAGCGGTGCGGTAATCT-3' (SEQ ID NO:166);

reverse primer: 5'-TTCTTTCCAGGTGCTCCAAC-3' (SEQ ID NO:167); and

5 hybridization probe: 5'- TGGGCACATACACGCAGGA-3' (SEQ ID NO:168).

[0091] Methods of the present disclosure may also be applied to multiplexed detection of fungal species in the case of fungal infections. Fungal species may be targeted in parallel with bacterial and/or host-response genes. Analogous to targeting the conserved regions of the 16S ribosomal subunit for PCR followed by specific and multiplexed hybridization to capture
10 polynucleotides with sequences that target the inter-spaced hypervariable regions (*e.g.*, V1, V6), the conserved and variable regions of the 18S ribosomal subunit gene can be targeted to identify different fungal species. Alternatively, the inter-transcribed spacer may be targeted for species identification. For example, fungal species of interest include *Fusarium sp.*, *Cladosporium sp.*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Candida albicans*, *Trichosporon asahii*, and
15 *Glomerella cingulate*. A non-limiting example of a primer and hybridization set for targeting the 18S ribosomal subunit gene with detection of *Aspergillus fumigatus* and *Candida albicans* is:

forward primer: TTGGTGGAGTGATTTGTCTGCT (SEQ ID NO:169);

reverse primer: TCTAAGGGCATCACAGACCTG (SEQ ID NO:170);

20 hybridization probe (*A. fumigatus*): TCGGCCCTTAAATAGCCCGGTCCGC (SEQ ID NO: 171);

hybridization probe (*C. albicans*): TTAACCTACTAAATAGTGCTGCTAGC (SEQ ID NO: 172).

[0092] Capture polynucleotides may contain spacer units between the sequence used for hybridization to amplicons and the functional group used for conjugation to the microbeads.
25 Spacer units may contain, for example, hexaethylene glycol, triethylene glycol, alkylene moieties (*e.g.*, dodecane-1,12-diyl, hexane-1,6-diyl), photo-cleavable spacers, reducible spacers, and other linking groups known in the art. *See, e.g.*, Hermanson, *Bioconjugate Techniques* (3rd Ed.), Academic Press, 2013. Spacer units may also contain nucleotides such as oligo(T) sequences

e.g., TTTTTTTT (SEQ ID NO:159). In some embodiments, the capture polynucleotides contain a 5'-amine group for conjugation to microbeads, as well as an optional spacer moiety linking the 5'-amine group to the amplicon hybridization sequence. As used herein the designation "5AmMC12" refers to a 5'-amine group having the formula $\text{NH}_2(\text{CH}_2)_{12}-$, which is bonded to the 5'-phosphate group of the capture polynucleotide. In some embodiments, the spacer unit is /5AmMC12/TTTTTTTT (SEQ ID NO:160).

B. Preparation of Microbeads

[0093] Microbeads of the present disclosure may include different types of lanthanide nanoparticles. As set forth above, the nanoparticles have at least one dimension (*e.g.*, length, width, or circumference) ranging from 1 to 1,000 nm. A microbead may include one or more different lanthanide nanoparticles, *e.g.*, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, or 10 or more, wherein each lanthanide nanoparticle has a different luminescence emission spectrum upon excitation. For example, in some embodiments, the microbeads disclosed herein may include from 1 to 10, from 2 to 10, from 3 to 10, from 4 to 10, from 5 to 10, from 6 to 10, from 7 to 10, from 8 to 10, or from 9 to 10 types of lanthanide nanoparticles, wherein each lanthanide nanoparticle has a different luminescence emission spectrum upon excitation. Signals from the combined luminescence spectra make up the spectral signature of a particular microbead, and are mapped to a unique spectral signature 'code' during code deconvolution.

[0094] Microbeads used in the methods described herein typically have capture polynucleotides immobilized to polymers in the microbead. In general, the capture polynucleotides are designed to contain predetermined sequences such that at least some of the capture polynucleotides comprise a sequence substantially complementary to a microbe-identifying sequence of one or more amplicons. Beads sets used in the methods generally contain many different predetermined sequences, although each copy of the capture polynucleotide on any one microbead will generally have the same predetermined sequence. In some embodiments, the plurality of microbeads comprises at least 5 (*e.g.*, at least 10, 15, 20, 25, 30, 35, 40, 45, or 50) different spectral signatures and immobilized capture polynucleotides comprising at least 5 (*e.g.*, at least 10, 15, 20, 25, 30, 35, 40, 45, or 50) different predetermined sequences. In some embodiments, the plurality of microbeads comprises at least 20 (*e.g.*, 20-25)

different spectral signatures and immobilized capture polynucleotides comprising at least 20 (*e.g.*, 20-25) different predetermined sequences. In some embodiments, the plurality of microbeads comprises at least 40 (*e.g.*, 40-50) different spectral signatures and immobilized capture polynucleotides comprising at least 40 (*e.g.*, 40-50) different predetermined sequences.

- 5 In some embodiments, the plurality of microbeads comprises 24 different spectral signatures and immobilized capture polynucleotides comprising 24 different predetermined sequences. In some embodiments, the plurality of microbeads comprises 48 different spectral signatures and immobilized capture polynucleotides comprising 48 different predetermined sequences.

[0095] Microbeads according to the present disclosure may contain as few as two different
10 predetermined sequences or as many as tens of thousands of predetermined sequences depending, in part, on the complexity of the samples intended for study. Accordingly, combining amplicons generated from the sample with the microbeads may include hybridizing amplicons to at least 1, 5, 10, 25, 50, 75, 100, 500, 1,000, 5,000, 10,000, or more microbeads having different spectral signatures and different capture polynucleotides. In some
15 embodiments, the combining in step (ii) comprises hybridizing amplicons to at least 50 microbeads (or 50 different populations of microbeads) having different spectral signatures and different capture polynucleotides. In some embodiments, the magnitude of signal detected from an individual microbead in step (iv) corresponds to the amount of the amplicons captured on the microbead.

20 [0096] Lanthanide spectral signatures employed in the methods of the present disclosure include emitted light in the range of 350–850 nm (*e.g.*, 400–800 nm), exhibiting one or more peaks associated with lanthanide luminescence. Lanthanide nanoparticle spectra are typically characterized by narrow emission bands (also referred to as “signals”) in the visible region, making one species of material easily distinguishable from another. A “specific” lanthanide
25 spectral signature in a microbead or other material can therefore be designed based on the particular identity and relative amounts of lanthanides in the microbead. In some embodiments, each of the lanthanide spectral signatures comprises an Eu signal, a Dy signal, an Sm signal, a Ce signal, a Tb signal, a La signal, a Pr signal, an Nd signal, a Gd signal, an Ho signal, an Er signal, a Tm signal, a Yb signal, a Pm signal, an Lu signal, or a combination thereof. The microbeads of
30 the present disclosure generally include one or more different lanthanide nanoparticles as

discussed herein and one or more polymers, copolymers, or combinations thereof. In some embodiments, each microbead further comprises a crosslinked polymer, wherein the capture polynucleotides are covalently bonded to the crosslinked polymer. In some embodiments, the crosslinked polymer is a hydrogel-forming polymer (*e.g.*, poly(ethylene glycol)) which can
5 evenly and irreversibly entrap the lanthanide nanoparticle materials within the microbead. The lanthanide nanoparticles themselves may be coated with a polymer such as poly(acrylic acid) as described in more detail below.

[0097] In some embodiments, the nanoparticles include a lanthanide and a host lattice.

Lanthanides which may be incorporated into the lanthanide nanoparticles include, for example,
10 Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, La, combinations thereof, compounds containing Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, La and combinations thereof, and ions of Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, La and combinations thereof. Host lattices employed in the nanoparticles generally contain constituent atoms packed in a regularly ordered, repeating pattern which can accommodate the incorporation of lanthanide atoms or ions.

15 The lattice can be crystalline, which a structure that is, *e.g.*, triclinic, monoclinic, orthorhombic, tetragonal, trigonal, hexagonal, or cubic. A crystalline lattice may contain one or more regions, *i.e.*, grains, with distinct crystal boundaries. The crystalline lattice may, in some instances, contain two or more crystal geometries. A number of suitable host lattices can be utilized in the lanthanide nanoparticles. For example, lanthanide dopants may be incorporated into a host lattice
20 to provide lanthanide-doped yttrium orthovanadate (YVO₄), lanthanide-doped oxides (*e.g.*, doped ZrO₂, doped TiO₂, doped BaTiO₃), lanthanide-doped halides (*e.g.*, doped LaF₃), lanthanide-doped phosphates (*e.g.*, doped LaPO₄, doped LuPO₄, or doped YbPO₄), and lanthanide-doped strontium borates (*e.g.*, SrB₄O₇, SrB₆O₁₀, and Sr₄B₁₄O₂₅), among others.

[0098] Lanthanide nanoparticles according to the present disclosure may be prepared using
25 methods such as those described by Xu et al. (*Solid State Communications*, 2004, 130:465-468), Choi et al. (*Journal of Luminescence*, 2010, 130:549-553), Wang et al. (*Angewandte Chemie International Edition*, 2008, 47:906-909), and Nguyen, et al. (2017) *Advanced Optical Materials*, 5(3): 1600548, the disclosures of which are incorporated by reference herein in their entirety. As a non-limiting example, one volume of aqueous lanthanide dopant solution (*e.g.*, Sm(NO₃)₃,
30 Dy(NO₃)₃, Eu(NO₃)₃; 0.1 M), can be combined with 10-20 volumes of an yttrium salt solution

(*e.g.*, $\text{Y}(\text{NO}_3)_3$, 0.1 M) and added portion-wise to an 10-100 additional volumes of osmogen solution (*e.g.*, ~2000 kDa PEG, 10% w/w), optionally containing a bismuth salt such as $\text{Bi}(\text{NO}_3)_3$. A solution of matrix material (*e.g.*, 10-100 volumes of Na_3VO_4 , 0.1 M) is added portion-wise prior to microwave heating (*e.g.*, 180 °C) for 5-120 min. Following heating, the resulting white material can be washed and resuspended (*e.g.*, in water with optional polyacrylic acid (1000-2000 kDa; 1-10% v/v)), with or without sonication and/or filtering (*e.g.*, through 0.45- μm PTFE filters) filters to obtain the final nanoparticles, 25-250 nm in size (*e.g.*, 30-160 nm), as milky white solutions with concentrations ranging from about 5 mg/mL to about 500 mg/mL.

[0099] Lanthanide nanoparticles according to the present disclosure may be up-converting or down-converting lanthanide nanoparticles. Suitable up-converting lanthanide nanoparticles may include, for example, $\text{NaGdF}_4:\text{Tm}$; $\text{NaGdF}_4:\text{Ln}$; NaGdF_4Yb ; NaGdF_4Er ; $\text{NaGdF}_4\text{Yb, Er}$; $\text{NaYF}_4:\text{Er}$; $\text{NaYF}_4:\text{Yb}$; $\text{NaYF}_4:\text{Er, Yb}$; $\text{NaYF}_4:\text{Tm, Yb}$; $\text{LaF}_3:\text{Yb, Tm}$; $\text{LaF}_3:\text{Yb, Er}$; and $\text{LaF}_3:\text{Yb, Ho}$ nanoparticles. Suitable down-converting lanthanide nanoparticles may include, for example, $\text{YVO}_4:\text{Eu}$; $\text{YVO}_4:\text{Dy}$; and $\text{YVO}_4:\text{Sm}$ nanoparticles. It should be noted that the above referenced lanthanides may be incorporated into the nanoparticles as their respective ions. Materials may be added during preparation of the lanthanide nanoparticles to increase their UV absorption, for downconverters, or IR absorption, for upconverters. For example, in some embodiments bismuth is incorporated into the lanthanide nanoparticles to increase their UV absorption.

[0100] In some embodiments, lanthanide nanoparticles as disclosed herein may be modified (*e.g.*, covered or coated) in a suitable material to facilitate formation of a stable colloid suspension of the lanthanide nanoparticles in a carrier fluid. Suitable materials may include materials which prevent aggregation of the lanthanide nanoparticles in the carrier fluid (*e.g.*, H_2O) and/or facilitate maintenance of a nanoparticle form of the lanthanide nanoparticles. For example, suitable materials which may be used to cover or coat the lanthanide nanoparticles may include polyethyleneimine (PEI), polyacrylic acid (PAA), sodium citrate, or citric acid. Polyethyleneimine (PEI) may be suitable for use, *e.g.*, as a coating material in order to make the nanophosphors more compatible with a monomer mixture. In some embodiments, the

nanoparticles are coated with PAA. Advantageously, PAA can enhance the photostability of the nanophosphors in addition to facilitating stable colloid formation.

[0101] Accordingly, some embodiments of the present disclosure provide a plurality of microbeads wherein each of the microbeads comprises a plurality of lanthanide nanoparticles. In some embodiments, the lanthanide nanoparticle comprises a lanthanide-doped host lattice. In some embodiments, the host lattice is yttrium orthovanadate, lanthanum phosphate, or a combination thereof.

[0102] The microbeads of the present disclosure can contain a variety of polymers. In some embodiments, the polymers form microbeads upon polymerization via, for example, a thermal- or photo-initiated polymerization process. Such polymers include, but are not limited to, polyacrylates, polyacrylamides, polymethacrylates, polymethacrylamides, polystyrenes, polythiol-enes, polyurethanes, epoxy resins, polysaccharides (such as agarose), as well as copolymers (*e.g.*, random copolymers or block copolymers) or combinations of two or more of the above. Suitable polymers also include polysiloxanes, polyethers (*e.g.*, polyethylene glycol (PEG)), polyvinylpyrrolidones, vinyl ethers, vinyl acetates, polyimides, polysulfones, polyamic acids, polyamides, polycarbonates, polyesters, and copolymers or combinations of two or more of the above. The polymers may be provided in monomer form during the microbead preparation process, and these monomers may be polymerized to form the above polymers, copolymers or combinations thereof in the spectrally encoded microbeads of the present disclosure. Suitable monomers may include those which can be polymerized *in situ* alone or with a cross-linking agent to form a cross-linked resin.

[0103] In some embodiments, polyacrylate or polyacrylamide microbeads can be prepared using monomers which contain functionalized PEGs. The functionalized PEG can contain a polymerizable functional group on each end of the PEG chain, *e.g.*, a PEG-diacrylate or a PEG-diacrylamide for formation of crosslinked polymers that contact the lanthanide nanoparticles (*e.g.*, a crosslinked PEG that contacts PAA-coated lanthanide nanoparticles). Alternatively, the functionalized PEG can contain a polymerizable functional group on one end of the PEG chain and an orthogonal reactive moiety on the other end of the PEG chain. The orthogonal reactive moiety can be used for the attachment of oligonucleotides or other elements (*e.g.*, dyes, labels, or the like). Examples of orthogonal reactive moieties include, but are not limited to, amines,

carboxylates, thiols, activated esters (*e.g.*, N-hydroxysuccinimidyl (NHS) esters, sulfo-NHS esters, and pentafluorophenyl (PFP) esters); carbodiimides; maleimides; halogenated acetamides; hydroxymethyl phosphines; aryl azides; imidoesters; isocyanates; vinyl sulfones; pyridyl disulfides; benzophenones; azides; alkynes (including linear alkynes and cycloalkynes); and tetrazines.

[0104] In some embodiments, a suitable monomer for use in preparation of the microbeads is selected from a PEG diacrylamide (PEG-DAM), a PEG monoacrylamide-monoamine (PEG-AM) and a PEG-monoacrylamide-monoBoc. Such monomers can contain any suitable branched or linear PEG. In some embodiments, the PEG is a linear polymer having a weight average molecular weight ranging from 500 g/mol to about 10,000 g/mol (*e.g.*, about 700 g/mol, about 2000 g/mol, or about 5,000 g/mol). If necessary, number average and weight average molecular weight values can be determined by gel permeation chromatography (GPC) using polymeric standards (*e.g.*, polystyrene or like material).

[0105] Additional monomers which may be utilized in the microbeads may include, *e.g.*, monomers which are capable of participating in thiol-ene thiol-yne reactions, *e.g.*, pentaerythritol tetrakis(3-mercaptopropionate) (TT); diallyl phthalate (DAP); 1,3,5-triallyl-1,3,5-triazine-2,4,6(1H,3H,5H)-trione (TTT); 1,7-octadiyne (OY); mercaptoacetic acid (MA); allylamine (AA), pentaerythritol triallyl ether (PTE) and propargylamine (PA). These monomers find use, for example, in photo-initiated polymerization processes. For additional discussion of thiol-ene thiol-yne reactions and monomers suitable for use therein, *see, e.g.*, Prasath et al. (*Polym. Chem.*, 2010 1: 685-692), the disclosure of which is incorporated by reference herein.

[0106] The microbeads can be prepared using microfluidic devices as described, for example, in U.S. Pat. No. 10,241,045, U.S. Pat. Appl. Pub. No. 2015/0192518, and Nguyen, et al. (*Adv Opt Mater.* 2017, 5, 1600548), which are incorporated herein by reference in their entirety.

Preparation of the microbeads may include, for example: (i) mixing at least two fluids into a first solution, wherein each fluid comprises a polymerizable component (*e.g.*, a polymer or monomer), a polymerization initiator, and a different lanthanide nanoparticle; (ii) forming droplets from the solution; and (iii) subjecting the droplets to polymerization conditions, thereby producing a set of polymeric microbeads embedded with at least two different lanthanide nanoparticles. In some embodiments, the relative concentrations of the lanthanide nanoparticles

are substantially equal (*i.e.*, not significantly different) among the polymeric microbeads in the set. Additional sets of microbeads can be prepared by mixing the fluids into additional solutions, wherein the concentration of at least one of the lanthanide nanoparticles in the addition solutions is different than the concentrations of the nanoparticles in (i) above, and conducting the droplet-forming steps and polymerization steps as set forth above.

[0107] The lanthanide nanoparticle contained in each fluid may be present at a concentration of from about 1 mg/mL to about 250 mg/mL, *e.g.*, from about 5 mg/mL to about 250 mg/mL, from about 10 mg/mL to about 250 mg/mL, from about 20 mg/mL to about 250 mg/mL, from about 30 mg/mL to about 250 mg/mL, from about 40 mg/mL to about 250 mg/mL, from about 50 mg/mL to about 250 mg/mL, from about 60 mg/mL to about 250 mg/mL, from about 70 mg/mL to about 250 mg/mL, from about 80 mg/mL to about 250 mg/mL, from about 90 mg/mL to about 250 mg/mL, from about 100 mg/mL to about 250 mg/mL, from about 150 mg/mL to about 250 mg/mL, or from about 200 mg/mL to about 250 mg/mL.

[0108] Where a polymerization method is utilized to form the spectrally encoded polymeric microbeads, a suitable polymerization initiator (*e.g.*, a photoinitiator or thermal initiator) may be utilized which is compatible with the polymerizable components and the polymerization conditions. For example, where a UV polymerization process is utilized, a suitable initiator may include a compound that, when exposed to UV light, undergoes a photoreaction, producing reactive species that are capable of initiating polymerization. Exemplary photoinitiators may include, *e.g.*, acetophenones, benzyl and benzoin compounds, benzophenone, cationic photoinitiators, and thioxanthenes. In some embodiments, a photoinitiator such as 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure® 2959) or lithium phenyl-2,4,6-trimethylbenzoylphosphine (LAP) is utilized. Suitable thermal initiators may include, for example, azo compounds, peroxides or hydroperoxides, persulfates, and the like.

[0109] The step of forming droplets from the solution may include, for example, contacting a hydrophilic solution containing the polymerizable component with a hydrophobic solvent (*e.g.*, mineral oil or water-immiscible organic solvent, *e.g.*, octanol) such that droplets are formed. Alternatively, a hydrophobic solvent (*e.g.*, mineral oil or water-immiscible organic solvent, *e.g.*, octanol) can be used to form the solution containing the polymerizable component, and droplets can be formed by contacting the solution with a hydrophilic carrier fluid (*e.g.*, water). These

steps may be accomplished, for example, by introducing the solution containing the polymerizable component into a flowing stream of the carrier fluid. Any suitable device and/or method for droplet formation may be utilized to form droplets in the context of the present disclosure, including, *e.g.*, the utilization of flow focusing nozzles. *See, e.g.*, Ward et al.

5 (*Electrophoresis*, 2005, 26:3716-3724), the disclosure of which is incorporated by reference herein. The droplet size may be modulated by adjusting the pressure used to form the droplet, *e.g.*, at the interface of the solution and the hydrophobic carrier fluid. In addition, droplet size may be modulated by adjusting the geometry, *e.g.*, size and shape, of the microfluidic device channels. One or more stabilizers or surfactants may be added to one or more of the fluids to
10 prevent droplet merging and sticking of droplets to the walls of the microfluidic device. Suitable surfactants may include, for example, Abil® EM90 (a silicon based emulsifier; CAS No. 144243-53-8) and Span™ 80 (CAS No. 1338-43-8), among others.

[0110] In some embodiments, monomer input solutions for microbead synthesis contain purified water, monomer (*e.g.*, 700 MW PEG-diacrylate, 40-60% w/w), initiator (*e.g.*,
15 photoinitiator Irgacure® 2959, 1-10% w/w), Sigma-Aldrich), and lanthanide nanoparticles at various concentrations. Droplets can be formed by introducing the input solutions in a continuous flowing stream of hydrophobic fluid (*e.g.*, light mineral oil), with or without surfactants to reduce or eliminate merging of droplets (*e.g.*, Abil® EM90 (Evonik Industries, Germany), Span™ 80 (Sigma-Aldrich), 0.05%-5% w/w).

20 [0111] The step of subjecting the droplets to polymerization conditions may include, for example, exposing the droplets to UV radiation or elevated temperatures to initiate polymerization. Other known polymerization methodology can be employed, provided that it is compatible with the polymers and/or monomer components to be polymerized. Examples of such methodology include, but are not limited to, thiol-ene polymerization, redox-initiated
25 polymerization, and controlled radical polymerization processes such as reversible addition-fragmentation chain transfer (RAFT) polymerization, atom transfer radical polymerization (ATRP), and nitroxide-mediated polymerization (NMP). *See also, e.g.*, Piskin et al. (*J. of Biomaterials Science Polymer Edition* 1994, 5: 451-471), the disclosure of which is incorporated by reference herein.

[0112] In some embodiments, droplets are exposed to radiation (e.g., UV radiation) by localizing the radiation exposure onto a microfluidic device such that the droplets are only irradiated after they have been formed on the microfluidic device and before they exit the microfluidic device. Radiation localization may be achieved using an inverted microscope by mounting the microfluidic device on the microscope stage. For example, UV illumination may occur through the objective onto a very small area and an additional aperture within the microscope UV light path may further restrict the UV irradiation to a specific area of the microfluidic device. Alternatively, UV illumination may occur after the droplets exit the microfluidic device.

[0113] Methods which do not require polymerization may also be used to form the spectrally encoded polymeric microbeads. For example, a polymer precipitation method may be utilized in which a pre-formed polymer (e.g., a mid- to high-molecular weight polymer) is dissolved in a suitable solvent (e.g., water) along with dispersed lanthanide nanoparticles. Droplets of this solution can be formed by introducing the solution into an immiscible carrier fluid (e.g., a hydrophobic carrier fluid, e.g., mineral oil). The immiscible carrier fluid and polymer should be selected such that the polymer does not dissolve in the immiscible carrier fluid, and the immiscible carrier fluid is capable of accepting the solvent leaching from the droplet as the polymeric microbead is formed through precipitation. Additional solvent-immiscible carrier fluid combinations may include, e.g., dichloromethane as a solvent and poly(vinyl alcohol) (PVA) as an immiscible carrier fluid. Microbead preparation methods utilizing a dichloromethane-poly(vinyl alcohol) (PVA) combination are described, for example, in Berkland et al. (*Journal of Controlled Release*, 2002, 73:59-74; *Journal of Controlled Release*, 2004, 94:129-141), the disclosures of which are incorporated by reference herein.

[0114] The steps of mixing at least two fluids can occur either before or after droplet formation depending on the particular microfluidic device architecture utilized. For example, where a herringbone type mixing architecture is utilized the two fluids may be mixed prior to droplet formation. Alternatively, where a zig-zag type mixing architecture is utilized droplets containing unmixed lanthanide nanoparticles may be formed and subsequently mixed to distribute the lanthanide nanoparticles within a droplet. Accurate programming of spectral codes for the spectrally encoded microbeads may be facilitated by precisely controlling the flow from each of

the lanthanide nanoparticle fluid inputs as previously described, for example, in U.S. Pat. No. 10,241,045.

C. Functionalization of Microbeads

[0115] In some embodiments, microbeads are functionalized with reactive groups for
5 conjugation of capture polynucleotides. Typically, a population of microbeads having the same spectral signature (*e.g.*, a population ranging up to thousands of beads) is carried through one or more functionalization steps such that (i) each microbead in the population having the same spectral signature is conjugated to capture polynucleotides having the same sequence, and (ii) the number of capture polynucleotides conjugated to each microbead in the population is
10 substantially the same. This process can be conducted, for example, with a microfluidic device having an in-line fraction collector for pooling of microbead populations by spectral signature. Sub-populations may be reserved for use in different chemical steps or for use in characterizing microbial populations. In some embodiments, for example, sub-populations of microbeads containing different spectral signatures may be combined such that a plurality of around 1,000
15 microbeads is used for combination with amplicons and each spectral signature is represented 50-100 times in the plurality.

[0116] In some embodiments, microbeads are functionalized with reactive groups that can then be conjugated to capture polynucleotides via one or more click reactions. As used herein, “click reaction” refers to a chemical reaction characterized by a large thermodynamic driving force that
20 usually results in irreversible covalent bond formation. Examples of click reactions include thiol-ene reactions, such as the Michael addition of a thiol to a maleimide or other unsaturated acceptor; [3+2] cycloadditions, such as the Huisgen 1,3-dipolar cycloaddition reaction of an azide and an alkyne; [4+1] cycloaddition reactions between an isonitrile and a tetrazine; the Staudinger ligation between an azide and an ester-functionalized phosphine or an alkanethiol-
25 functionalized phosphine; Diels-Alder reactions (*e.g.*, between a furan and a maleimide); and inverse electron demand Diels-Alder reactions (*e.g.*, between a tetrazine and a dienophile such as a strained transcyclooctene).

[0117] In some embodiments, the microbeads contain carboxylate groups for bonding to amine-functionalized capture polynucleotides. For example, acrylate groups in microbeads can
30 be coupled to a thiol-functionalized carboxylic acid (*e.g.*, 3-mercaptopropionic acid) in the

presence of a base such as pyridine, *N,N*-diisopropylamine (DIPEA), or 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU). The carboxylates can then be reacted with amine-functionalized oligonucleotides using one or more coupling reagents such as a carbodiimide (*e.g.*, *N,N'*-dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), a phosphonium salt (HOBt, PyBOP, HOAt, etc.), an aminium/uronium salt, a pyridinium coupling reagent (*e.g.*, Mukaiyama's reagent, pyridinium tetrafluoroborate coupling reagents, etc.), a polymer-supported reagent (*e.g.*, polymer-bound carbodiimide, polymer-bound TBTU, polymer-bound 2,4,6-trichloro-1,3,5-triazine, polymer-bound HOBt, polymer-bound HOSu, polymer-bound IIDQ, polymer-bound EEDQ, etc.), or the like (*see, e.g.*, El-Faham, *et al. Chem. Rev.*, 2011, 111(11): 6557–6602; Han, *et al. Tetrahedron*, 2004, 60:2447-2467).

[0118] In some embodiments, the capture oligonucleotide includes a clickable moiety for reaction with a complementary clickable moiety on a microbead. As used herein, a “clickable moiety” refers to a functional group that is capable of forming a covalent bond via a click reaction, such as an azide, an alkyne, a phosphine, a thiol, a maleimide, an isonitrile, or a tetrazine. In some embodiments, each clickable moiety is independently selected from the group consisting of an azide, an alkyne, and a phosphine. In some embodiments, the microbeads contain an alkyne moiety for bonding to azide-functionalized capture polynucleotides. For example, the amine groups in microbeads formed using PEG monoacrylamide-monoamine (PEG-AM) can be coupled to a carboxylate-functionalized alkyne (*e.g.*, 4-pentynoic acid) using a carbodiimide reagent such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC). The alkynes can then be reacted with azide-functionalized oligonucleotides using a suitable catalyst (*e.g.*, a copper salt such as copper (II) acetate, copper (II) sulfate, copper (I) bromide, or copper (I) iodide) and optional reducing agents (*e.g.*, sodium ascorbate) and ligands (*e.g.*, tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA), tris(2-benzimidazolylmethyl)amine, tris(3-hydroxypropyltriazolylmethyl)amine, or the like).

[0119] In some embodiments, a crosslinking reagent is used for attachment of the capture polynucleotides to the microbeads. The crosslinking reagents can react to form covalent bonds with functional groups in the capture oligonucleotides and on the microbead surfaces (*e.g.*, a primary amine, a thiol, a carboxylate, a hydroxyl group, or the like). Crosslinkers useful for attaching capture oligonucleotides to microbeads include homobifunctional crosslinkers, which

react with the same functional group in the oligonucleotide and the bead, as well as heterobifunctional crosslinkers, which react with functional groups in the bead and the oligonucleotide wherein the functional groups differ from each other.

[0120] Examples of homobifunctional crosslinkers include, but are not limited to, amine-reactive homobifunctional crosslinkers (*e.g.*, dimethyl adipimidate, dimethyl suberimidate, dimethyl pimilimidate, disuccinimidyl glutarate, disuccinimidyl suberate, bis(sulfosuccinimidyl) suberate, bis(diazo-benzidine), ethylene glycobis(succinimidylsuccinate), disuccinimidyl tartrate, disulfosuccinimidyl tartrate, glutaraldehyde, dithiobis(succinimidyl pro-pionate), dithiobis(sulfosuccinimidyl propionate), dimethyl 3,3'-dithiobispropionimide, bis 2-(succinimidyl-oxycarbonyloxy)ethyl-sulfone, and the like) as well as thiol-reactive homobifunctional crosslinkers (*e.g.*, bismaleidohexane, 1,4-bis-[3-(2-pyridyldithio)propionamido]butane, and the like). Examples of heterobifunctional crosslinkers include, but are not limited to, amine- and thiol-reactive crosslinkers (*e.g.*, succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester, succinimidyl-4-(*p*-maleimidophenyl)butyrate, *N*-(γ -maleimidobutyryloxy)succinimide ester, *N*-succinimidyl(4-iodoacetyl) aminobenzoate, 4-succinimidyl oxycarbonyl- α -(2-pyridyldithio)-toluene, sulfosuccinimidyl-6- α -methyl- α -(2-pyridyldithio)-toluamido-hexanoate, *N*-succinimidyl-3-(2-pyridyldithio) propionate, *N*-hydroxysuccinimidyl 2,3-dibromopropionate, and the like).

[0121] Reaction mixtures for attaching capture oligonucleotides can contain additional reagents of the sort typically used in bioconjugation reactions. For example, in certain embodiments, the reaction mixtures can contain buffers (*e.g.*, 2-(*N*-morpholino)ethanesulfonic acid (MES), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), 3-morpholinopropane-1-sulfonic acid (MOPS), 2-amino-2-hydroxymethyl-propane-1,3-diol (TRIS), potassium phosphate, sodium phosphate, phosphate-buffered saline, sodium citrate, sodium acetate, and sodium borate), cosolvents (*e.g.*, dimethylsulfoxide, dimethylformamide, ethanol, methanol, tetrahydrofuran, acetone, and acetic acid), salts (*e.g.*, NaCl, KCl, CaCl₂, and salts of Mn²⁺ and Mg²⁺), detergents/surfactants (*e.g.*, a non-ionic surfactant such as *N,N*-bis[3-(*D*-gluconamido)propyl]cholamide, polyoxyethylene (20) cetyl ether, dimethyldecylphosphine oxide, branched octylphenoxy poly(ethyleneoxy)ethanol, a polyoxyethylene-polyoxypropylene block copolymer, *t*-octylphenoxypolyethoxyethanol, polyoxyethylene (20) sorbitan monooleate,

and the like; an anionic surfactant such as sodium cholate, *N*-lauroylsarcosine, sodium dodecyl sulfate, and the like; a cationic surfactant such as hexdecyltrimethyl ammonium bromide, trimethyl(tetradecyl) ammonium bromide, and the like; or a zwitterionic surfactant such as an amidosulfobetaine, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate, and the like), chelators (*e.g.*, ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), 2-(2-[bis(carboxymethyl)amino]ethyl) (carboxymethyl)amino)acetic acid (EDTA), and 1,2-bis(o-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA)), and reducing agents (*e.g.*, dithiothreitol (DTT), β -mercaptoethanol (BME), and tris(2-carboxyethyl)phosphine (TCEP)). Buffers, cosolvents, salts, detergents/surfactants, chelators, and reducing agents can be used at any suitable concentration, which can be readily determined by one of skill in the art. In general, buffers, cosolvents, salts, detergents/surfactants, chelators, and reducing agents are included in reaction mixtures at concentrations ranging from about 1 μ M to about 1 M. For example, a buffer, a cosolvent, a salt, a detergent/surfactant, a chelator, or a reducing agent can be included in a reaction mixture at a concentration of about 1 μ M, or about 10 μ M, or about 100 μ M, or about 1 mM, or about 10 mM, or about 25 mM, or about 50 mM, or about 100 mM, or about 250 mM, or about 500 mM, or about 1 M.

[0122] The reactions are conducted under conditions sufficient to install the capture oligonucleotides on the microbeads. The reactions can be conducted at any suitable temperature. In general, the reactions are conducted at a temperature of from about 4°C to about 40°C. The reactions can be conducted, for example, at about 25°C or about 37°C. The reactions can be conducted at any suitable pH. In general, the reactions are conducted at a pH of from about 4.5 to about 10. The reactions can be conducted, for example, at a pH of from about 5 to about 9. The reactions can be conducted for any suitable length of time. In general, the reaction mixtures are incubated under suitable conditions for anywhere between about 1 minute and several hours. The reactions can be conducted, for example, for about 1 minute, or about 5 minutes, or about 10 minutes, or about 30 minutes, or about 1 hour, or about 2 hours, or about 4 hours, or about 8 hours, or about 12 hours, or about 24 hours, or about 48 hours, or about 72 hours. Other reaction conditions may be employed in the methods of the invention, depending on the composition of the microbeads and the reagents used for installing the capture oligonucleotides.

D. Detection and Identification of Hybridized Polynucleotides

[0123] Amplicons are combined with the microbeads such that at least some amplicons are captured onto beads by the capture polynucleotides. Labeled amplicons (*e.g.*, Alexa Fluor 647-labeled amplicons, or ethidium bromide-stained amplicons, as described above) can then be located by visualizing the microbeads under an appropriate filter, *e.g.*, a fluorescence microscope equipped with a BrightLine® Pinkel filter set (Semrock, Rochester NY). In some embodiments, the methods including separating uncaptured amplicons from beads having captured amplicons. The microbeads can be dispersed on a microscope slide for detection of labeled amplicons and recording of lanthanide spectral signatures. Alternatively, the microbeads can be distributed in a well-plate or like apparatus having addressable positions prior to detection. As an alternative to microscopy, beads having labeled, hybridized amplicons can be detected using a flow cytometer with fluorescence-activated particle sorting.

[0124] Following identification of beads containing duplexes formed by amplicons and capture polynucleotides on microbead surfaces, microbe-identifying sequences in the captured amplicons are determined by examining the lanthanide spectral signatures of individual microbeads. In some embodiments, beads with and without detectable amplicons can be imaged so as to identify the spectral code and to quantify the amount of amplicon bound to the beads. In this manner, the absence of particular microbial polynucleotides in a given sample can also be characterized. In general, each of the spectral signatures contains signals generated from predetermined amounts of one or more lanthanides (*e.g.*, one or more Eu-, Dy-, Sm-, Ce-, Tb-, La-, Pr-, Nd-, Gd-, Ho-, Er-, Tm-, Yb-containing nanoparticles). Lanthanides in the microbeads can be excited with UV light (*e.g.*, 275 nm, 285 nm, or 292 nm) and emitted luminescent signals can be detected in the range of 400-800 nm (*e.g.*, 435 nm, 474 nm, 527 nm, 536 nm, 546 nm, 572 nm, 620 nm, 630 nm, 650 nm, or 780 nm). As a non-limiting example, a set of unique spectral signatures can be prepared with microparticles that contain europium-doped yttrium orthovanadate (YVO₄:Eu) to generate a reference signal and varying amounts of YVO₄:Dy, YVO₄:Sm, YVO₄:Tm, and LaPO₄:CeTb. Other lanthanide-containing materials, such as YVO₄:Ho, YVO₄:Er, NaYF₄:YbEr, and NaYF₄:YbTm, can be used in this fashion.

[0125] Codes can be read upon imaging of the excited microbeads at different wavelengths chosen to best discriminate between individual emission spectra. The raw images can be

converted to intensity images for each lanthanide by linear unmixing to determine the most likely linear combination of lanthanide nanoparticles to have produced the observed spectra at each pixel in an image. Individual codes can then be reported as the ratio of intensities of each coding LN relative to the YVO4:Eu reference signal. A transformation matrix can be applied to register the measured ratios onto the known input ratios, as described by Nguyen et al. ((2017) Advanced Optical Materials, 5, 1600548), and a Gaussian mixture model (GMM) can be used to fit the mean ratios and covariance matrices that describe each code cluster, and then assign each bead to its most likely code cluster.

[0126] Also provided herein are microbeads comprising a lanthanide spectral signature and a plurality of copies of a capture polynucleotide immobilized on the microbead, including all of the embodiments described above. Each capture polynucleotide in a plurality of the microbeads comprises a predetermined sequence and each capture polynucleotide is paired with a lanthanide spectral signature, such that the predetermined sequence can be identified based on the lanthanide spectral signature. In some embodiments, each capture polynucleotide comprises a nucleic acid sequence that hybridizes to a bacterial 16S rRNA gene sequence. In other embodiments, each capture polynucleotide comprises a nucleic acid sequence that hybridizes to a bacterial 23S rRNA gene sequence. In some embodiments, the capture polynucleotides comprise a nucleic acid sequence that hybridizes to a V3 variable region, a V4 variable region, or a V6 variable regions in a bacterial 16S rRNA gene sequence. In some embodiments, each of the capture polynucleotides comprises a nucleic acid sequence able to hybridize a *Pseudomonas* gene sequence, a *Streptococcus* gene sequence, *Staphylococcus* gene sequence, a *Neisseria* gene sequence, an *Acinetobacter* gene sequence, an *Escherichia* gene sequence, an *Enterobacter* gene sequence, or a *Klebsiella* gene sequence. In some embodiments, each of the lanthanide spectral signatures comprises a europium (Eu) signal, a dysprosium (Dy) signal, a samarium (Sm) signal, a cerium (Ce) signal, a terbium (Tb) signal, a lanthanum (La) signal, a praseodymium (Pr) signal, a neodymium (Nd) signal, a gadolinium (Gd) signal, a holmium (Ho) signal, an erbium (Er) signal, a thulium (Tm) signal, an ytterbium (Yb) signal, or a combination thereof. In some embodiments, each of the microbeads comprises a plurality of lanthanide nanoparticles. In some embodiments, the lanthanide nanoparticles comprise a lanthanide-doped host lattice. In some embodiments, the host lattice is yttrium orthovanadate, lanthanum phosphate, or a combination thereof. In some embodiments, each of the microbeads further comprises a crosslinked polymer

, wherein the capture polynucleotides are covalently bonded to the crosslinked polymer. In some embodiments, a plurality of the microbeads contains at least 100 lanthanide spectral signatures. In some embodiments, the plurality of microbeads contains at least 1000 lanthanide spectral signatures.

5 **E. Identification of Pathogens using Patterns of Hybridization**

[0127] It will be appreciated that although a predetermined sequence in a capture polynucleotide can be designed for hybridization to a particular target sequence in amplicons (e.g., a sequence specific to a particular microbe of interest), the same predetermined sequence may hybridize at varying levels to other sequences under certain conditions. Accordingly, amplicons generated from a sequence-agnostic target (including polynucleotides from a previously unknown or uncharacterized bacterium) could hybridize to multiple capture probes present in a panel of capture probes at different levels, thereby producing a pattern showing different magnitudes of hybridization to individual capture probes. The hybridization pattern can be evaluated in conjunction with the spectral codes associated with the individual capture probes. The measured pattern of hybridization can be compared to a predicted pattern of hybridization. To generate the predicted pattern, the hybridization binding level of an amplicon to capture polynucleotides can be predicted using thermodynamic models as described, for example by Aghazadeh, et al. (*Sci. Adv.* 2016; 2 : e1600025). In one aspect, the hybridization binding level can be predicted based on the Gibbs free energy of hybridization (Alexander et al. (2015, "Evaluation of the Gibbs Free Energy Changes and Melting Temperatures of DNA/DNA Duplexes Using Hybridization Enthalpy Calculated by Molecular Dynamics Simulation", *J. Phys. Chem. B* 2015, 119, 49, 15221–15234, Tulpan et al. (2010), "Free energy estimation of short DNA duplex hybridizations", *BMC Bioinformatics*. 2010; 11: 105). In another embodiment, the hybridization binding level can be predicted based on melting temperatures (T_m) or the Helmholtz Free Energy (Markham and Zuker (2004), "DINAMelt web server for nucleic acid melting prediction", *Nucleic Acids Res.*, 33, W577-W581; Dimitrov and Zuker (2004), "Prediction of hybridization and melting for double-stranded nucleic acids", *Biophys. J.*, 87, 215-226; SantaLucia (1998), "A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics", *PNAS*, 95, 1460-1465; Schmitta and Knotts (2011), "Thermodynamics of DNA hybridization on surfaces", *J. Chem. Phys.* 134, 205105). In yet

another embodiment, the hybridization binding level can be predicted using similarity metrics, such as E-score (Anderson & Brass (1998), "Searching DNA databases for similarities to DNA sequences: when is a match significant?", *Bioinformatics*, 14 (4), 349–356; Johnson et al. (2008, "NCBI BLAST: a better web interface", *Nucleic Acids Research*, 36 (Web server issue), W5–W9). The predicted hybridization pattern and the measured hybridization pattern can then be used in an analysis to determine which pathogen from the panel is most likely present in a sample.

[0128] Thus, in one embodiment, a hybridization pattern analysis is performed by comparing the measured hybridization pattern to the predicted hybridization pattern. In one approach, for an amplicon of a given species, such as an amplicon of the V6 region of *Acinetobacter baumannii*, the Gibbs free energy of hybridization is calculated for the amplicon binding to each capture polynucleotide in the panel, where the panel is comprised of a plurality of capture polynucleotides. This calculation may be performed for all amplicons of all species the panel is designed to detect. In one approach, the assay of the present disclosure is then performed on a sample where the pathogen is unknown. In one aspect, a correlation coefficient is calculated to measure the correlation between the measured hybridization pattern and the predicted hybridization patterns based on the Gibbs free energy of hybridization. In one embodiment, the Pearson correlation coefficient, known as r , R or Pearson's r , is calculated for this analysis. However, any type of correlation coefficient that presents a numerical measure of correlation can be used to perform the analysis.

[0129] In one approach, the Pearson correlation coefficient for the measured hybridization signals and the predicted hybridization energies is calculated for every pathogen amplicon the panel is designed to detect. By analyzing which pathogen amplicon has the highest Pearson correlation coefficient (R) and, thus, is best correlated with the measured unknown pathogen, it can be determined which pathogen from the panel is most likely present in the sample. In one aspect, negative Pearson correlation coefficients are omitted from consideration in this analysis. In one embodiment, the analysis is performed by considering all capture polynucleotide. In another embodiment, the analysis is performed by setting a certain threshold to only consider capture polynucleotide with hybridization energies above or below this threshold. For example, the correlation analysis may be performed using only capture polynucleotide predicted to bind to

the target amplicon with a Gibbs free energy less than -7 kcal/mol. In another example, the correlation analysis may be performed using only capture polynucleotide predicted to bind to the target amplicon with a Gibbs free energy less than -10 kcal/mol.

[0130] In one embodiment, the assay of the present disclosure targets multiple variable

5 regions, such as the V3 and the V6 within the 16S ribosomal DNA for a particular species. This can prove useful if there is sequence similarity between multiple species in one region, such as in V6, but sequences in a different region, such as V3, show differences and, therefore, may be used to discriminate these species. In one embodiment, the Pearson correlation coefficient analysis described above is performed for all V3 amplicons and for all V6 amplicons separately.

10 In another embodiment the Pearson correlation coefficient analysis is performed by combining the predictions and measurements for V3 and V6 to calculate a single Pearson correlation coefficient representing the combined measurement. In one embodiment, measurement of other regions, such as the V5 region, are combined with measurements for V3 and V6.

[0131] In one embodiment, the Pearson correlation coefficient analysis described above is
15 performed by correlating measurements of an unknown sample with measurements of known pathogens. For example, the analysis may be performed with measurements of known pathogens in the place of predicted hybridization signatures.

[0132] In a further embodiment, the hybridization pattern analysis described above can be used
20 for detecting the presence of pathogens that the capture polynucleotides were not specifically designed to probe for. In one approach, this analysis is performed similarly as described above by using amplicons generated from a sequence-agnostic target to calculate the predicted Gibbs free energy of hybridization with the existing capture polynucleotides in the panel. The presence of the sequence-agnostic target can be established when the predicted pattern is observed, i.e. by determining whether the measured hybridization pattern is well correlated (showing a high
25 Pearson correlation coefficient, R) with the predicted hybridization pattern of the unknown species.

F. Specificity Analysis and Optimization of Capture Polynucleotide Panels

[0133] In one embodiment, the specificity of the assay according to the present disclosure when using the above described correlation analysis is assessed by performing an area under a

receiver operating characteristic curve (AUROC) analysis. For example, this can be performed by setting a threshold of the Pearson correlation coefficient (R) to call a species (or amplicon) present and analyzing how many (or what fraction of) species would be detected as true positives or false positives. The curve may be generated by scanning this threshold through all possible values for R (0 to 1), and plotting the fraction of true positive and false positive detected species at each detection threshold.

[0134] In one embodiment, a similar correlation analysis as described can be used to select an optimal panel of capture polynucleotides. In one approach, for an amplicon of a given species, the Gibbs free energy of hybridization is calculated for the amplicon binding to each capture polynucleotide in the panel, where the panel is comprised of a plurality of capture polynucleotide. This calculation may be done for all amplicons of all species the panel is designed to detect. To assess the predicted specificity of a panel, the Pearson correlation coefficient is calculated to measure the correlation of the predicted hybridization patterns for a given amplicon, with the predicted hybridization patterns for all other amplicons, as well as itself. In one aspect, this analysis is performed by considering all capture polynucleotides. In another aspect, the analysis is performed by setting a threshold to only consider capture polynucleotides with hybridization energies above or below this threshold. For example, the correlation analysis may be performed using only capture polynucleotides predicted to bind to the target amplicon with a Gibbs free energy less than -7 kcal/mol. In one embodiment, negative Pearson correlation coefficients are omitted for the analysis.

[0135] In one embodiment, the Pearson correlation coefficient values are then used to generate a receiver operating characteristic curve by setting a threshold of the Pearson correlation coefficient (R) to call a species (or amplicon) present and analyzing how many (or what fraction of) species would be detected as true positives or false positives. In one approach, the curve is generated by scanning this threshold through all possible values for R (0 to 1), and plotting the fraction of true positive and false positive detected species at each detection threshold. In one aspect, the area under the receiver operating characteristic curve (AUROC) is calculated as a metric of specificity. In one embodiment, an optimal capture polynucleotide panel can be found by iteratively creating panels of capture polynucleotides from the multitude of candidate capture polynucleotides, and maximizing the predicted AUROC.

III. Examples

Example 1. Preparation of lanthanide-encoded beads.

[0136] Beads were prepared using a microfluidic bead synthesizer device that has been previously described. *See*, Gerver, et al. (2012) *Lab on a Chip*, 12, 4716–4723; Nguyen, et al. (2017) *Advanced Optical Materials*, 5, 1600548; Harink, et al. (2019) *PLOS One*, 14(3): e0203725; Nguyen, et al. (2018) *bioRxiv* 306779; Brower, et al. (2017) *J. Vis. Exp.* (119), e55276. The microfluidic device was fabricated in polydimethylsiloxane (PDMS, RTV 615, Momentive Performance Materials, Albany, NY) by multi-layer soft lithography using 4” test-grade silicon wafers (University Wafer, South Boston, MA) coated with multiple layers of SU-8 (Microchem Corp., Newton, MA) and AZ50 XT photoresists (Capitol Scientific, Austin, TX) patterned by standard photolithography processes. Lanthanide nanophosphors were synthesized using microwave synthesis in a Biotage Initiator (Biotage AB, Uppsala, Sweden), using methods previously published [1,2,4]. *See*, Gerver, et al. (2012); Nguyen, et al. (2017); Nguyen, et al. (2018); *supra*.

[0137] Encoded beads were generated by varying ratios of four input solutions each containing different lanthanide nanoparticles. The four monomer input solutions used in the microfluidic bead synthesizer all contained purified water with 42.8% v/v 700 MW PEG-diacrylate (Sigma-Aldrich), 3% v/v lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) photoinitiator dissolved in water at 19.6 mg/mL, and 5% v/v YVO₄:Eu. A first input solution also contained 21.3% v/v YVO₄:Dy; a second input solution contained 21.3% v/v YVO₄:Sm; and a third input solution contained 21.3% v/v YVO₄:Tm. Droplets were formed by flowing the aqueous streams into a T-junction for entry into a continuous flowing stream of HFE 7500 oil (3M Novec) that contained 2% v/v ionic Krytox as surfactant to eliminate droplet merging and sticking to the PDMS walls. The droplets were polymerized into beads by exposing the channel (downstream of the T-junction) with the stream of droplets to UV light with a liquid lightguide. Beads from each code were collected into a 96 well plate before being washed as described in the bead functionalization section.

Example 2. Design of hybridization probes for bead-oligonucleotide functionalization

An algorithm for the identification of hybridization oligonucleotides that target specific DNA sequences

[0138] A program was developed to aid in finding hybridization oligonucleotides that would be specific to a target DNA sequence in the presence of other similar DNA sequences (FIG. 2).

- 5 Given a pool of sequences, provided as a file in FASTA format, the program outputs candidate hybridization oligonucleotides that meet designated design specifications. The program uses a k-mer based approach (where a k-mer represents a sub-string of DNA bases within the sequence provided) that works in the following way: (i) given a set of sequences, the minimum k-mer length that splits all sequences up into unique, unambiguous k-mers is identified; (ii) all
- 10 sequences are broken into unique k-mers using the identified value of k; (iii) all k-mers that occur multiple times within the collection of sequences of interest are removed from consideration (such that only unique kmers are considered); (iv) a matrix of Hamming distances between all unique k-mers within a single sequence and all other k-mers from all other sequences is generated; (v) all rows that contain a Hamming distance of 1 for any k-mer pair are removed
- 15 (unique k-mers must be at least 2 mutations from any other k-mer in the pool); (vi) Hamming distances are mapped to similarity scores S by calculating $S = k - \text{Hamming distance}$ and minimize the sum of the squared similarity scores; (vii) k-mers are found within the original sequences; (viii) sliding window primer design is performed for oligonucleotides of varying size until desired T_m and other properties are achieved to generate a pool of potential hybridization
- 20 oligos; (ix) the oligo pool is pruned to a single representative oligo based on pruning properties; and (x) the hybridization oligos are reported in a machine/human readable format.

An algorithm for the identification of species-specific hybridization oligonucleotides

[0139] For the identification of species-specific hybridization oligonucleotides a program was developed that uses k-mers which uniquely distinguish a hypervariable region associated with a particular species of interest from all other species in the pool. In this approach, k-mers for each

25 species are required to have a specified Hamming distance from the hyper variable region sequences of other species. Furthermore, this algorithm does not disqualify k-mers that appear multiple times in a particular species' sequence. The algorithm explores a greater search space by not requiring that a selected panel of probes contains k-mers of the same length, and instead

30 considering all combinations of probes that are within a certain length range. The algorithm

further optimises for experimental assay performance by using RNAcofold (from the Vienna RNA package 2.4.14; Lorenz et al. (2011), ViennaRNA Package 2.0. Algorithms for molecular biology, 6(1), 26) to calculate the affinities between probes and amplicons. Capture polynucleotides that are predicted to exhibit strong, species-specific binding are ultimately
 5 selected.

[0140] The approach works in the following way: (i) each hypervariable region is split into 20-30 nucleotide subsequences; (ii) all k -mers between lengths 20 and 30 are identified for each species, concordant with commonly used probe length ranges for fluorescence in situ hybridization and hybridization-based sequencing (Biliveau et al. (2017), “OligoMiner provides
 10 a rapid, flexible environment for the design of genome-scale oligonucleotide in situ hybridization probes”. *PNAS* 115(10), 2183-2192); (iii) redundant k -mers are removed from consideration; (iv) a matrix of Hamming distances between all unique k -mers within a single sequence and all other k -mers from all other sequences is generated; (v) k -mers that contain a Hamming distance less than 2 to any other k -mer from other sequences are removed (unique k -mers must be at least 2
 15 mutations from any other k -mer in the pool; Letowski et al. (2004), “Designing better probes: effect of probe size, mismatch position and number on hybridization in DNA oligonucleotide microarrays”, *Journal Microbiol Methods*, 57(2), 269-278); (vi) the pool is further narrowed by limiting probes to a melting temperature range of 55 to 65 degrees Celsius; (vii) the predicted Gibbs free energy of hybridization between each pair of capture polynucleotide candidate and
 20 amplicon is calculated using the program RNAcofold; (viii) the absolute value of this binding energy is maximized for species-specific pairs and minimized for non-specific combinations of amplicon and capture polynucleotide, as the overall Gibbs free energy change is a strong predictor of both hybridization efficiency and specificity (Yilamz et al. (2004), “Mechanistic approach to the problem of hybridization efficiency in fluorescent in situ hybridization”, *Appl. Environ. Microbiol.*, 70(12), 7126-7139; Matveeva et al. (2018), “Sequence characteristics
 25 define trade-offs between on-target and genome-wide off-target hybridization of oligoprobes”, *PloS one*, 13(6)); (ix) one or two capture polynucleotides with optimal binding energetics per species are selected for assay inclusion.

[0141] Table 5 shows the pathogens targeted in Example 2, 4, and 5 include a variety of
 30 pathogens related to sepsis and hospital acquired infections. The inclusion of *Neisseria*

gonorrhoeae demonstrates the utility and feasibility of using this spectrally encoded multiplexed assay for the detection of co-infections.

Table 5. Targeted Pathogens

Organism	Characteristics
<i>Escherichia coli</i>	Gram-negative; found in lower intestine; cause of foodborne illness.
<i>Staphylococcus aureus</i>	Gram-positive; found in nose, respiratory tract, skin; leads to abscesses, respiratory infections, and food poisoning; can be methicillin-resistant.
<i>Pseudomonas aeruginosa</i>	Gram-negative; can be multidrug-resistant; associated with ventilator-associated pneumonia
<i>Acinetobacter baumannii</i>	Gram-negative; opportunistic pathogen; associated with nosocomial infections.
<i>Enterococcus faecium</i>	Gram-positive; alpha-hemolytic; commensal in gastrointestinal tract, but can cause meningitis or endocarditis; can be vancomycin-resistant.
<i>Enterobacter cloacae</i>	Gram-negative; gut flora; known to cause obesity in germfree mice under certain conditions.
<i>Klebsiella pneumoniae</i>	Gram-negative; lactose-fermenting; seen in people with a weakened immune system; associated with bronchitis.
<i>Neisseria gonorrhoeae</i>	Gram-negative; causes the sexually transmitted infection gonorrhea as well as septic arthritis.
<i>Staphylococcus epidermis</i>	Gram-positive; part of normal skin flora; forms biofilms on instruments and leads to hospital acquired infections.
<i>Streptococcus pneumoniae</i>	Gram-positive; major cause of pneumonia and meningitis.

5 Example 3. Bead-oligonucleotide functionalization

[0142] To prepare beads for oligo conjugation, the beads were carboxylated via Michael addition to acrylate groups. Beads (up to 30,000) were collected in filter bottom syringe tubes (PP-Reactor 2 mL with PTFE frit, catalog number V020TF051, Biotage LLC). Each separate reaction tube contained beads of a single spectral code. The beads were washed 3 times by adding 1 mL dimethylformamide (DMF) and then draining the solvent through the filter which retains the beads. This washing procedure was repeated with dichloromethane (DCM), methanol, and finally DMF again. The beads were then resuspended in 1.5 mL DMF, and 54 μ L of 3-

mercaptopropionic acid and 50 μ L of pyridine were added. The reaction vessel was capped and incubated for 24 hours at room temperature. Mixing of the beads in the reaction solutions was achieved by rotating the tubes on an end-over-end rotator, or alternatively by shaking on vortex mixer (Catalog number S006VO002, Biotage LLC). For this procedure, 1 ng of beads was
5 estimated to amount to approximately 20,000 beads, and available sites for carboxylation were estimated to be present at 0.32 mmol/g. At least 20 equivalents of 3-mercaptopropionic acid and pyridine, with respect to the carboxylation sites on the beads, were used in the reactions.

[0143] Keeping the beads in the filter tubes used during carboxylation, the beads were washed 3 times by adding 1 mL dimethylformamide (DMF) and then draining the solvent through the
10 filter which retains the beads. This washing procedure was repeated with dichloromethane (DCM), methanol, and finally DMF again. The DMF was drained and the beads were washed 3 times in dimethyl sulfoxide (DMSO). After the final wash, the beads were resuspended in 1 mL DMSO prior to addition of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; 20 μ L of 300 mM stock solution in DMSO); 1-hydroxy-7-azabenzotriazole (HOAT; 20 μ L of 60 mM stock
15 solution in DMSO), and *N,N*-diisopropylethylamine (DIPEA; 20 μ L of 300 mM stock solution in DMSO). The beads were incubated in this EDC, HOAT, and DIPEA solution for 15 minutes on a shaker (or rotator) for mixing. After 15 minutes, 10 μ L (from 100 μ M stock solution) of oligonucleotides modified at the 5' end with an amino-terminated 12-carbon spacer (the "5AmMC12" modification from Integrated DNA Technologies) were added, as well as an
20 additional 20 μ L of EDC, HOAT, and DIPEA. At this step, specific oligonucleotides were added to specific reaction tubes containing beads of a desired spectral code. As non-limiting examples, hybridization oligonucleotides for the V6 variable region in *Escherichia coli* 16S ribosomal DNA ("v6Eco") were added to reactions with beads of code 1, and hybridization oligonucleotides for *Pseudomonas aeruginosa* were added to reactions with beads of code 2. The
25 reaction mixtures were incubated at room temperature for 8-24 hours (*e.g.*, 16 hours) on a shaker or rotator. The reaction mixtures were then neutralized by adding 50 μ L ethanolamine (500 mM stock solution in DMSO) to each mixture and incubating for 1 hour. Following conjugation, the beads were washed with phosphate buffered saline (PBS) containing 0.1% (v/v) Tween 20. The beads were finally resuspended in PBS with 0.1% Tween 20 and stored (*e.g.*, at 4 $^{\circ}$ C). The beads
30 were kept according to their separate codes, or they were pooled together into a combined code set. In order to reduce bead-to-bead variation in conjugation of fluorescently labeled oligos,

carboxylation and conjugation reactions were performed with up to about 60,000 beads (e.g., around 1000 beads) per reaction. Conjugation reactions were also performed with input oligonucleotide concentrations ranging from 10 nM to 200 μ M to control the amount of oligonucleotide coupled to the bead (FIG. 3D).

5 **Example 4. Multiplexed Pathogen Detection Assay**

[0144] To test the sensitivity of the assay in detecting low abundance pathogens in the presence of blood (FIG. 4), *E. coli* was added to 5 mL whole blood at concentrations of 10 cfu/ml to 10,000 cfu/ml. DNA from whole blood was purified using the QIAamp DNA Blood Midi Kit (Qiagen), and eluted into 500 μ L tris buffer. From this 500 μ L sample, 10 μ L was
 10 added to each PCR reaction.

PCR Amplification

[0145] Primers were selected to amplify the V3, V4 and V6 variable regions of the 16S ribosomal subunit gene (Table 1 above). Forward primers were labeled at the 5' end with a fluorophore (e.g., Alexa647 or Cy5), and reverse primers were phosphorylated. PCR was
 15 performed using the Invitrogen Platinum II Taq Hot-Start DNA Polymerase (Thermo Fisher Scientific) following the manufacturer guidelines. Specifically, PCR reactions contained 10 μ L 5x Platinum II Taq Host-Start DNA Polymerase Master Mix, 1 μ L dNTPs (10mM), 1 μ L V6 Forward primer (from 10 μ M stock solution, resulting in 200 nM final concentration), 1 μ L V6
 20 Reverse primer, 1 μ L V3 Forward primer, 1 μ L V3 Reverse primer, 0.4 μ L Platinum II Taq Host-Start DNA Polymerase, 24.6 μ L water, and 10 μ L of solution containing sample template (e.g., *E. coli* DNA at 1 ng/mL concentration, or human DNA purified from whole blood). PCR was performed by heating this reaction mixture for 2 minutes at 95 $^{\circ}$ C followed by 35 cycles of 10 s at 95 $^{\circ}$ C and 30 s at 60 $^{\circ}$ C. Reactions were also performed with the forward primer at 500
 25 nM and 750 nM, in excess of the reverse primer, in order to generate single stranded products for downstream hybridization. In an alternative protocol, real-time quantitative PCR (qPCR) is performed to measure the abundance of bacterial DNA present in a sample prior to multiplexed detection of pathogen sequences. Quantitative PCR is performed using the iTaq Universal SYBR Green Supermix (BioRad), as 10 μ L 2x Supermix, 3 μ L water, 1 μ L Forward Primer (from 10
 30 μ M stock), 1 μ L Reverse Primer, and 5 μ L solution with sample template molecules.

[0146] In order to produce single stranded DNA for hybridization, a lambda exonuclease treatment was performed to digest the phosphorylated strand of the PCR amplicon (created by the phosphorylated reverse primer). A master mix containing 18 μ L water, 5 μ L lambda exonuclease buffer (New England Biolabs, Catalog # M0262S), and 1 μ L lambda exonuclease (New England Biolabs, Catalog # M0262S) for each reaction was prepared, and then 25 μ L was added to each tube (*e.g.*, 200 μ L strip tubes or 96 well plates) containing the product from the PCR reaction. The exonuclease reaction was then performed by incubating at 37 °C for 30 minutes, followed by a heat inactivation of the lambda exonuclease at 75 °C for 10 minutes. The products of this reaction were used for hybridization.

10 *Hybridization to Encoded Beads*

[0147] A pooled library of spectrally encoded beads functionalized with hybridization oligonucleotides was added to the products of the exonuclease reactions. Employment of approximately 100 beads per code can be particularly useful in the analysis of clinical samples, although 1 to 1000 beads per code can typically be used within operational limits. Robust hybridization was observed using a variety of commercially available buffers including PerfectHyb Plus Hybridization Buffer (Sigma-Aldrich), and SSC buffers. For the experiments shown in FIG. 5, beads were initially treated with PBS containing 2% bovine serum albumin and 0.1% Tween to prevent non-specific adsorption of biomolecules to the beads. This was followed by washing away this blocking solution with a hybridization buffer containing 6X SSC, 0.1% salmon sperm DNA, 0.6% SDS, and 2% BSA. This hybridization buffer and set of encoded beads was added to the PCR/exonuclease reaction products prior to hybridization. During hybridization, the reaction mixture was heated to 80 °C and then cooled at 1 °C /min to room temperature while the beads were mixed on a thermal-shaker (Eppendorf). Hybridization time was reduced by rapidly ramping the temperature from 80 °C to 65 °C over 1 minute on a Peltier element PCR thermocycler, followed by a 4 minute incubation at 65 °C, then a 4 minute incubation at 60 °C (which is the designed T_m of the hybridization oligos, and is further stabilized at this temperature by using the Platinum II Taq Polymerase PCR kit from Invitrogen), then a 4 minute incubation at 55 °C (below probe T_m), all while the beads were mixed within the solution. Finally, the beads were cooled to room temperature and washed by centrifuging the beads, removing the supernatant, and adding a wash buffer. The beads were then washed 3 times

by spinning down the beads to pellet them at the bottom of the reaction tube and removing the supernatant before adding wash buffer again. This can alternatively be done with a filter system. The wash buffer (0.1% SSC, 0.5% SDS) was designed to be low-salt for added stringency and reducing the energetic likelihood that a hybridized oligonucleotide would unbind and then re-
 5 bind to the wrong probe. Though less ideal, the beads may alternatively be washed with 0.1% Tween 20 in PBS.

Bead Imaging

[0148] The beads are then imaged on a glass slide by microscopy with standard epi-
 10 fluorescence excitation to quantify the amount of dye-labeled DNA that has hybridized to each bead, and with UV excitation and collection of emitted light at multiple wavelengths to quantify emitted lanthanide luminescence and thus identify the embedded spectral code. Due to the 1:1 linkage between spectral codes and hybridization probes sequences, this spectral code of each bead is used to identify which oligonucleotide sequence is presented on each bead, and the
 15 fluorescence signal from each bead is a measure of the amount of complementary target DNA present in the sample. By using the spectral code of the beads to track the identity of each oligonucleotide sequence being assayed, many oligonucleotides may be tracked within a single reaction volume. The methods for imaging and performing image analysis have been previously presented (*see*, Gerver (2012), Nguyen (2017), Harink (2019), Nguyen (2018), *supra*).

Analysis

[0149] Hybridization probes are assessed for similarity by E-score (FIG. 2B), by using BLASTn (NCBI). The Gibbs free energy of hybridization (FIG. 6) was calculated using the UNAFold through “The DINAMelt Web Server” from the University of Albany. These
 25 affinities and sequence similarities can be used to predict the extent of cross-reactivity that a particular pathogen sequence will have to multiple hybridization probes, and this affinity matrix A can be used to determine the presence of specific combinations of pathogens, x , even in the case of multiple hybridization oligonucleotides showing signal, b , by solving for x in $Ax = b$. The presence of pathogens may also be assessed using Bayesian approaches. A student t-test
 30 was performed to assess the presence of bacteria in the multiplexed pathogen assay by looking at the significance of the difference between bead populations in reactions with sample added and beads in negative control reactions with not template added (no-template control, “NTC”).

Measuring both the V3 and V6 region can be used to increase confidence in calling a pathogen detected, and can be used in logical combination to identify the presence of pathogens even if detection in one variable region shows affinity to other hybridization oligos.

Example 5. Illustrative example for identifying species in the assay according to the present disclosure using pattern of hybridization analysis.

[0150] The identification of a pathogen present in the assay according to the present disclosure can be performed by comparing the measured hybridization signal to the predicted amplicon-capture polynucleotide hybridization energies. FIG. 9 shows an illustrative example of such an analysis. An amplicon may be predicted to bind to multiple capture polynucleotides with different affinities (referred to as predicted hybridization patterns, or signatures), and may be measured to bind to multiple capture polynucleotide with different affinities (referred to as measured hybridization patterns, or signatures). The process then uses the Pearson correlation coefficient calculated from the thermodynamic predictions of amplicon-capture polynucleotide affinities (Gibbs free energy of hybridization) to identify species in the assay.

[0151] The calculation was performed with an example of three species that bind with different affinities to three capture polynucleotides (FIG. 9A). For each species (e.g. "sp1"), the measured hybridization signal was plotted against the predicted hybridization energy for itself (e.g. "sp1 vs sp1") and for all other species (e.g. "sp1 vs sp2"), and the Pearson correlation coefficient (r) was calculated (FIG. 9B). A heatmap was generated to summarize the Pearson correlation coefficients (FIG. 9C). Negative correlations were ignored.

[0152] A similar plot to FIG. 9B can be generated plotting the predicted hybridization energies against the predicted hybridization energies. This provides a predicted matrix of how well hybridization patterns are correlated between different species. This can be used as a preview (or as a predictor) of how the measured versus predicted correlation heatmap will appear after performing the experimental measurement. This can be useful for optimizing a capture polynucleotide panel for specificity before running the assay according to the present disclosure. A threshold can be set for one set of the predicted hybridization affinities where only hybridization affinities greater than this threshold (e.g. a change of Gibbs free energy less than -7 kcal/mol) is considered. This would make the predicted versus predicted matrix more

representative of the measured versus predicted matrix, where low affinity capture probe-amplicon interactions are not detected, or are detected but the signal does not correlate with binding energy below this threshold.

[0153] A similar analysis as described above was performed combining hybridization affinities for two different regions (V3 and V6 variable region amplicons from the 16S ribosomal DNA). A heatmap was generated to summarize the predicted hybridization affinities for different species with a model containing four species, where two of them are identical in V3 and two of them are identical in V6 (FIG. 9D). Correlations for each of the species considering V3 and V6 together were calculated and are shown in FIG. 9E. Combining V3 and V6 information resolves ambiguity in detecting species 2, 3, and 4 with only one variable region. A heatmap was generated to summarize the Pearson correlation coefficients for each of the species considering the combined measurements for V3 and V6 (FIG. 9F).

Example 6. The assay according to the present disclosure combined with a pattern of hybridization analysis can identify *Acinetobacter baumannii* in a sample

[0154] The assay according to the present disclosure was performed on *Acinetobacter baumannii*, targeting the V3 and V6 variable region, and the fluorescence signal from the beads was measured (FIG. 10A). The Gibbs free energy of hybridization was calculated for the amplicon of the V3 and V6 region of *Acinetobacter baumannii* binding to each capture polynucleotide in the panel (where the panel was comprised of 48 capture polynucleotides; see Table 7). This calculation was performed for all amplicons of all species in the panel.

[0155] Table 6 shows the targeted pathogens and the capture polynucleotide sequences used in the experiments presented in Examples 6-9.

Table 6. Targeted Pathogens in Examples 6-9

Species
<i>Acinetobacter baumannii</i>
<i>Haemophilus influenzae</i>
<i>Neisseria meningitidis</i>
<i>Pseudomonas aeruginosa</i>
<i>Enterobacter cloacae</i>
<i>Escherichia coli</i>

<i>Klebsiella oxytoca</i>
<i>Klebsiella pneumoniae</i>
<i>Proteus mirabilis</i>
<i>Serratia marcescens</i>
<i>Enterococcus faecalis</i>
<i>Enterococcus faecium</i>
<i>Listeria monocytogenes</i>
<i>Staphylococcus epidermidis</i>
<i>Staphylococcus aureus</i>
<i>Streptococcus agalactiae</i>
<i>Streptococcus pneumoniae</i>
<i>Streptococcus pyogenes</i>

Table 7. Sequences for Hybridization Capture Polynucleotides used in the experiments presented in Examples 6, 7, and 9.

Description	Sequence	Variable region
v3Aba_4_190815	GCTTATTCTGCGAGTAACGTCCACTATCTCTAGGT	V3
v3Ecl_3_190815	GCGGGTAACGTCAATTGCTGCGGTTATT	V3
v3Eco_1_190815	GCGGGTAACGTCAATGAGCAAAGGTATTAAC TTT	V3
v3Efa_4_190815	GTCAGGGGACGTTTCAGTTACTAACGT	V3
v3Efa_2_190815	GTCAAGGGATGAACAGTTACTCTCATCCTTGTT	V3
v3Hin_3_190515	TAGCCGGTGCTTCTTCTGTATTTAACGTCAATTTG	V3
v3Kox_4_190515	GGTGCTTCTTCTGCGGGTAACGTCAATGAATAAG	V3
V3Kpn_5_190515	GCGGGTAACGTCAATCGATGAGGTTATTAACC	V3
v3Lmo_2_190515	GGTTAGATACCGTCAAGGGACAAGCAGTTACTCTT	V3
v3Pae_3_190515	GTCAAAACAGCAAGGTATTAAC T TACTGCCCTT	V3
v3Pmi_1_190515	GGGTATTAACCTTATCACCTTCCTCCCCGCTG	V3
v3Sag_2_190515	GTCAC T TGGTAGATTTTCCACTCCTACCAACGTT	V3
v3Sau_5_190515	GATGTGCACAGTTACTTACACATATGT	V3
v3Sep_1_190515	GTCAAGACGTGCATAGTTACTTACACATTTGTT	V3
v3Sma_3_190515	GCGAGTAACGTCAATTGATGAGCGTATTAAGCTCA	V3
v3Spn_2_190515	GTCACAGTGTGAAC T TCCACTCTCACACTCGTT	V3
v3Spy_4_190515	GT TGCCCCCATTGCCGAAGATT	V3
v6Aba_2_190815	GCACCTGTATCTAGATTCCCGAAGG	V6
v6Eco_2_190815	TGAAAACTTCCGTGGATGTCAAGACCAGGTAAGG	V6
v6Efa_3_190815	GAAGGGGAAGCTCTATCTCTAGAGTGGTCAAAGG	V6
v6Hin_2_190815	TGCAAACTTCTTAGGATGTCAAGAGTAGGTAAGG	V6

v6Kox_4_190815	AGCATCTCTGCTAAATTCTCTGGATGTCAAGAG	V6
v6Lmo_3_190815	CTCCAGAGTGGTCAAAGGATGTCAAGACCTGG	V6
v6Pae_3_190815	GGAAAGTTCTCAGCATGTCAAGGC	V6
v6Pmi_5_190815	TCCTCTATCTCTAAAGGATTCGCTGGATGTCAAG	V6
v6Sag_2_190815	GAAGAGAAAGCCTATCTCTAGGCCGGTCAGAAGG	V6
v6Sau_1_190815	GGGGAAGGCTCTATCTCTAGAGTTGTCAAAGGATG	V6
v6Sep_5_190815	GGGGAAGGCTCTATCTCTAGAGGGGTCAGAGGATG	V6
v6Sma_1_190815	GGAAAGTTCTCTGGATGTCAAGAGTAGGTAAGG	V6
v6Spn_5_190815	GAAGGAAAAGTCTATCTCTAGAGCGGTCAGAGGG	V6
v6Spy_4_190815	GAAGTAAAAGTCTATCTCTAGAGCGGGCATCGGG	V6
v5Aba_2_190815	GCTGCGCCACTAAAGCCTCAAAGGC	V5
v5Ecl_1_190815	GGTCGATTTAACGCGTTAGCCTCCGGAAG	V5
v5Eco_1_190815	GGCGGTCGACTTAACGCGTTANNTCCGGAAG	V5
v5Hin_2_190815	GGCGGTCGATTTATCACGTTAGCTACGGG	V5
v5Kox_4_190815	AACGCGTTAGCTCCGGAAGCCACTC	V5
v5Pae_1_190815	GTTAGCTGCGCCACTAAGATCTCAAGGATC	V5
v5Pmi_3_190815	GAAGCCACGGTTCAAGACCACAACCTCTAAAT	V5
v5Spn_1_190815	GGAGTGCTTAATGCGTTAGCTACGGCACTAAA	V5
v5Spy_1_190815	GAGTGCTTAATGCGTTAGCTCCGGCACTAAG	V5

Table 8. Sequences with 5' amine modifications and carbon and oligo spacers for Hybridization Capture Polynucleotides used in the experiments presented in Example 6, 7, 5 and 9.

Description	Sequence
v3Aba_4_190815	/5AmMC12/TTTTTTTGCTTATTCTGCGAGTAACGTCCACTATCTCTAGGT
v3Ecl_3_190815	/5AmMC12/TTTTTTTGCGGGTAACGTCAATTGCTGCGGTTATT
v3Eco_1_190815	/5AmMC12/TTTTTTTGCGGGTAACGTCAATGAGCAAAGGTATTAACCTT
v3Efa_4_190815	/5AmMC12/TTTTTTTGTCAGGGGACGTTTCAGTTACTAACGT
v3Efa_2_190815	/5AmMC12/TTTTTTTGTCAGGGATGAACAGTTACTCTCATCCTTGTT
v3Hin_3_190515	/5AmMC12/TTTTTTTAGCCGGTGCTTCTTCTGTATTAAACGTCAATTG
v3Kox_4_190515	/5AmMC12/TTTTTTTGGTGCTTCTTCTGCGGGTAACGTCAATGAATAAG
V3Kpn_5_190515	/5AmMC12/TTTTTTTGCGGGTAACGTCAATCGATGAGGTTATTAACC
v3Lmo_2_190515	/5AmMC12/TTTTTTTGGTTAGATACCGTCAAGGGACAAGCAGTTACTCTT
v3Pae_3_190515	/5AmMC12/TTTTTTTGTCAAAACAGCAAGGTATTAACCTTACTGCCCTT
v3Pmi_1_190515	/5AmMC12/TTTTTTTGGGTATTAACCTTATCACCTTCCTCCCCGCTG
v3Sag_2_190515	/5AmMC12/TTTTTTTGTCACCTGGTAGATTTTCCACTCCTACCAACGTT
v3Sau_5_190515	/5AmMC12/TTTTTTTGATGTGCACAGTTACTTACACATATGT

v3Sep_1_190515	/5AmMC12/TTTTTTTGTCAAGACGTGCATAGTTACTTACACATTTGTT
v3Sma_3_190515	/5AmMC12/TTTTTTTGCAGTAACGTCAATTGATGAGCGTATTAAGCTCA
v3Spn_2_190515	/5AmMC12/TTTTTTTGTACAGTGTGAACTTCCACTCTCACACTCGTT
v3Spy_4_190515	/5AmMC12/TTTTTTTGTGCCCCATTGCCGAAGATT
v6Aba_2_190815	/5AmMC12/TTTTTTTGCACCTGTATCTAGATTCCCGAAGG
v6Eco_2_190815	/5AmMC12/TTTTTTTGA AAAACTTCCGTGGATGTCAAGACCAGGTAAGG
v6Efa_3_190815	/5AmMC12/TTTTTTTGAAGGGGAAGCTCTATCTCTAGAGTGGTCAAAGG
v6Hin_2_190815	/5AmMC12/TTTTTTTGC AA ACTTCTTAGGATGTCAAGAGTAGGTAAGG
v6Kox_4_190815	/5AmMC12/TTTTTTTAGCATCTCTGCTAAATCTCTGGATGTCAAGAG
v6Lmo_3_190815	/5AmMC12/TTTTTTTCTCCAGAGTGGTCAAAGGATGTCAAGACCTGG
v6Pae_3_190815	/5AmMC12/TTTTTTTGGAAAGTTCTCAGCATGTCAAGGC
v6Pmi_5_190815	/5AmMC12/TTTTTTTTCCTCTATCTCTAAAGGATTGCTGGATGTCAAG
v6Sag_2_190815	/5AmMC12/TTTTTTTGAAGAGAAAGCCTATCTCTAGGCCGGTCAGAAGG
v6Sau_1_190815	/5AmMC12/TTTTTTTGGGGAAGGCTCTATCTCTAGAGTTGTCAAAGGATG
v6Sep_5_190815	/5AmMC12/TTTTTTTGGGGAAA ACTCTATCTCTAGAGGGGTCAGAGGATG
v6Sma_1_190815	/5AmMC12/TTTTTTTGGAAAGTTCTCTGGATGTCAAGAGTAGGTAAGG
v6Spn_5_190815	/5AmMC12/TTTTTTTGAAGGAAA ACTCTATCTCTAGAGCGGTCAGAGGG
v6Spy_4_190815	/5AmMC12/TTTTTTTGAAGTAAA ACTCTATCTCTAGAGCGGGCATCGGG
v5Aba_2_190815	/5AmMC12/TTTTTTTGCTGCGCCACTAAAGCCTCAAAGGC
v5Ecl_1_190815	/5AmMC12/TTTTTTTGGTCGATTTAAACGCGTTAGCCTCCGGAAG
v5Eco_1_190815	/5AmMC12/TTTTTTTGGCGGTCGACTTAACGCGTTANNTCCGGAAG
v5Hin_2_190815	/5AmMC12/TTTTTTTGGCGGTCGATTTATCACGTTAGCTACGGG
v5Kox_4_190815	/5AmMC12/TTTTTTTAAACGCGTTAGCTCCGGAAGCCACTC
v5Pae_1_190815	/5AmMC12/TTTTTTTGT TAGCTGCGCCACTAAGATCTCAAGGATC
v5Pmi_3_190815	/5AmMC12/TTTTTTTGAAGCCACGGTTCAAGACCACAACCTCTAAAT
v5Spn_1_190815	/5AmMC12/TTTTTTTGGAGTGCTTAATGCGTTAGCTACGGCACTAAA
v5Spy_1_190815	/5AmMC12/TTTTTTTGAGTGCTTAATGCGTTAGCTCCGGCACTAAG

[0156] After performing the assay according to the present disclosure on a sample (this could be a sample where the pathogen is unknown), the Pearson correlation coefficient for the measured hybridization signal and the predicted hybridization energies based on the Gibbs free energy was calculated. This calculation was performed for every pathogen amplicon the panel was designed to detect. As an example, FIG. 10B illustrates the Pearson correlation of measured hybridization signal intensities with the predicted hybridization energies that was calculated for V3 (top row) and V6 (bottom row) *Acinetobacter baumannii* amplicons. Each dot represents the median hybridization signal intensity of all beads of that spectral code, and the error bars are

standard errors of them mean. The cognate capture polynucleotide for each amplicon shows the strongest hybridization signal, however there is hybridization signal on other beads, the intensity of which correlates with predicted hybridization affinity. The highlighted plots on the left show the correlations of hybridization measurements for *Acinetobacter baumannii* with the predicted hybridization energies for the *Acinetobacter baumannii* V3 or V6 amplicon against all probes. The plots to the right of these show how well the measurements of *Acinetobacter baumannii* correlate with the predicted hybridization affinities to the capture polynucleotide for V3 or V6 amplicons from different species. By analyzing which pathogen amplicon is best correlated (the highest Pearson correlation coefficient, R) with the measured unknown pathogen sample, it can be determined which pathogen (from the panel) is most likely present in the sample. In this analysis, a threshold was applied to omit low affinity capture polynucleotides from correlation analyses. Specifically, the correlation analysis was performed using only capture polynucleotides predicted to bind to the target amplicon with a Gibbs free energy less than -10 kcal/mol. Negative Pearson correlation coefficients were omitted from consideration during pathogen identification.

[0157] The specificity of the assay when using this above described correlation analysis can be assessed by analyzing the area under a receiver operating characteristic curve (AUROC). This is generated by setting a threshold of the Pearson correlation coefficient (R) to call a species (or amplicon) present and analyzing how many (or what fraction of) species would be detected as true positives or false positives. In particular, the curve is generated by scanning this threshold through all possible values for R (0 to 1), and plotting the fraction of true positive and false positive detected species at each detection threshold.

Example 7. Using the assay of the present disclosure for targeting species for which the panel was not designed

[0158] Hybridization affinities, and subsequent capture polynucleotide panel hybridization patterns, can be predicted for species for which the capture polynucleotide were not originally designed for. This allows testing for species that were not originally targeted in the capture polynucleotide panel design. A heatmap was generated displaying the Gibbs free energy of hybridization (FIG. 11). The heatmap shows the hybridization energies for each capture polynucleotide (y-axis) plotted against all species (x-axis). In this case, the energy of the V6 amplicon binding to the capture polynucleotide was calculated, and the energy of the V6

amplicon hybridizing with other V6 amplicons was subtracted and multiplied by -1. While the existing capture polynucleotides may not be able to clearly distinguish all bacteria, it is possible to distinguish groups of bacteria, for example based on a similar sequence or phylogeny.

Example 8. Hybridization pattern analysis for the selection of an optimal panel of capture polynucleotide

[0159] The correlation analysis as described above was used to select an optimal panel of capture polynucleotides. Two different panels were tested. For an amplicon of a given species (here an amplicon of the V6 region of *Acinetobacter baumannii*), the Gibbs free energy of hybridization was calculated for the amplicon binding to each capture polynucleotide in the panel (where the panel is comprised of a plurality of probes). This calculation was done for all amplicons of all species the panel was designed to detect. To assess the predicted specificity of a panel, the Pearson correlation coefficient was calculated to measure the correlation of the predicted hybridization patterns for a given amplicon, with the predicted hybridization patterns for all other amplicons, as well as itself (FIG. 12). Summary heatmaps of correlations between measured hybridization signal and predicted hybridization affinity were generated for each of the panels (FIG. 12A and FIG. 12C). The scale bar represents Pearson correlation coefficients (R) and frames highlight the cognate pairing of measured and predicted species. The “dG threshold” is the value of ΔG (the Gibbs free energy) for a capture polynucleotide:amplicon hybridization for which the capture polynucleotides with lower affinity are omitted from the Pearson Correlation analysis. This analysis was performed using only capture polynucleotides predicted to bind to the target amplicon with a Gibbs free energy less than -10 kcal/mol. Negative Pearson correlation coefficients were omitted.

[0160] Prediction matrices were then generated, showing that the two panels of capture polynucleotides present different hybridization patterns, with one of the panels (FIG. 12D) exhibiting improved discrimination of species compared to the other panel (FIG. 12B). Comparison of the heatmap illustrating the measured hybridization signature vs. predicted hybridization signature correlations with the heatmap illustrating the predicted hybridization signature vs. predicted hybridization signature correlations reveals that the measured panel performs similar to what is predicted.

[0161] The performance of the assay was then evaluated for a particular panel of capture polynucleotides. The Pearson correlation coefficient values were used to generate a receiver operating characteristic curve (AURAC) by setting a threshold of the Pearson correlation coefficient (R) to call a species (or amplicon) present and analyzing how many (or what fraction of) species would be detected as true positives or false positives (FIG. 13). The curve was generated by scanning this threshold through all possible values for R (0 to 1), and plotting the fraction of true positive and false positive detected species at each detection threshold. This was done for the V3 and V6 amplicons separately (FIG. 13B and FIG. 13C, respectively), or from the combined V3 and V6 correlations (FIG. 13A). The area under the receiver operating characteristic curve (AUROC) was calculated as a metric of specificity. An optimal capture polynucleotide panel can be found by iteratively creating panels of capture polynucleotides from the multitude of candidate probes and maximizing the predicted AUROC.

Table 9. Sequences with 5' amine modifications and carbon and oligo spacers for Hybridization Probes used in the experiments presented in Example 8.

Description	Sequence
v6Acinetobacter_baumannii_28	/5AmMC12/TTTTTTTGTTCCTAGTATGTCAAGGCCA GGTAA
v6Enterobacter_cloacae_7	/5AmMC12/TTTTTTTAATCCATCTCTGGAAAGTTCT GTG
v6Enterococcus_faecalis_0	/5AmMC12/TTTTTTTCCCCGAAGGGAAAGCTCTA
v6Enterococcus_faecium_2	/5AmMC12/TTTTTTTCTTTGCCCCGAAGGGG
v6Escherichia_coli_0	/5AmMC12/TTTTTTTCATTCTCATCTCTGAAAACCT CCGTG
v6Haemophilus_influenzae_41	/5AmMC12/TTTTTTTCATCTCTGCAAACCTCTTAGG ATGTC
v6Klebsiella_oxytoca_24	/5AmMC12/TTTTTTTCAAAGCATCTCTGCTAAATTC TCTGG
v6Listeria_monocytogenes_0	/5AmMC12/TTTTTTTGTCTCCAGAGTGGTCAAAGG ATG
v6Proteus_mirabilis_8	/5AmMC12/TTTTTTTCACTCCTCTATCTCTAAAGGA TTCGC
v6Pseudomonas_aeruginosa_0	/5AmMC12/TTTTTTTATCTCTGGAAAGTTCTCAGCA TGTC
v6Serratia_marcescens_0	/5AmMC12/TTTTTTTGAAAGTTCTCTGGATGTCAAG AGTAG
v6Staphylococcus_aureus_23	/5AmMC12/TTTTTTTAGGCTCTATCTCTAGAGTTGT CAAAG

v6Staphylococcus_epidermidis_11	/5AmMC12/TTTTTTTCTATCTCTAGAGGGGTCAGAGGAT
v6Streptococcus_agalactiae_11	/5AmMC12/TTTTTTTGTCACCTCTGCTCCGAAGAGAGAA
v6Streptococcus_pneumoniae_10	/5AmMC12/TTTTTTTCTGTCCCGAAGGAAACTCTATCT
v6Streptococcus_pyogenes_29	/5AmMC12/TTTTTTTCCGATGTACCGAAGTAAAC TCTATC
v3Acinetobacter_baumannii_106	/5AmMC12/TTTTTTTACGTCCACTATCTCTAGGTAT TAACT
v3Enterobacter_cloacae_8	/5AmMC12/TTTTTTTCGGTTATTAACCACAACACCT TCC
v3Enterococcus_faecalis_29	/5AmMC12/TTTTTTTGTTTCAGTTACTAACGTCCTTG TTCTT
v3Enterococcus_faecium_17	/5AmMC12/TTTTTTTGTCAAGGGATGAACAGTTAC TCTCAT
v3Escherichia_coli_40	/5AmMC12/TTTTTTTGGTATTAACCTTACTCCCTTC CTCCC
v3Haemophilus_influenzae_153	/5AmMC12/TTTTTTTTCTCAATACCGAAAGAACT TTACA
v3Klebsiella_oxytoca_56	/5AmMC12/TTTTTTTGAATAAGGTTATTAACCTCAC TCCCT
v3Klebsiella_pneumoniae_20	/5AmMC12/TTTTTTTGATGAGGTTATTAACCTTAAC GCCTT
v3Listeria_monocytogenes_102	/5AmMC12/TTTTTTTGCAGTTACTCTTATCCTTGTT CTCTCT
v3Proteus_mirabilis_64	/5AmMC12/TTTTTTTAGGGTATTAACCTTATCACCT TCCTC
v3Pseudomonas_aeruginosa_45	/5AmMC12/TTTTTTTTCAAACAGCAAGGTATTAA CTTACT
v3Serratia_marcescens_17	/5AmMC12/TTTTTTTAACGTCAATTGATGAGCGTAT TAAGC
v3Staphylococcus_aureus_40	/5AmMC12/TTTTTTTGTCAAGATGTGCACAGTTACT TACAC
v3Staphylococcus_epidermidis_36	/5AmMC12/TTTTTTTGACGTGCATAGTTACTTACAC ATTTG
v3Streptococcus_agalactiae_4	/5AmMC12/TTTTTTTTAGATTTTCCACTCCTACCAA CGTTC
v3Streptococcus_pneumoniae_14	/5AmMC12/TTTTTTTCACTCTCACACTCGTTCTTCT CTTA
v3Streptococcus_pyogenes_13	/5AmMC12/TTTTTTTCCACCATCATCTTCTCTAAC AACAG

Example 9. Clustering of capture polynucleotide candidates and bacterial species based on hybridization affinities

[0162] Capture polynucleotide-to-amplicon hybridization affinities were used to cluster capture polynucleotide candidates and bacterial species into genus and Gram stain status (positive or negative). A heatmap was generated illustrating the clustering (FIG. 14). The heatmap shows the hybridization affinities for each capture polynucleotide (x-axis). Capture polynucleotides may be selected for species specificity or broader categorization such as Gram status using hierarchical clustering. For example, if a capture polynucleotide is shown to bind to all bacteria of a particular Gram type (i.e., Gram positive or Gram negative), it may be selected as a probe to test for Gram type. Similarly, if a capture polynucleotide binds to all bacteria of the Viridians group, it may be selected as a more general probe for bacteria species in this group.

[0163] Although the foregoing has been described in some detail by way of illustration and example for purposes of clarity and understanding, one of skill in the art will appreciate that certain changes and modifications can be practiced within the scope of the appended claims. In addition, each reference provided herein is incorporated by reference in its entirety to the same extent as if each reference was individually incorporated by reference.

INFORMAL SEQUENCE LISTING:

SEQ ID NO:1

/A647/GTGCCAGCMGCCGCGGTAA

SEQ ID NO:2

/Phos/GGACTACHVGGGTWTCTAAT

SEQ ID NO:3

GTGCCAGCMGCCGCGGTAA

SEQ ID NO:4

GGACTACHVGGGTWTCTAAT

SEQ ID NO:5

/A647/TGGAGCATGTGGTTTAATTCGA

SEQ ID NO:6

TGGAGCATGTGGTTTAATTCGA

SEQ ID NO:7

/Phos/AGCTGACGACANCCATGCA

SEQ ID NO:8

AGCTGACGACANCCATGCA

SEQ ID NO:9

/A647/CCAGACTCCTACGGGAGGCAG

SEQ ID NO:10

CCAGACTCCTACGGGAGGCAG

SEQ ID NO:11

/Phos/CGTATTACCGCGGCTGCTG

SEQ ID NO:12

CGTATTACCGCGGCTGCTG

SEQ ID NO:13

/5AmMC12/TTTTTTTCTAGAGTTGTCAAAGGATGTCAAGATTTGGTAAG

SEQ ID NO:14

S. aureus

CTAGAGTTGTCAAAGGATGTCAAGATTTGGTAAG

SEQ ID NO:15

/5AmMC12/TTTTTTTACCTGTGTCTGAGTTCCCGAAGG

SEQ ID NO:16

P. aeruginosa

ACCTGTGTCTGAGTTCCCGAAGG

SEQ ID NO:17

/5AmMC12/TTTTTTTGGAAAGTTCTCAGCATGTCAAGGC

SEQ ID NO:18

P. aeruginosa

GGAAAGTTCTCAGCATGTCAAGGC

SEQ ID NO:19

/5AmMC12/TTTTTTTGCCGTATTAATACTAAATCCTCCTCGCTTAAAG

SEQ ID NO:20

A. baumannii

GCCGTATTAATACTAAATCCTCCTCGCTTAAAG

SEQ ID NO:21

/5AmMC12/TTTTTTTGGAAAGTTTCTAGTATGTCAAGGCCAGG

SEQ ID NO:22

A. baumannii

GGAAAGTTTCTAGTATGTCAAGGCCAGG

SEQ ID NO:23

/5AmMC12/TTTTTTTGACCTGTATCTAGATTCCCGAAGG

SEQ ID NO:24

A. baumannii

GCACCTGTATCTAGATTCCCGAAGG

SEQ ID NO:25

/5AmMC12/TTTTTTTGCTAAGTTCTCTGGATGTCAAGAGTAGG

SEQ ID NO:26

E. cloacae

GCTAAGTTCTCTGGATGTCAAGAGTAGG

SEQ ID NO:27

/5AmMC12/TTTTTTTGACCTGGTAAGGTTCTTTTCGCGTTG

SEQ ID NO:28

E. faecium

GACCTGGTAAGGTTCTTTCGCGTTG

SEQ ID NO:29

/5AmMC12/TTTTTTTAGAGTGGTCAAAGGATGTCAAGA

SEQ ID NO:30

E. faecium

AGAGTGGTCAAAGGATGTCAAGA

SEQ ID NO:31

/5AmMC12/TTTTTTTGTCTCACAGTTCCCGAAGGCA

SEQ ID NO:32

K. pneumoniae

GTCTCACAGTTCCCGAAGGCA

SEQ ID NO:33

/5AmMC12/TTTTTTTGGAAAGTTCTGTGGATGTCAAGA

SEQ ID NO:34

K. pneumoniae

GGAAAGTTCTGTGGATGTCAAGA

SEQ ID NO:35

/5AmMC12/TTTTTTTGTCACTCTGTCCCCCGAAGG

SEQ ID NO:36

S. epidermis

GTCACCTCTGTCCCCCGAAGG

SEQ ID NO:37

/5AmMC12/TTTTTTTGAGGGGTCAGAGGATGTCAAGATTG

SEQ ID NO:38

S. epidermis

GAGGGGTCAGAGGATGTCAAGATTG

SEQ ID NO:39

/5AmMC12/TTTTTTTGTACCTCTGTCCCGAAGGAAA

SEQ ID NO:40

S. pneumoniae

GTCACCTCTGTCCCGAAGGAAA

SEQ ID NO:41

/5AmMC12/TTTTTTTAGAGCGGTCAGAGGGATGTCAAG

SEQ ID NO:42

S. pneumoniae

AGAGCGGTCAGAGGGATGTCAAG

SEQ ID NO:43

/5AmMC12/TTTTTTTGAGCAAAGGTATTAACCTTACTCCCTTCC

SEQ ID NO:44

E. coli

GAGCAAAGGTATTAACCTTACTCCCTTCC

SEQ ID NO:45

/5AmMC12/TTTTTTTGATGTGCACAGTTACTTACACATATGT

SEQ ID NO:46

S. aureus

GATGTGCACAGTTACTTACACATATGT

SEQ ID NO:47

/5AmMC12/TTTTTTTGTATTAACCTTACTGCCCTTCCTCCCAAC

SEQ ID NO:48

P. aeruginosa

GTATTAACCTTACTGCCCTTCCTCCCAAC

SEQ ID NO:49

/5AmMC12/TTTTTTTCTATCTCTAGGTAGCCGTATTAATAAA

SEQ ID NO:50

A. baumannii

CTATCTCTAGGTAGCCGTATTAATAAA

SEQ ID NO:51

/5AmMC12/TTTTTTTAGGGATGAACAGTTACTCTCATCCT

SEQ ID NO:52

E. cloacae

AGGGATGAACAGTTACTCTCATCCT

SEQ ID NO:53

/5AmMC12/TTTTTTTCGGCAGGGTTATTAACCCTGTCGC

SEQ ID NO:54

E. faecium

CGGCAGGGTTATTAACCCTGTCGC

SEQ ID NO:55

/5AmMC12/TTTTTTTCGACAAGGTTATTAACCTTATCG

SEQ ID NO:56

K. pneumoniae

CGACAAGGTTATTAACCTTATCG

SEQ ID NO:57

/5AmMC12/TTTTTTTCGGCCGCCGATATTGGCAACGGCCTT

SEQ ID NO:58

N. gonorrhoeae

CGGCCGCCGATATTGGCAACGGCCTT

SEQ ID NO:59

/5AmMC12/TTTTTTTGACGTGCATAGTTACTTACACATTTG

SEQ ID NO:60

S. epidermis

GACGTGCATAGTTACTTACACATTTG

SEQ ID NO:61

/5AmMC12/TTTTTTTCAGTGTGAACTTTCCACTCTCACACTC

SEQ ID NO:62

S. pneumoniae

CAGTGTGAACTTTCCACTCTCACACTC

SEQ ID NO:63

GGACTACAGGGGTATCTAATCCCTTTTG

SEQ ID NO:64

GGTGACAGGGGTTTACAATCCGAG

SEQ ID NO:65

GCGTTGCACCATCAGGGTTT

SEQ ID NO:66

CGGCTTCATGCAGTCGAGTTG

SEQ ID NO:67

CAGTGTACTCCGCTCCGAAGAG

SEQ ID NO:68

AGTAACTCCGAACAACGCTTGC

SEQ ID NO:69

GAACTGTGGCTGGGTTTGATGAGATT

SEQ ID NO:70

CATGCAGGCGAGTTTCAGCC

SEQ ID NO:71

GTTTCGCTCCCCTTTGTCCCAA

SEQ ID NO:72

GTCAATTCATTTGAGTTTTAACCTTGCG

SEQ ID NO:73

AGCTCGCCAGTTTTGGATGCAG

SEQ ID NO:74

AGTCGACATCGTTTACGGCGTG

SEQ ID NO:75

GAACTGAGATGGCTTTTGGAGATTCTG

SEQ ID NO:76

GAGCCAGGATCAAACCTCTCAGGTTT

SEQ ID NO:77

GTCTGGCCGACATCGTTTACGG

SEQ ID NO:78

TTAGCTTTTGTAGCCTTTTTCCCTGCTG

SEQ ID NO:79

CGAACTCGAGTTTTGCAGTATCTAAAGC

SEQ ID NO:80

AGGAATTCCGCTTGCTTTTCCCG

SEQ ID NO:81

CAGTTACTAGTTTTACCCTAGGCAGC

SEQ ID NO:82

GAACTGAGATAGTGTTTAAGGGATTCTGC

SEQ ID NO:83
ACGTAGAAGGGTTTATTCCCAAACAAAA

SEQ ID NO:84
TACGACCGGTTTTTCGGGATTGG

SEQ ID NO:85
ATGTCAAAGGTGGGTAAGGTTTTTCG

SEQ ID NO:86
GACTACGACCGGTTTTTCGGGATTG

SEQ ID NO:87
GATCGCTCCCTTTTACCTCTCGG

SEQ ID NO:88
GGTAAGGTTCTTCGGTTTGCATCGAATT

SEQ ID NO:89
GTACTCCAGTCCGACGGTTTCG

SEQ ID NO:90
TGCAGCACCTGTGTTTAGGTT

SEQ ID NO:91
GTTCTAGCAAGCTAGCACTCTCATATTT

SEQ ID NO:92
CGGACTACGACGAGTTTTTTGGGATT

SEQ ID NO:93
ATAGGGCTCGGCTTCATGCG

SEQ ID NO:94
ATATCACTATAGGGCTCGGCTTCATGC

SEQ ID NO:95
TATAGGGCTCGGCTTCATGCG

SEQ ID NO:96
GAGACCGGCTTTTTGGGATTTC

SEQ ID NO:97
AACTGAGACCGGCTTTTTGGGATTG

SEQ ID NO:98

tCiCCTTTCAGGAAGAGGCCCCCTTTT

SEQ ID NO:99

CATCAGTCCTTTTTCCCCGACAAAAGG

SEQ ID NO:100

CCATCAGTCCTTTTTCCCCGACAAAAG

SEQ ID NO:101

tCiCGGTTGAGCCGTGGTATTTTACGC

SEQ ID NO:102

tCGCTACTGATCGTCGCCATGGTAAG

SEQ ID NO:103

TTTCCTTACTCACCATGCAGTAAGTAAT

SEQ ID NO:104

tCiCGTGCGCCAGTGTACTCTGCT

SEQ ID NO:105

GTGCAGCACCTGTCTTTAGGTTCTTG

SEQ ID NO:106

CGTGGACGGTAGCCTATTTAGCATT

SEQ ID NO:107

ATGTTTTAGAGATTTGCTCCACCTCGC

SEQ ID NO:108

GCGGTATTGCATCTTTTTGTCCTT

SEQ ID NO:109

AGCCGTGCAGCACCTGTTTT

SEQ ID NO:110

tCCGAAGTGAAGAGAAGGTTTTGAGATTAGC

SEQ ID NO:111

ACGGCCGGCTTTTTGCGA

SEQ ID NO:112

GAGTTAAGCTCCAGGTTTTCACGC

SEQ ID NO:113

TACGGCCGGCTTTTTCGATT

SEQ ID NO:114

TACATTTAGTTTTTCTCCCTGCACCATG

SEQ ID NO:115

GTACATTTAGTTTTTCTCCCTGCACCAT

SEQ ID NO:116

tCAAAGTTGAGCTTCGGCTTTTCAC

SEQ ID NO:117

ACTGACAGAGTTTTACACCCCAAGGG

SEQ ID NO:118

CGATCCGAAGTGAAGAATAGGTTTAAGAG

SEQ ID NO:119

CAGCGTGCTGATCTGCGTTTACTAG

SEQ ID NO:120

GCAACTCCCATTTTTGGGTTGGATG

SEQ ID NO:121

TGAAGTGAAGGACGGTTTTATGGATTTGC

SEQ ID NO:122

GAGCGTATGCGGTATTAGCGTAAGTTT

SEQ ID NO:123

ACGGTATTAGCGCAACCCCTTT

SEQ ID NO:124

GTTTGGCAACCCCTTTGTACCGA

SEQ ID NO:125

ATGTCAAGGGTAGGTAAGGTTTTTCG

SEQ ID NO:126

GAACTACGAACAGCTTTTTGAGATTCGC

SEQ ID NO:127

ATAATATCCGGTTTTAGCACTCCTTTCG

SEQ ID NO:128

AAGCCCTCCTTTAGCACCTTAGTTTT

SEQ ID NO:129

ATAGAGCTCGGCTTCATGCGGTATTAG

SEQ ID NO:130

TATAGAGCTCGGCTTCATGCGGTATTAG

SEQ ID NO:131

ATATCACTATAGAGCTCGGCTTCATGCG

SEQ ID NO:132

GAACTGAGACGCACTTTTAGAGGTTGG

SEQ ID NO:133

TGCAGCACCTTGTTTCAGGTCATTG

SEQ ID NO:134

tCGCGTTTTCTTCCCAGATAAAAGCAGTTT

SEQ ID NO:135

CatAAATTCCAtgTaCCcCatCGGc

SEQ ID NO:136

CCGGAAACTTTCACCGCTGAC

SEQ ID NO:137

tgTcAcATTCCgCCTaCCTCTAgtGTAt

SEQ ID NO:138

cGTCAGTATCAAggGCAcTgCgata

SEQ ID NO:139

CTcCGaccgTCTAGCcTca

SEQ ID NO:140

TCGGTCTTTAGCATCGGACTTGAGAG

SEQ ID NO:141

GgAATTctACCcCCCTCTACGagAC

SEQ ID NO:142

GGGTATTATCCCCAGTTTTCCGGG

SEQ ID NO:143

GCCTTGGTGGGCTTTTACCCC

SEQ ID NO:144

GAACTGAGAACGGTTTTTTCCGATTAGC

SEQ ID NO:145

ATTCGACAGGGGTTTACGATCCGAA

SEQ ID NO:146

CGGTATTAGCGGTCGTTTCCAACC

SEQ ID NO:147

CGAAGGAACGGACTATCTCTAGTCTTTT

SEQ ID NO:148

GTTAAGCTGTGAGTTTTTACGAACAACG

SEQ ID NO:149

GCCATGCAGCACCTTGTTTCATG

SEQ ID NO:150

GCTACTTGTGGAATTCCATTTTCTCCGT

SEQ ID NO:151

GGTACCGCCGCTTTTGAGAG

SEQ ID NO:152

GAACTGGGGCGCGCTTTTTTG

SEQ ID NO:153

TGAGCCAGGATCAAACCTCTCCAGTTTTT

SEQ ID NO:154

TTGCATGTCAAGCCTTGGTAAGGTTTTT

SEQ ID NO:155

GAACTGGCGCCTCTTTTAAGGATTIG

SEQ ID NO:156

GGCTTCGGCACCGATAGGTTTT

SEQ ID NO:157

TGAGGACGGCTTTTTTGGGATTGG

SEQ ID NO:158

TGAGACTGGTTTTTTTGAGATTAGCTTGG

SEQ ID NO:159

TTTTTTT

SEQ ID NO:160

/5AmMC12/TTTTTTT

SEQ ID NO:161

CATTGATCGCAACGTTCAATTTAAT

SEQ ID NO:162

TGGTCTTTCTGCATTCCTGGA

SEQ ID NO:163

CTATGATCCCAATCTAACTTCCACATACC

SEQ ID NO:164

AGRGTTYGATYMTGGCTCAG

SEQ ID NO:165

RGYTACCTTGTTACGACTT

SEQ ID NO:166

TCTAAGCGGTGCGGTAATCT

SEQ ID NO:167

TTCTTTCCAGGTGCTCCAAC

SEQ ID NO:168

TGGGCACATACACGCAGGA

SEQ ID NO:169

TTGGTGGAGTGATTTGTCTGCT

SEQ ID NO:170

TCTAAGGGCATCACAGACCTG

SEQ ID NO:171

TCGGCCCTTAAATAGCCCGGTCCGC

SEQ ID NO:172

TTAACCTACTAAATAGTGCTGCTAGC

WHAT IS CLAIMED IS:

1 1. A method for characterizing a population of microbial strains in a sample,
2 wherein different microbial strains in the population comprise polynucleotides with different
3 microbe-identifying sequences, the method comprising:
4 (i) amplifying polynucleotides obtained from the sample to form a plurality of
5 amplicons, wherein the amplicons comprise microbe-identifying sequences;
6 (ii) combining the amplicons with a plurality of microbeads,
7 wherein each of the microbeads has a lanthanide spectral signature and a plurality
8 of copies of a capture polynucleotide immobilized on the microbead,
9 wherein each of the capture polynucleotides comprises a predetermined sequence,
10 wherein each predetermined sequence is paired with a specific lanthanide spectral
11 signature, such that the capture polynucleotide immobilized on a microbead can be identified
12 based on the lanthanide spectral signature of the microbead,
13 wherein at least some of the capture polynucleotides comprise a sequence
14 substantially complementary to a microbe-identifying sequence of one or more amplicons,
15 wherein the combining is conducted under conditions in which at least some
16 amplicons are captured onto the beads by the capture polynucleotides, thereby producing
17 captured amplicons, and
18 wherein the amplicons are labeled with one or more signal-generating moieties
19 prior to, simultaneously with, or after being captured onto the beads, such that microbeads
20 comprising immobilized signal-generating moieties are produced;
21 (iii) detecting signal from immobilized signal-generating moieties of individual
22 microbeads from step (ii); and
23 (iv) determining the lanthanide spectral signatures of the individual microbeads
24 from step (ii), thereby determining the microbe-identifying sequences of the captured amplicons
25 and characterizing the population of microbial strains in the sample.

1 2. The method of claim 1, wherein step (iii) occurs prior to step (iv).

1 3. The method of claim 1, wherein step (iii) occurs simultaneously with step (iv).

1 4. The method of claim 1, wherein step (iii) occurs after step (iv).

1 5. The method of any one of claims 1-4, wherein the amplicons are captured by
2 hybridization of microbe-identifying sequences in the amplicons to the substantially
3 complementary sequences in the capture polynucleotides.

1 6. The method of any one of claims 1-5, wherein the polynucleotides in step (i)
2 are DNA.

1 7. The method of any one of claims 1-5, wherein the polynucleotides in step (i)
2 are RNA.

1 8. The method of claim 1, wherein the plurality of microbeads comprises at least
2 50 different spectral signatures and immobilized capture polynucleotides comprising at least 50
3 different predetermined sequences.

1 9. The method of claim 1 or claim 8, wherein the combining in step (ii)
2 comprises hybridizing amplicons to at least 50 microbeads having different spectral signatures
3 and different capture polynucleotides.

1 10. The method of any one of claims 1-9, wherein the signal-generating moiety
2 produces a fluorescent or chemiluminescent signal.

1 11. The method of any one of claims 1-10, wherein the amplicons are labeled
2 during the amplifying in step (i).

1 12. The method of any one of claims 1-11, wherein the magnitude of signal
2 detected from an individual microbead in step (iii) corresponds to the amount of the amplicons
3 captured on the microbead.

1 13. The method of any one of claims 1-12, wherein the population of microbial
2 strains comprises bacterial strains.

1 14. The method of any one of claims 1-13, wherein at least one of the microbe
2 identifying sequences in the amplicons comprises a bacterial 16S ribosomal RNA (rRNA) gene
3 sequence.

1 15. The method of claim 14, wherein the amplification is conducted using primer
2 pairs that hybridize to conserved regions flanking one or more variable regions in a bacterial 16S
3 rRNA gene sequence.

1 16. The method of claim 14 or claim 15, wherein the amplification in step (i)
2 comprises amplifying a V3 variable region, a V4 variable region, or a V6 variable region in a
3 bacterial 16S rRNA gene sequence.

1 17. The method of claim 1, wherein at least one of the microbe indentifying
2 sequenes in the amplicons comprises an antibiotic resistance gene sequence.

1 18. The method of any one of claims 1-15, wherein the microbial strains comprise
2 one or more species selected from the group consisting of *Pseudomonas*, *Streptococcus*,
3 *Staphylococcus*, *Neisseria*, *Acinetobacter*, *Escherichia*, *Enterobacter*, and *Klebsiella*.

1 19. The method of any one of claims 1-18, wherein each of the lanthanide spectral
2 signatures comprises a europium (Eu) signal, a dysprosium (Dy) signal, a samarium (Sm) signal,
3 a cerium (Ce) signal, a terbium (Tb) signal, a lanthanum (La) signal, a praseodymium (Pr) signal,
4 a neodymium (Nd) signal, a gadolinium (Gd) signal, a holmium (Ho) signal, an erbium (Er)
5 signal, a thulium (Tm) signal, an ytterbium (Yb) signal, or a combination thereof.

1 20. The method of any one of claims 1-19, wherein each of the microbeads
2 comprises a plurality of lanthanide nanoparticles.

1 21. The method of claim 20, wherein the lanthanide nanoparticle comprises a
2 lanthanide-doped host lattice.

1 22. The method of claim 21, wherein the host lattice is yttrium orthovanadate,
2 lanthanum phosphate, or a combination thereof.

1 23. The method of any one of claims 20-22, wherein each of the microbeads
2 further comprises a crosslinked polymer, and wherein the capture polynucleotides are covalently
3 bonded to the crosslinked polymer.

1 24. The method of any one of claims 1-23, wherein the plurality of microbeads
2 are dispersed on a microscope slide prior to step (iii) or step (iv).

1 25. The method of any one of claims 1-24, further comprising separating
2 uncaptured amplicons from microbeads having captured amplicons.

1 26. The method of any one of claims 1-25, further comprising separating
2 microbeads having immobilized signal-generating moieties from microbeads not having
3 immobilized signal-generating moieties.

1 27. The method of any one of claims 1-26, wherein the immobilized signal-
2 generating moieties are fluorescent labels.

1 28. The method of claim 27, wherein the immobilized signal-generating moieties
2 are detected in step (iii) via fluorescence microscopy.

1 29. The method of any one of claims 1-28, wherein the sample is a biological
2 sample from an animal, a food sample, a beverage sample, or an environmental sample.

1 30. The method of claim 29, wherein the biological sample is a blood sample, an
2 intestinal sample, a stool sample, a urine sample, a saliva sample, or a sputum sample.

1 31. A plurality of microbeads, each of the microbeads comprising a lanthanide
2 spectral signature and plurality of copies of a capture polynucleotide immobilized on the
3 microbead, wherein each capture polynucleotide comprises a predetermined sequence and each
4 capture polynucleotide is paired with a lanthanide spectral signature, such that the predetermined
5 sequence can be identified based on the lanthanide spectral signature.

1 32. The plurality of microbeads according to claim 31, wherein each capture
2 polynucleotide comprises a nucleic acid sequence that hybridizes to a bacterial 16S rRNA gene
3 sequence.

1 33. The plurality of microbeads according to claim 32, wherein the capture
2 polynucleotides comprise a nucleic acid sequence that hybridizes to a V3 variable region, a V4
3 variable region, or a V6 variable regions in a bacterial 16S rRNA gene sequence.

1 34. The plurality of microbeads according to any one of claims 31-33, wherein
2 each of the capture polynucleotides comprises a nucleic acid sequence able to hybridize a
3 *Pseudomonas* gene sequence, a *Streptococcus* gene sequence, *Staphylococcus* gene sequence, a
4 *Neisseria* gene sequence, an *Acinetobacter* gene sequence, an *Escherichia* gene sequence, an
5 *Enterobacter* gene sequence, or a *Klebsiella* gene sequence.

1 35. The plurality of microbeads according to any one of claims 31-34, wherein
2 each of the lanthanide spectral signatures comprises one or more signals selected from a
3 europium (Eu) signal, a dysprosium (Dy) signal, a samarium (Sm) signal, a cerium (Ce) signal, a
4 terbium (Tb) signal, a lanthanum (La) signal, a praseodymium (Pr) signal, a neodymium (Nd)
5 signal, a gadolinium (Gd) signal, a holmium (Ho) signal, an erbium (Er) signal, a thulium (Tm)
6 signal, and an ytterbium (Yb) signal.

1 36. The plurality of microbeads according to any one of claims 31-35, wherein
2 each of the microbeads comprises a plurality of lanthanide nanoparticles.

1 37. The plurality of microbeads according to claim 36, wherein the lanthanide
2 nanoparticles comprise a lanthanide-doped host lattice.

1 38. The plurality of microbeads according to claim 37, wherein the host lattice is
2 yttrium orthovanadate, lanthanum phosphate, or a combination thereof.

1 39. The plurality of microbeads according to any one of claims 36-38, wherein
2 each of the microbeads further comprises a crosslinked polymer, and wherein the capture
3 polynucleotides are covalently bonded to the crosslinked polymer.

1 40. The plurality of microbeads according to any one of claims 31-39, comprising
2 at least 20 lanthanide spectral signatures.

1 41. The plurality of microbeads according to any one of claims 31-40, comprising
2 at least 1000 lanthanide spectral signatures.

1 42. A method for characterizing a population of microbial strains in a sample,
2 wherein different microbial strains in the population comprise polynucleotides with different
3 microbe-identifying sequences, the method comprising:

4 (i) amplifying polynucleotides obtained from the sample to form a plurality of
5 amplicons, wherein the amplicons comprise microbe-identifying sequences;

6 (ii) combining the amplicons with a panel of microbeads,
7 wherein each of the microbeads has a lanthanide spectral signature and a plurality
8 of copies of a capture polynucleotide immobilized on the microbead,

9 wherein each of the capture polynucleotides comprises a predetermined sequence,

10 wherein each predetermined sequence is paired with a specific lanthanide spectral
11 signature, such that the capture polynucleotide immobilized on a microbead can be identified
12 based on the lanthanide spectral signature of the microbead,

13 wherein at least some of the capture polynucleotides comprise a sequence
14 substantially complementary to a microbe-identifying sequence of one or more amplicons,

15 wherein the combining is conducted under conditions in which at least some
16 amplicons hybridize to the capture polynucleotides, thereby producing captured amplicons, and

17 wherein the amplicons are labeled with one or more signal-generating moieties
18 prior to, simultaneously with, or after being captured onto the beads, such that microbeads
19 comprising immobilized signal-generating moieties are produced; wherein the magnitude of
20 signal detected from an individual microbead corresponds to the amount of the amplicons
21 captured on the microbead.

22 (iii) measuring the magnitude of the signal from immobilized signal-generating
23 moieties of individual microbeads from step (ii);

24 (iv) determining the lanthanide spectral signatures of the individual microbeads
25 from step (ii), thereby determining the microbe-identifying sequences of the captured amplicons;

26 (v) generating a pattern of measured amount of hybridization of the captured
27 amplicons to individual capture polynucleotides;

(vi) comparing the pattern of hybridization measured in (v) to a predicted pattern of hybridization of amplicons to each capture oligonucleotide, wherein predicting hybridization of the amplicons to a capture oligonucleotide comprises calculating the Gibbs free energy of hybridization; and

(vii) correlating the pattern of hybridization measured in (v) to the predicted pattern of hybridization for each species to identify the species that has a predicted pattern of hybridization that has the strongest correlation with the pattern of hybridization measured in (v).

43. The method of claim 42, wherein step (iii) occurs prior to step (iv).

44. The method of claim 42, wherein step (iii) occurs simultaneously with step (iv).

45. The method of claim 42, wherein step (iii) occurs after step (iv).

46. The method of any one of claims 42-45, wherein the polynucleotides in step (i) are DNA.

47. The method of claim 42, wherein the plurality of microbeads comprises at least 50 different spectral signatures and immobilized capture polynucleotides comprising at least 50 different predetermined sequences.

48. The method of claim 42, wherein the combining in step (ii) comprises hybridizing amplicons to at least 50 microbeads having different spectral signatures and different capture polynucleotides.

49. The method of any one of claims 42-48, wherein the signal-generating moiety produces a fluorescent or chemiluminescent signal.

50. The method of any one of claims 42-49, wherein the amplicons are labeled during the amplifying in step (i).

51. The method of any one of claims 42-50, wherein the population of microbial strains comprises bacterial strains.

1 52. The method of any one of claims 42-51, wherein at least one of the microbe
2 identifying sequences in the amplicons comprises a bacterial 16S ribosomal RNA (rRNA) gene
3 sequence.

1 53. The method of claim 52, wherein the amplification is conducted using primer
2 pairs that hybridize to conserved regions flanking one or more variable regions in a bacterial 16S
3 rRNA gene sequence.

1 54. The method of claim 52 or claim 53, wherein the amplification in step (i)
2 comprises amplifying one or more of a V3 variable region, a V4 variable region, or a V6 variable
3 region in a bacterial 16S rRNA gene sequence.

1 55. The method of claim 54, wherein the amplification step (i) comprises
2 amplifying the V3 variable region and the V6 variable region.

1 56. The method of any one of claims 42-55, wherein the microbial strains
2 comprise one or more species selected from the group consisting of *Pseudomonas*,
3 *Streptococcus*, *Staphylococcus*, *Neisseria*, *Acinetobacter*, *Escherichia*, *Enterobacter*, *Klebsiella*,
4 *Haemophilus*, *Proteus*, *Serratia*, *Enterococcus*, and *Listeria*.

5 57. The method of any one of claims 42-56, wherein each of the lanthanide
6 spectral signatures comprises a europium (Eu) signal, a dysprosium (Dy) signal, a samarium
7 (Sm) signal, a cerium (Ce) signal, a terbium (Tb) signal, a lanthanum (La) signal, a
8 praseodymium (Pr) signal, a neodymium (Nd) signal, a gadolinium (Gd) signal, a holmium (Ho)
9 signal, an erbium (Er) signal, a thulium (Tm) signal, an ytterbium (Yb) signal, or a combination
10 thereof.

1 58. The method of any one of claims 42-57, wherein each of the microbeads
2 comprises a plurality of lanthanide nanoparticles.

1 59. The method of claim 58, wherein the lanthanide nanoparticle comprises a
2 lanthanide-doped host lattice.

1 60. The method of claim 59, wherein the host lattice is yttrium orthovanadate,
2 lanthanum phosphate, or a combination thereof.

1 61. The method of any one of claims 59-60, wherein each of the microbeads
2 further comprises a crosslinked polymer, and wherein the capture polynucleotides are covalently
3 bonded to the crosslinked polymer.

1 62. The method of any one of claims 42-61, wherein the plurality of microbeads
2 are dispersed on a microscope slide prior to step (iii) or step (iv).

1 63. The method of any one of claims 42-62, further comprising separating
2 uncaptured amplicons from microbeads having captured amplicons.

1 64. The method of any one of claims 42-63, further comprising separating
2 microbeads having immobilized signal-generating moieties from microbeads not having
3 immobilized signal-generating moieties.

1 65. The method of any one of claims 42-64, wherein the immobilized signal-
2 generating moieties are fluorescent labels.

1 66. The method of claim 65, wherein the immobilized signal-generating moieties
2 are measured in step (iii) via fluorescence microscopy.

1 67. The method of any one of claims 42-66, wherein the sample is from blood,
2 cerebrospinal fluid, lymph, or urine.

1

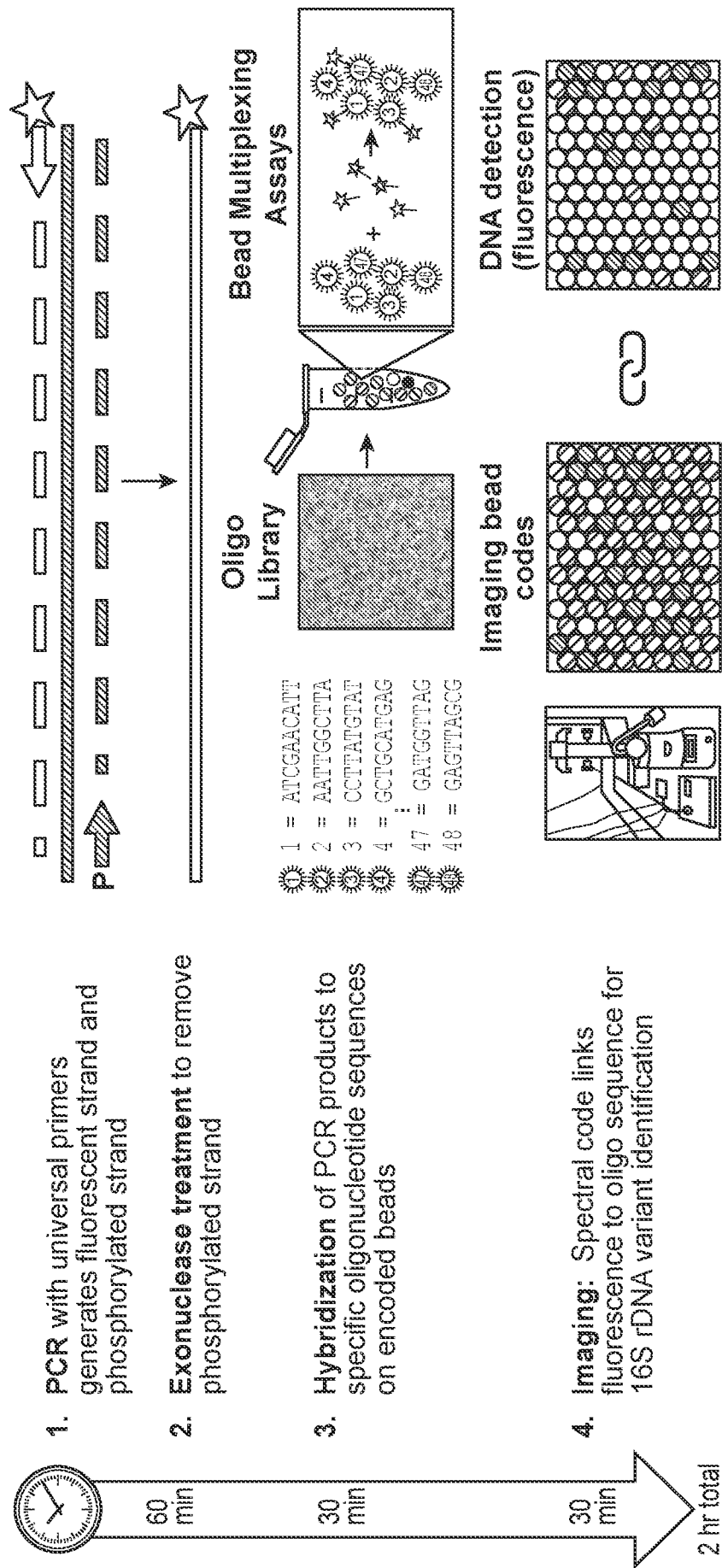


FIG. 1

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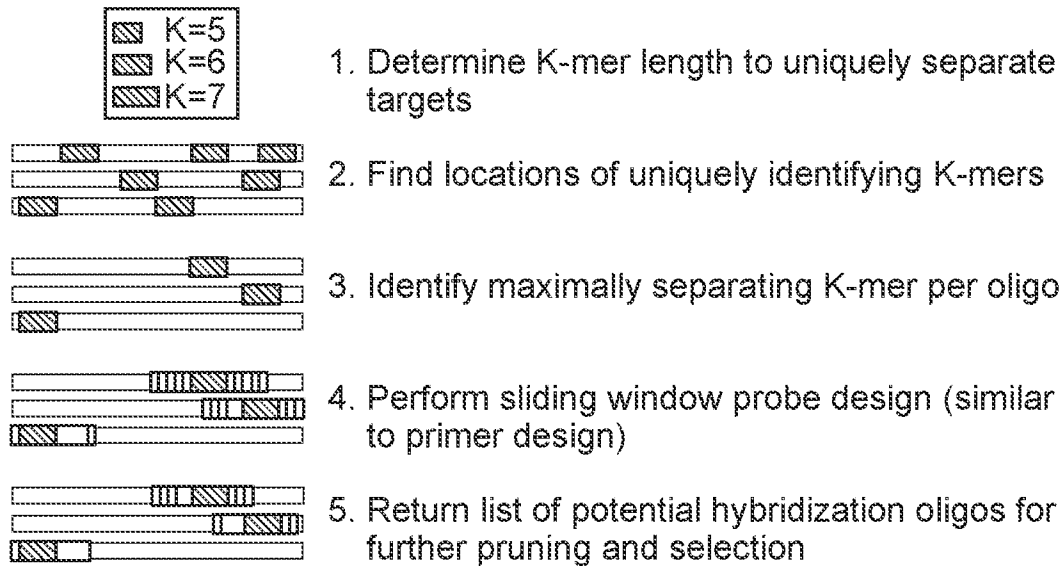


FIG. 2A

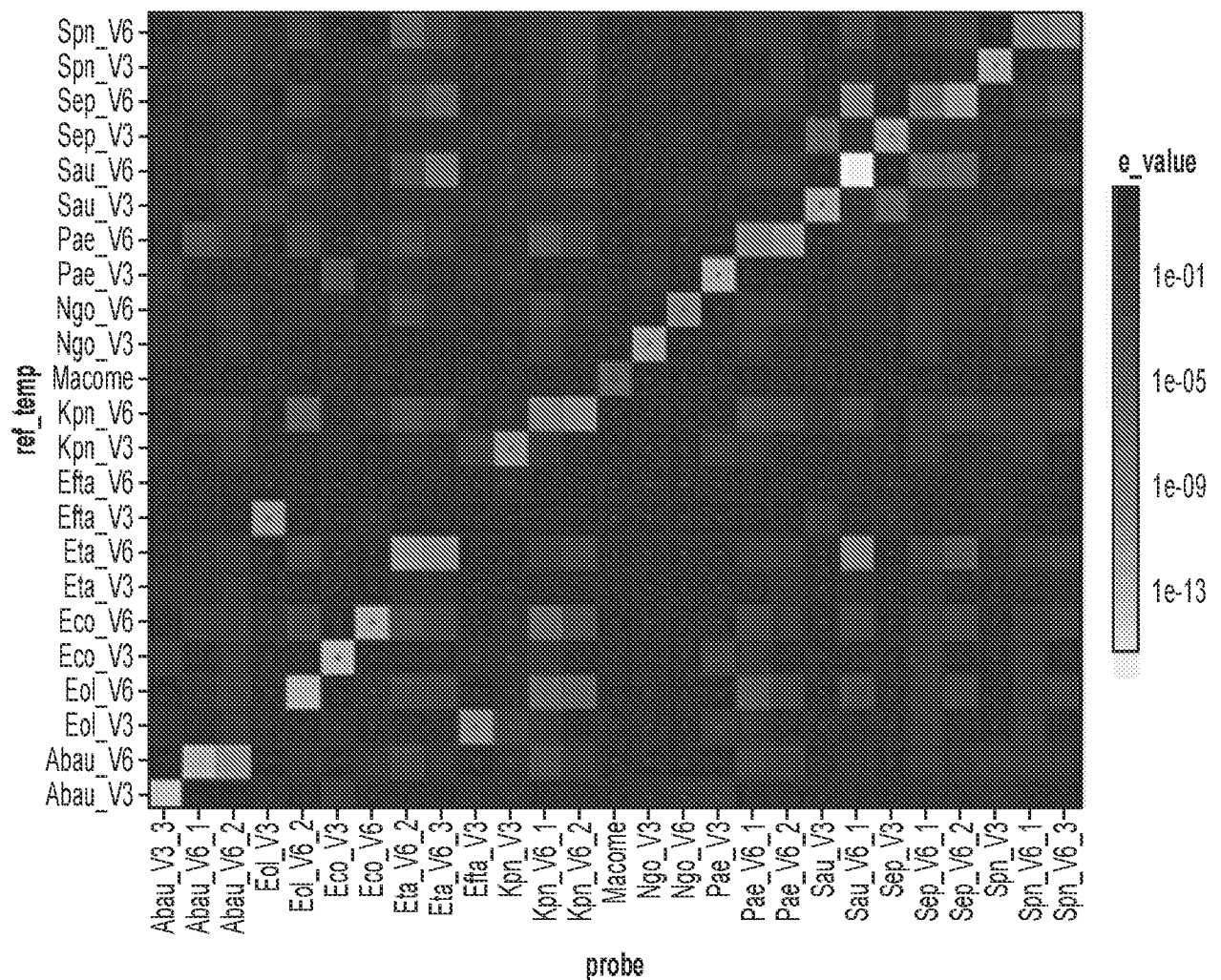


FIG. 2B

Pseudomonas_aeruginosa_V6	TGGAGCATGTGGTTTAATTTCGATGCAACGGG--AAGAAGCTTACCTGGCTTGACAT--gcttagaactttccgagatggaatttgcctttccctttc
Streptococcus_pneumoniae_V6	TGGAGCATGTGGTTTAATTTCGATGCAACGGG--AAGAAGCTTACCTGGCTTGACAT--gcttagaactttccgagatggaatttgcctttccctttc
Staphylococcus_epidermis_V6	TGGAGCATGTGGTTTAATTTCGATGCAACGGG--AAGAAGCTTACCTGGCTTGACAT--gcttagaactttccgagatggaatttgcctttccctttc
Enterococcus_faecium_V6	TGGAGCATGTGGTTTAATTTCGATGCAACGGG--AAGAAGCTTACCTGGCTTGACAT--gcttagaactttccgagatggaatttgcctttccctttc
Staphylococcus_aureus_V6	TGGAGCATGTGGTTTAATTTCGATGCAACGGG--AAGAAGCTTACCTGGCTTGACAT--gcttagaactttccgagatggaatttgcctttccctttc
Neisseria_gonorrhoeae_V6	TGGAGCATGTGGTTTAATTTCGATGCAACGGG--AAGAAGCTTACCTGGCTTGACAT--gcttagaactttccgagatggaatttgcctttccctttc
Acinetobacter_baumannii_V6	TGGAGCATGTGGTTTAATTTCGATGCAACGGG--AAGAAGCTTACCTGGCTTGACAT--gcttagaactttccgagatggaatttgcctttccctttc
Escherichia_coli_V6	TGGAGCATGTGGTTTAATTTCGATGCAACGGG--AAGAAGCTTACCTGGCTTGACAT--gcttagaactttccgagatggaatttgcctttccctttc
Enterobacter_cloacae_V6	TGGAGCATGTGGTTTAATTTCGATGCAACGGG--AAGAAGCTTACCTGGCTTGACAT--gcttagaactttccgagatggaatttgcctttccctttc
Klebsiella_pneumoniae_V6	TGGAGCATGTGGTTTAATTTCGATGCAACGGG--AAGAAGCTTACCTGGCTTGACAT--gcttagaactttccgagatggaatttgcctttccctttc

FIG. 2C

4 / 19

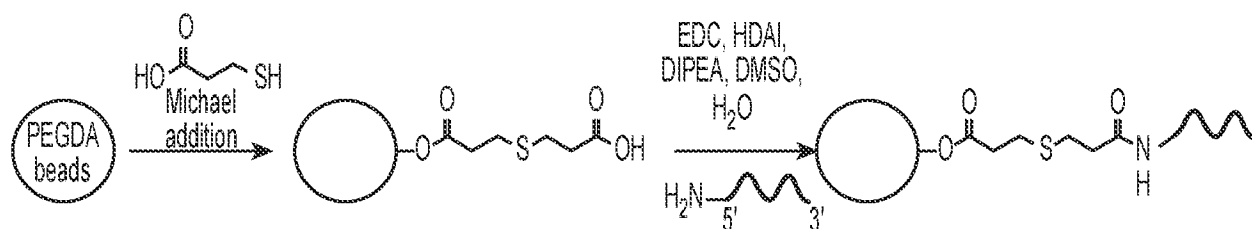


FIG. 3A

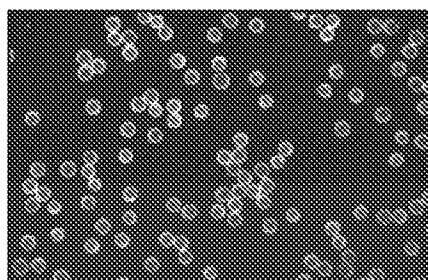


FIG. 3B

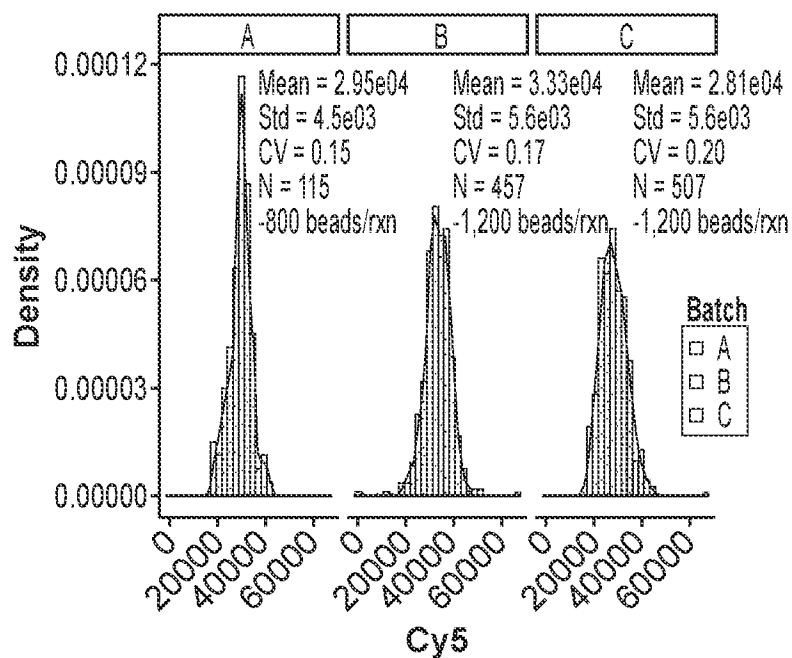


FIG. 3C

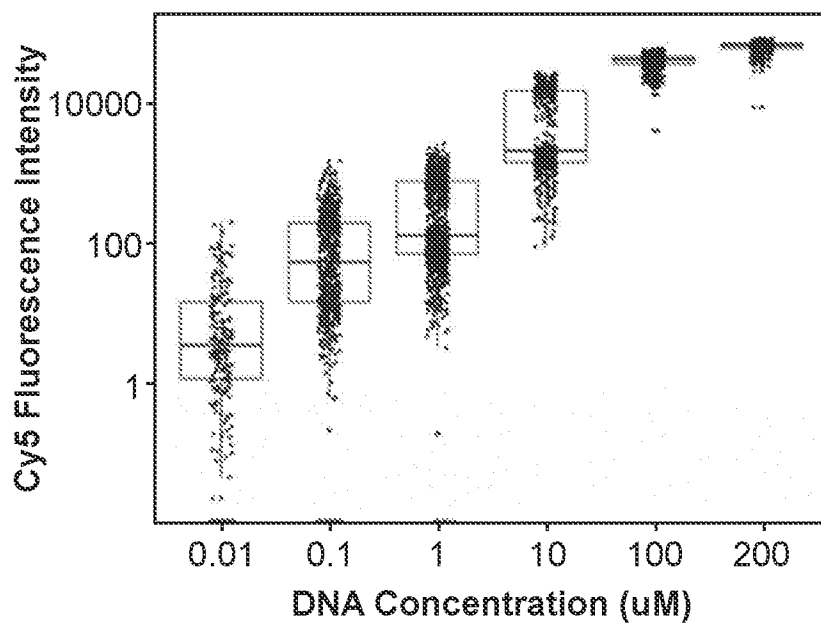


FIG. 3D

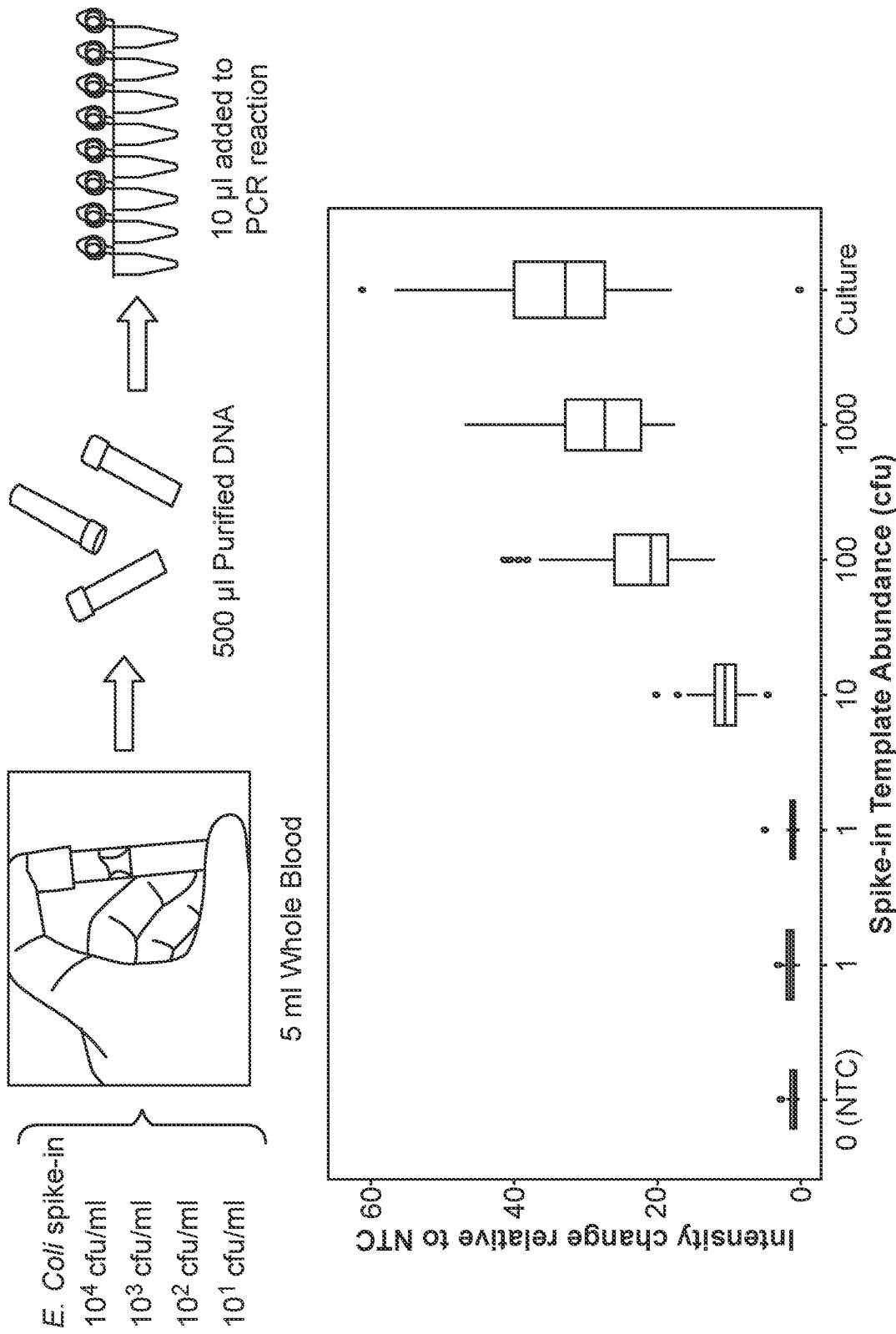


FIG. 4

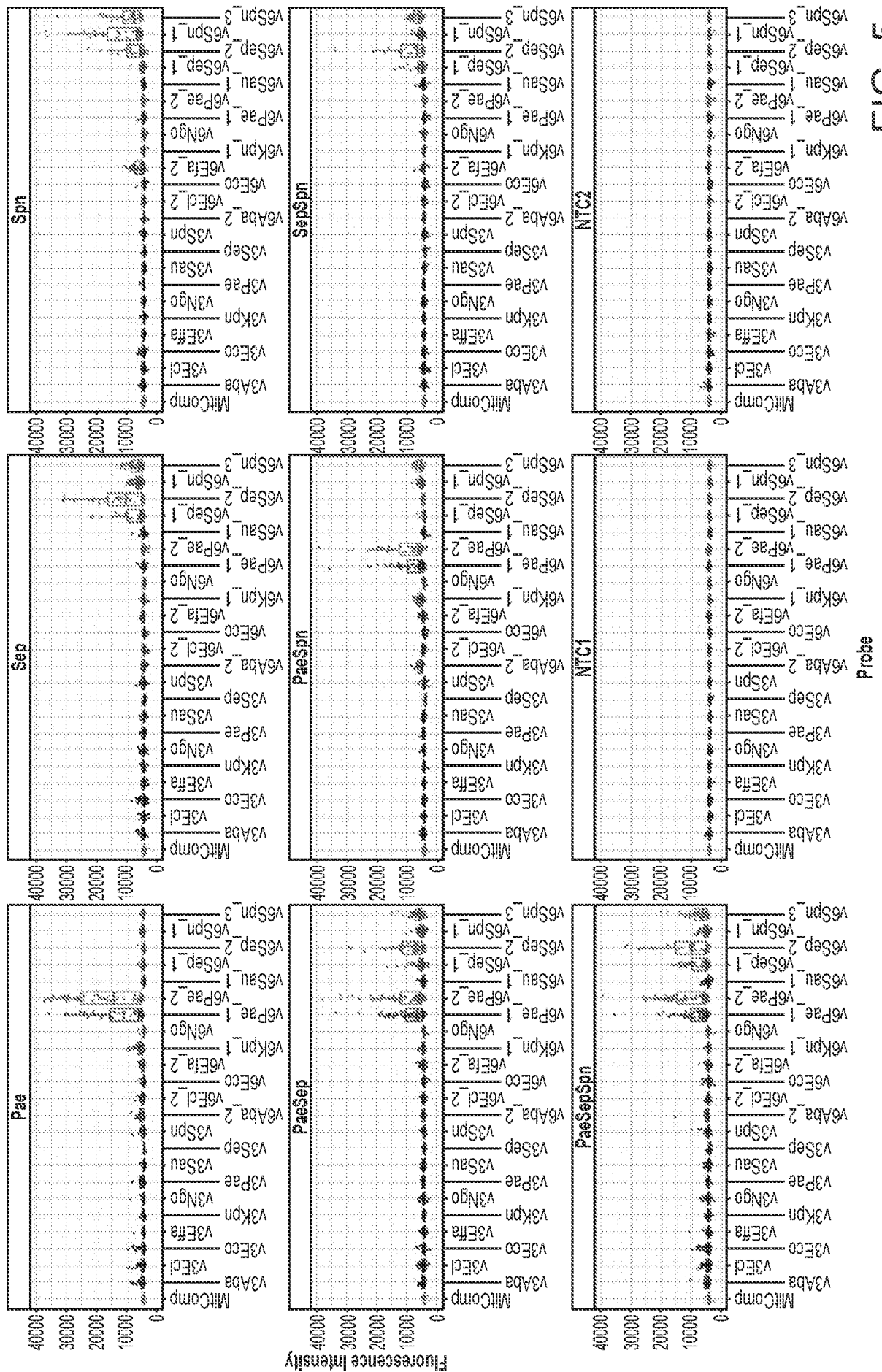


FIG. 5

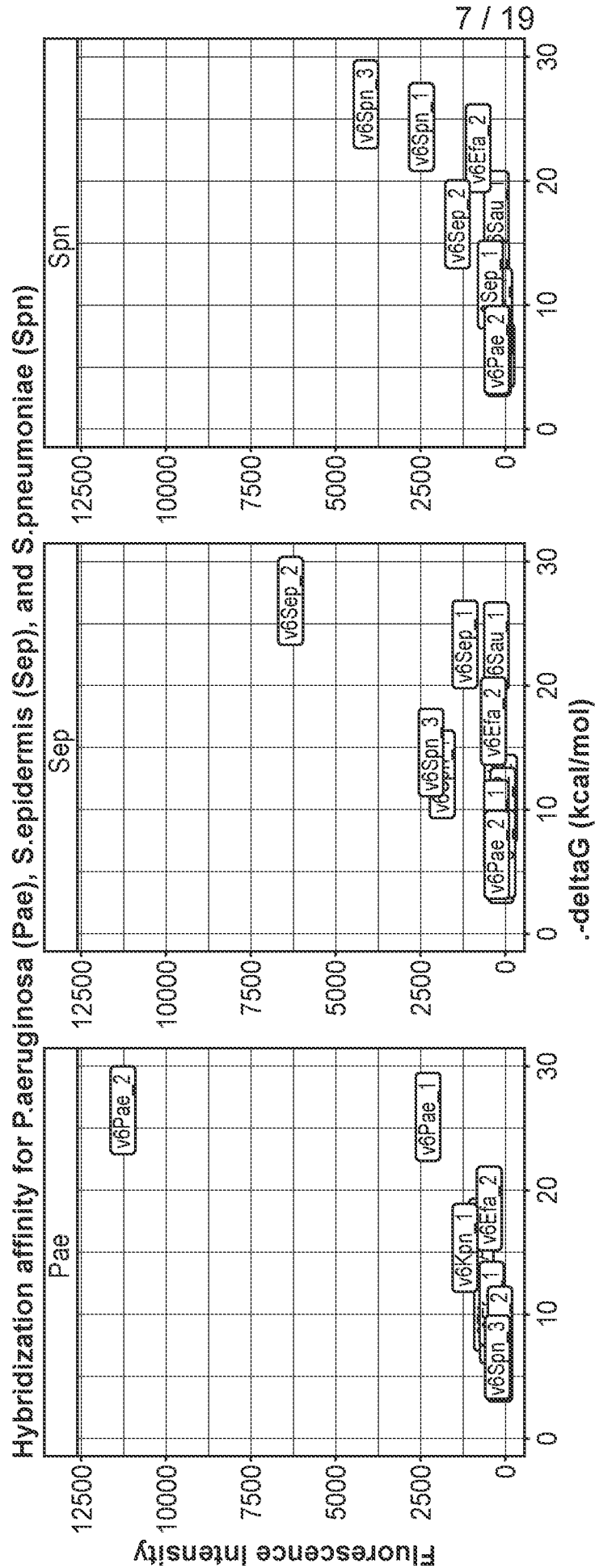


FIG. 6

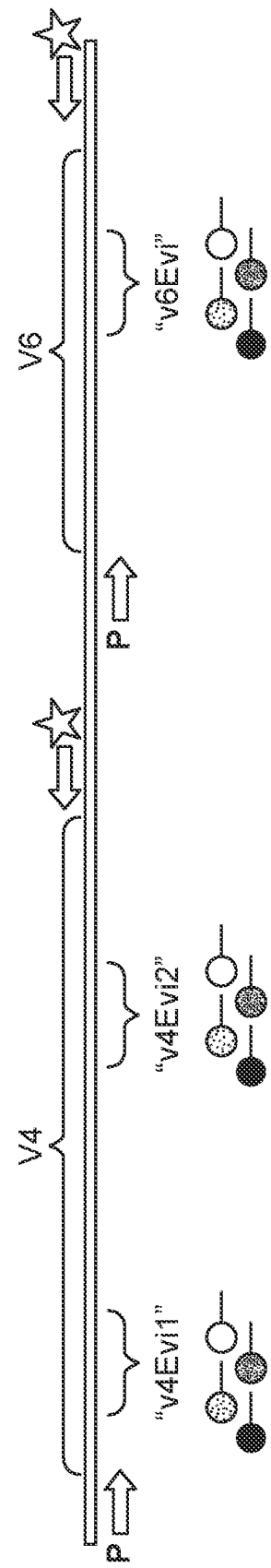


FIG. 7

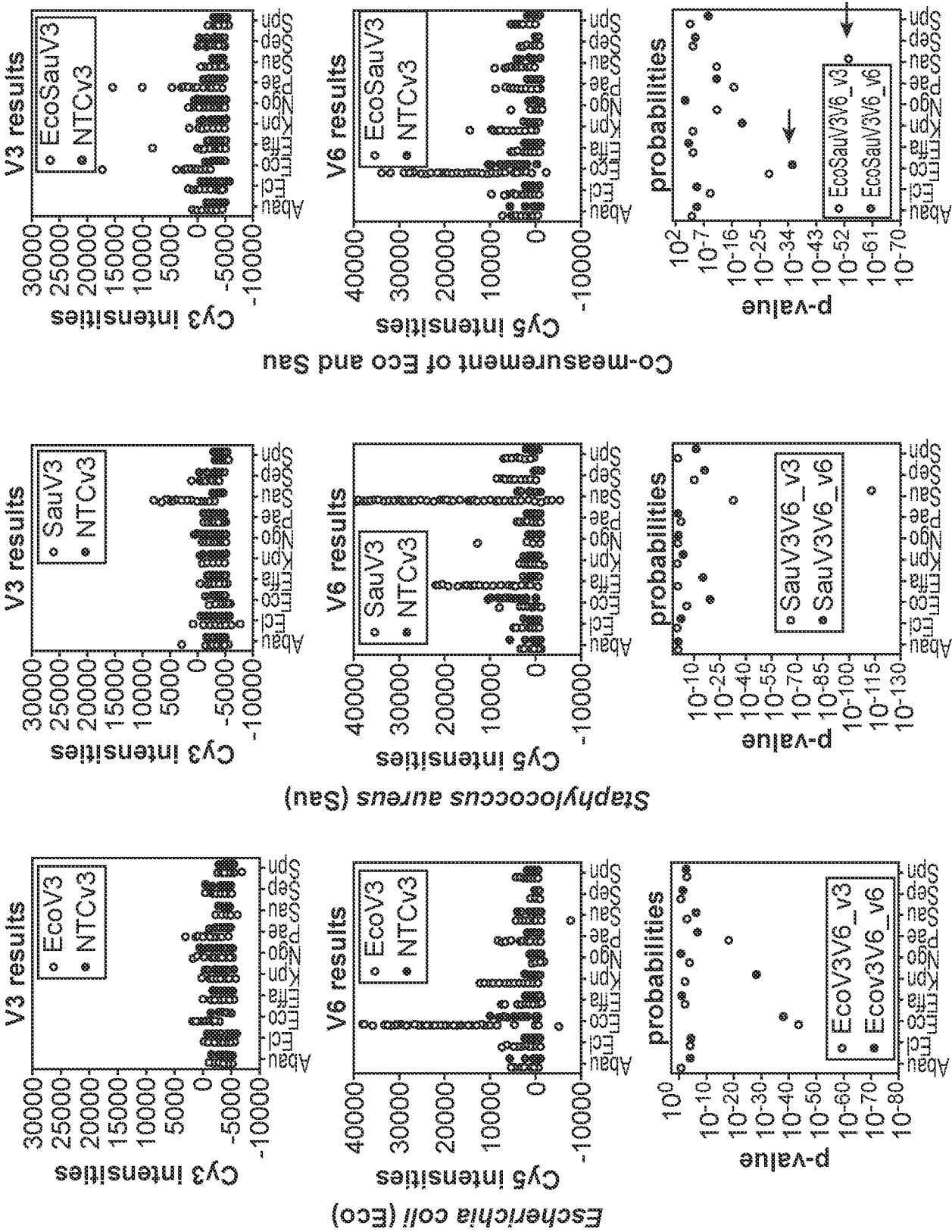
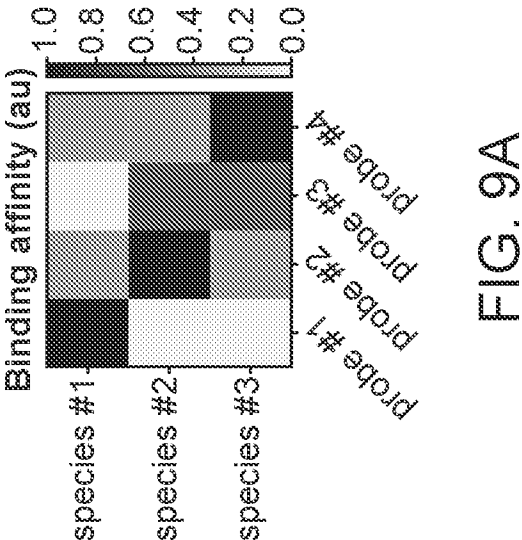
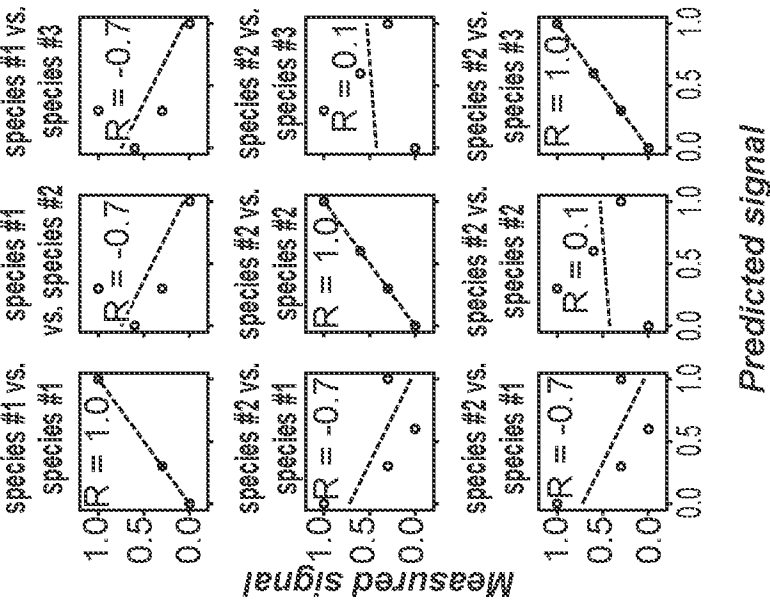
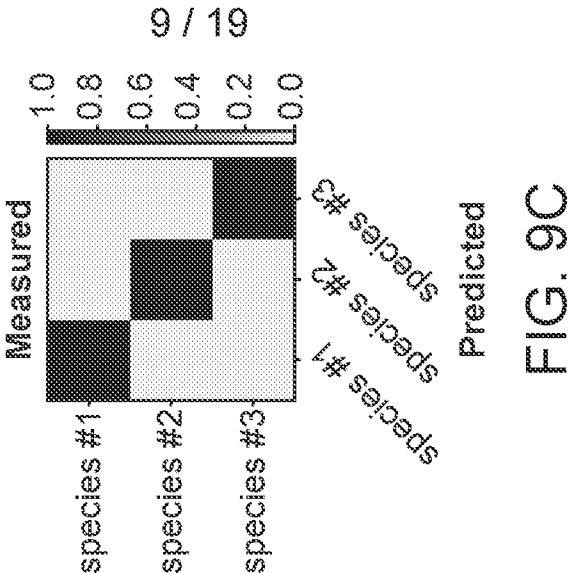
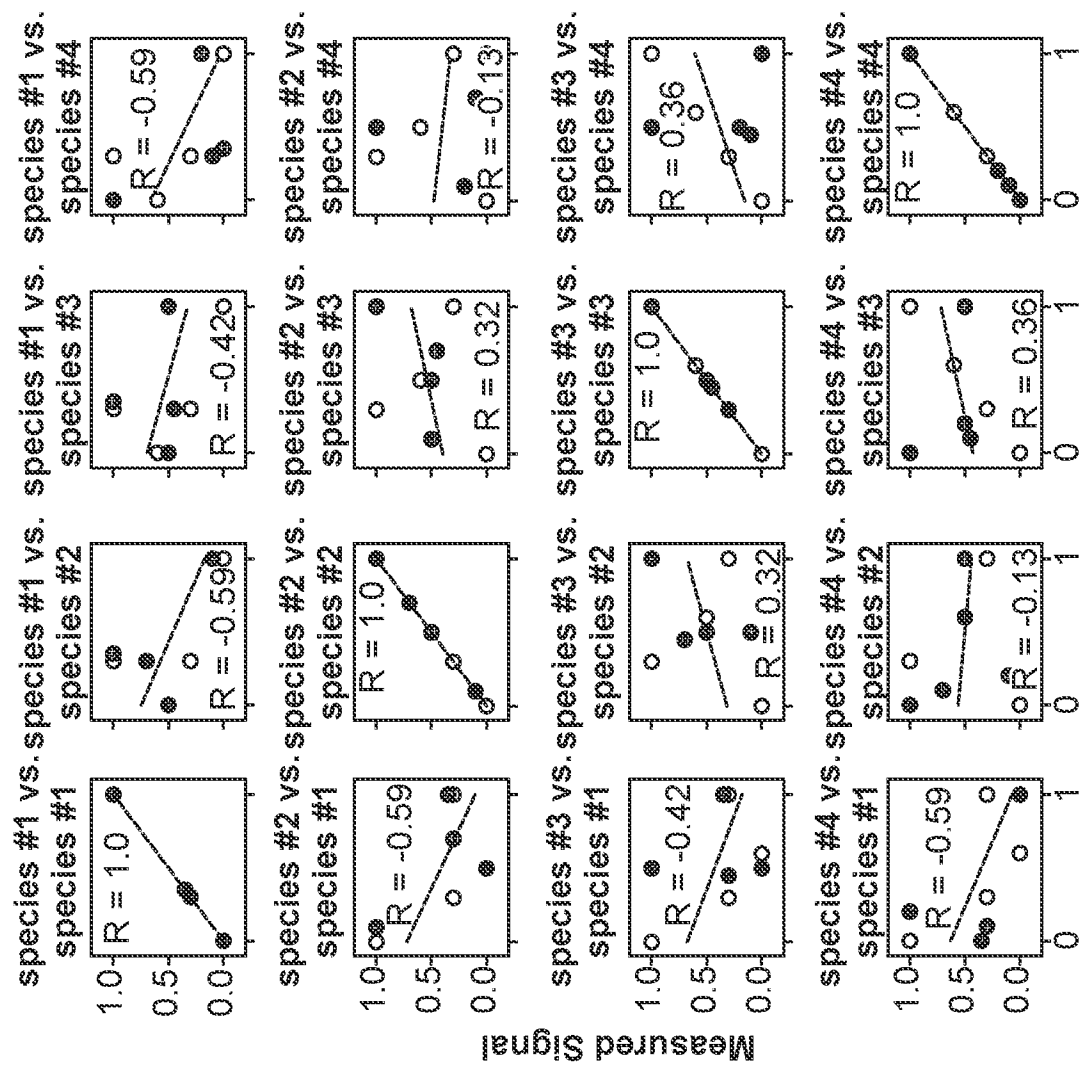


FIG. 8





Predicted Signal

FIG. 9E

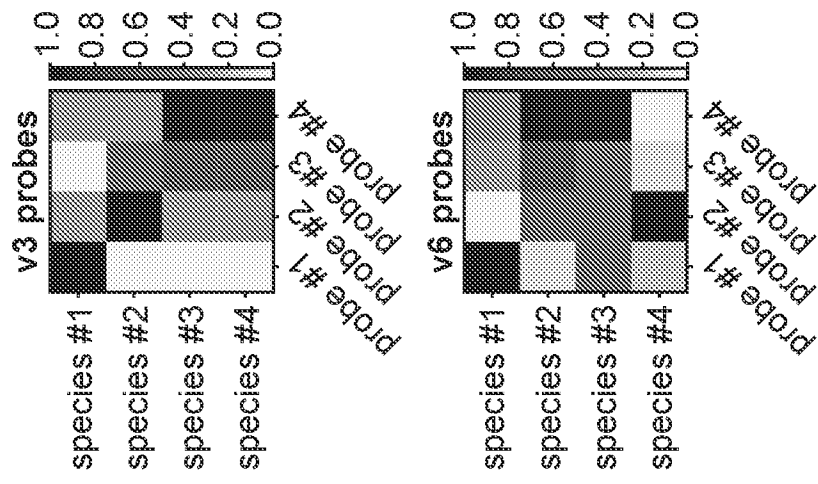


FIG. 9D

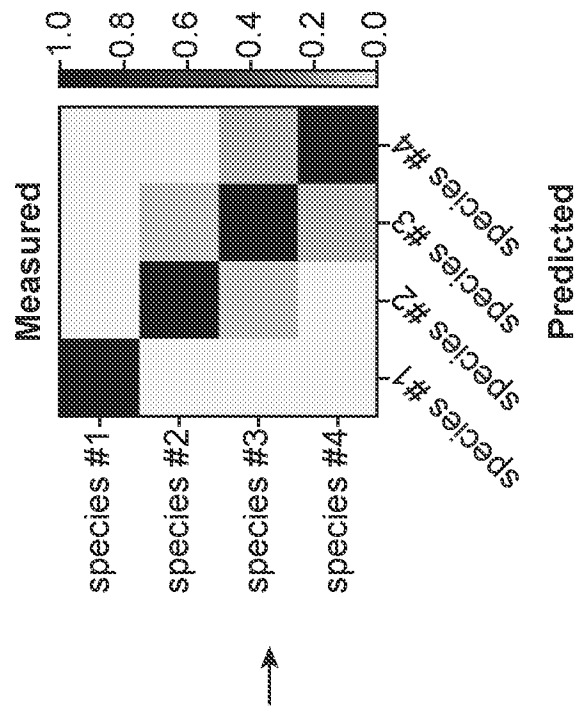


FIG. 9F

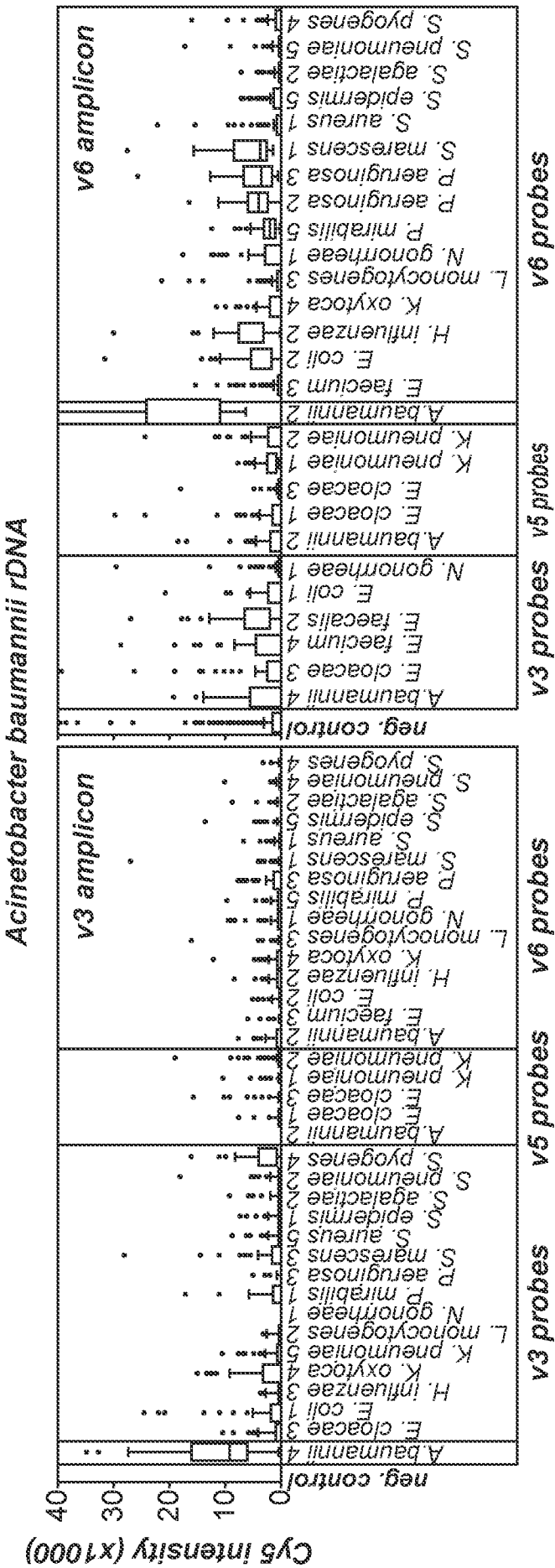


FIG. 10A

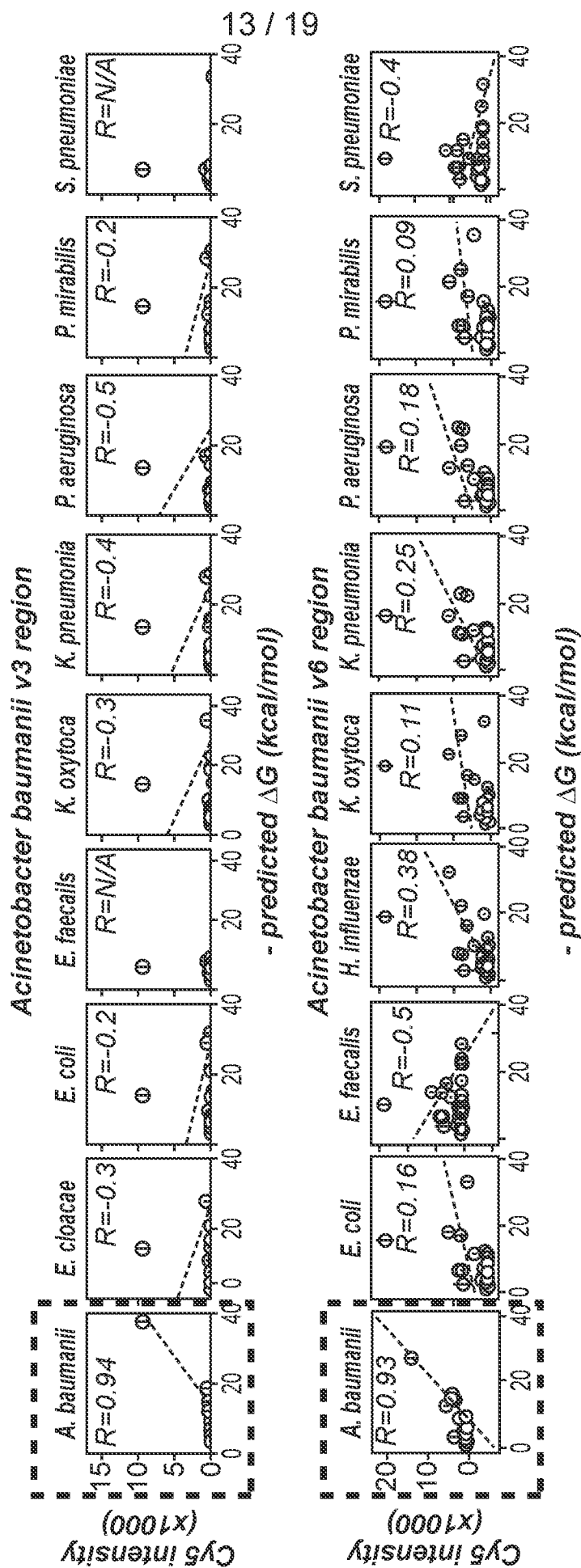
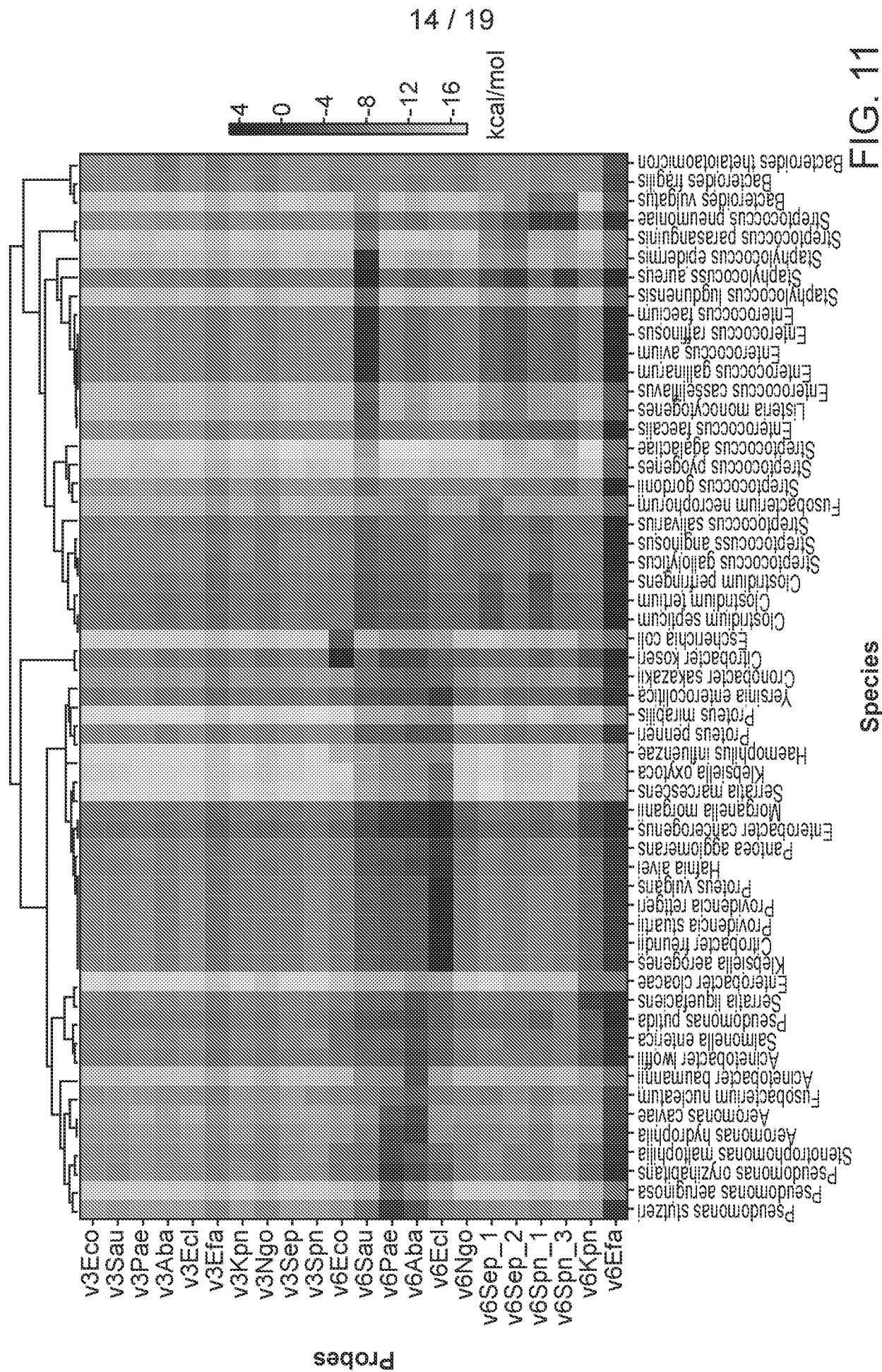


FIG. 10B



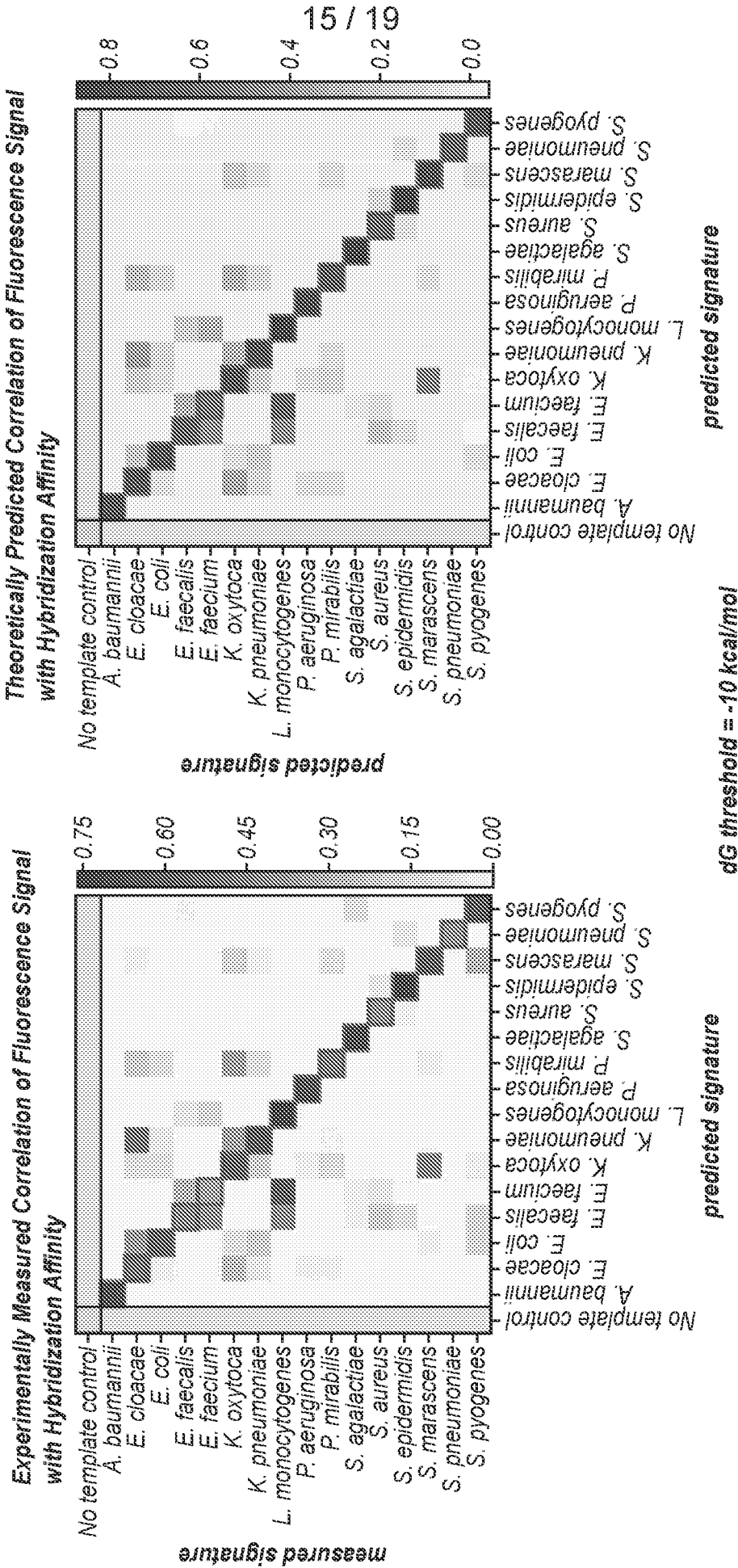


FIG. 12A

FIG. 12B

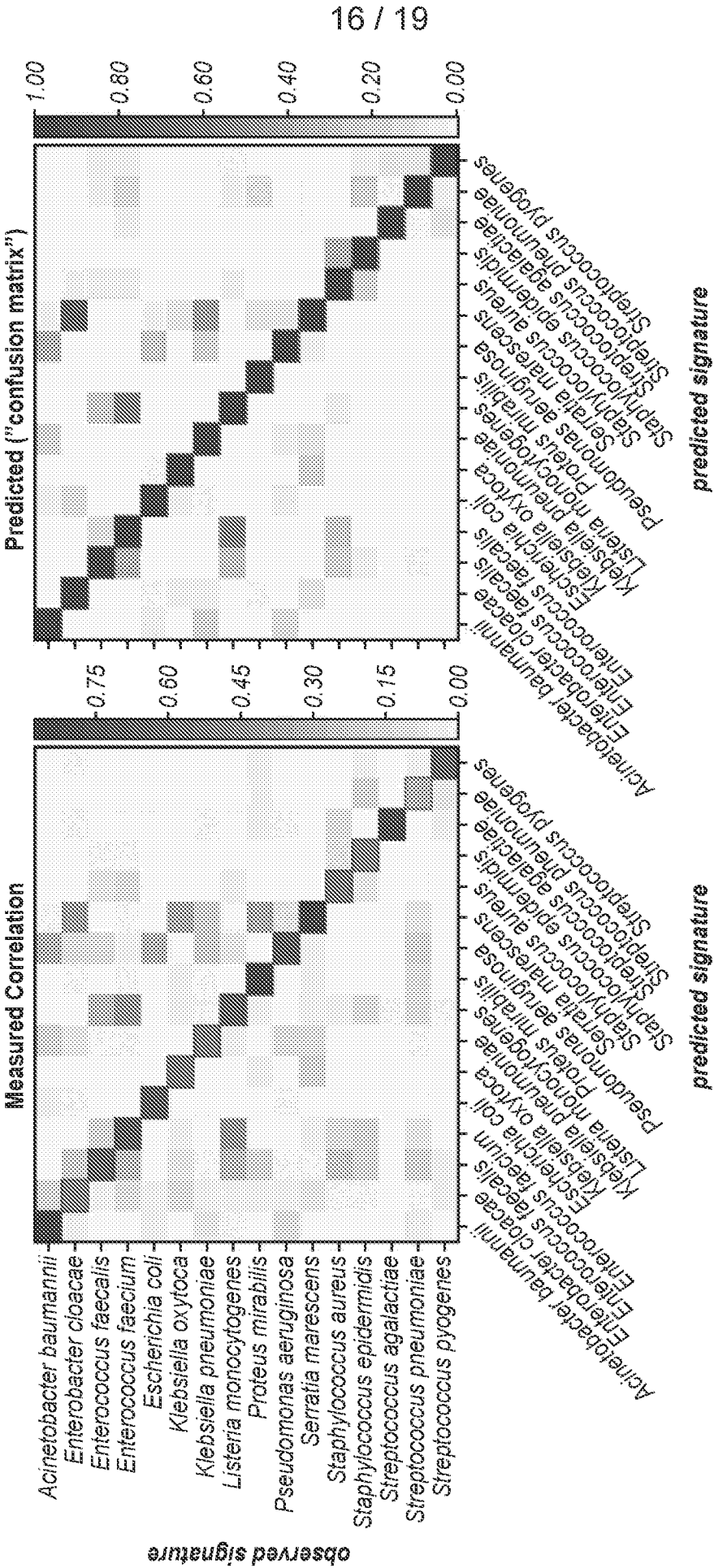


FIG. 12C

FIG. 12D

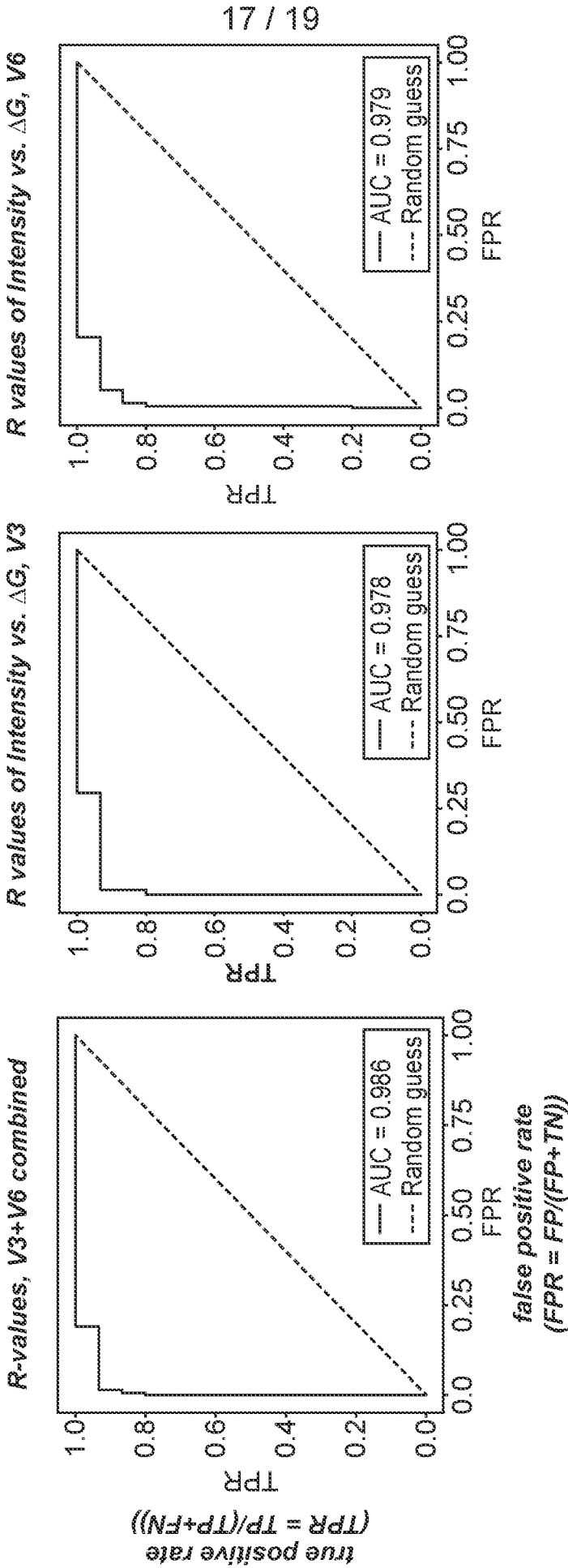


FIG. 13A

FIG. 13B

FIG. 13C

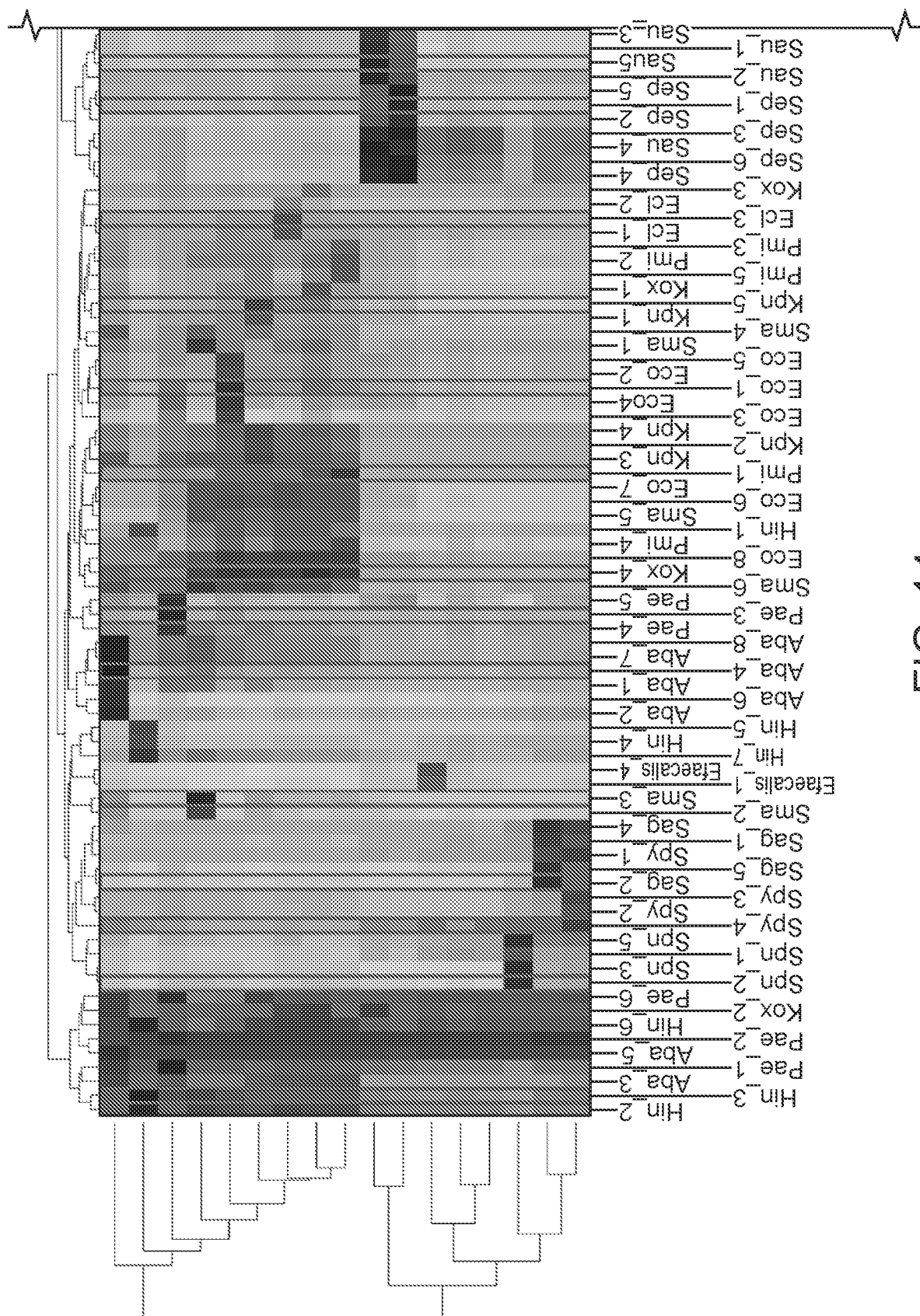


FIG. 14

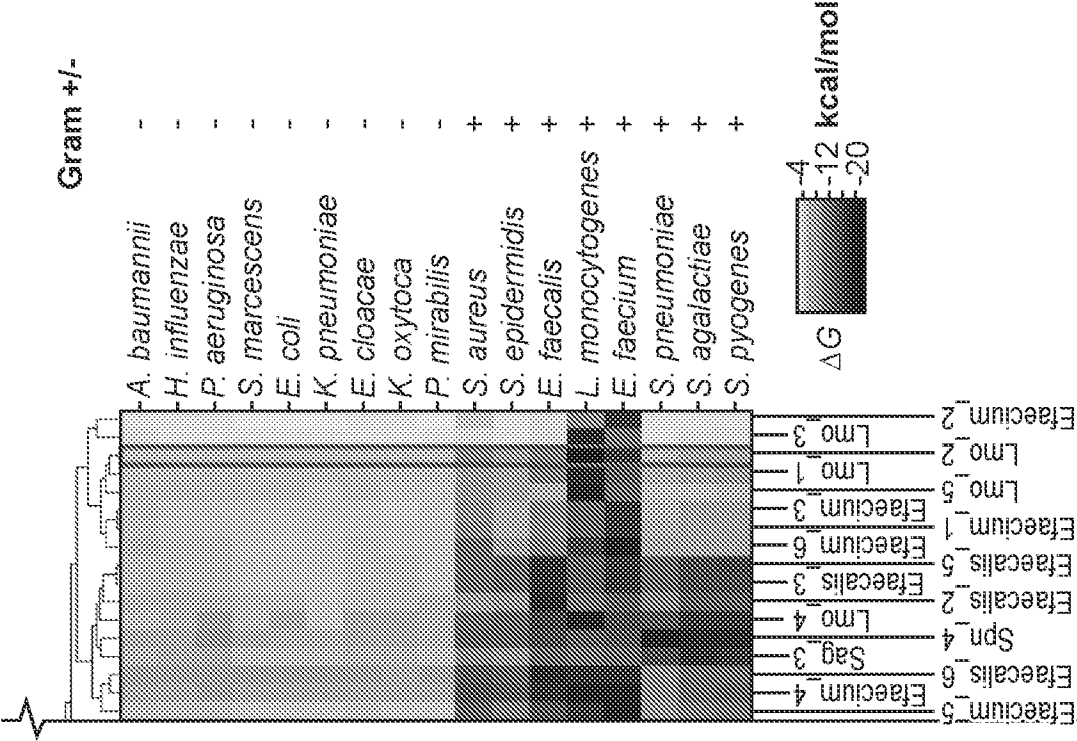


FIG. 14 (Cont.)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/34747

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12Q 1/6804; C12Q 1/6816; C12Q 1/6823 (2020.01)

CPC - C12Q 1/6804; C12Q 1/6816; C12Q 1/6823; G01N 2458/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y --- A	✓ Klamp, et al. Highly Rapid Amplification-Free and Quantitative DNA Imaging Assay. Sci. Rep. 3, 1852 16 May 2013 (16.05.2013), entire document esp pg 2, pg 5, Figure 1	1-5, 8-9, 17 ----- 42-48
Y --- A	✓ Harink et al. 'An open-source software analysis package for Microspheres with Ratiometric Barcode Lanthanide Encoding (MRBLEs)' PLoS ONE 14(3), 22 March 2019 (22.03.2019), entire document esp pg 1, 2, 4, 12	1-5, 8-9, 17 ----- 42-48
A	WO 2018/213604 A2 (LOCUS AGRICULTURE IP COMPANY, LLC) 22 November 2018 (22.11.2018), entire document esp abstract, pg 21	42-48
A	US 9,170,197 B2 (Geddes et al.) 27 October 2015 (27.10.2015), entire document	1-5, 8-9, 17, 42-48

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

12 August 2020

Date of mailing of the international search report

22 OCT 2020

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Lee Young

Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/34747

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 6-7, 10-16, 18-30, 35-41, 49-67
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I: Claims 1-5, 8-9, 17, 42-48, directed to a method for characterizing a population of microbial strains in a sample, wherein different microbial strains in the population comprise polynucleotides with different microbe-identifying sequences.

Group II: Claims 31-34, directed to a plurality of microbeads, each of the microbeads comprising a lanthanide spectral signature and plurality of copies of a capture polynucleotide immobilized on the microbead.

The group of inventions listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

*****See supplemental box below*****

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-5, 8-9, 17, 42-48

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Box III Lack of Unity

Special Technical Features:

Group I requires the special technical feature of a method for characterizing a population of microbial strains in a sample, wherein different microbial strains in the population comprise polynucleotides with different microbe-identifying sequences, not required by Group II.

Group II requires the special technical feature of a plurality of microbeads, each of the microbeads comprising a lanthanide spectral signature and plurality of copies of a capture polynucleotide immobilized on the microbead, not required by Groups I.

Common technical features:

Groups I-II share the technical feature of microbeads comprising a lanthanide spectral signature and plurality of copies of a capture polynucleotide immobilized on the microbead, wherein each capture polynucleotide comprises a predetermined sequence and each capture polynucleotide is paired with a lanthanide spectral signature, such that the predetermined sequence can be identified based on the lanthanide spectral signature.

These shared technical features, however, do not provide a contribution over the prior art, as being obvious over the article titled 'An open-source software analysis package for Microspheres with Ratiometric Barcode Lanthanide Encoding (MRBLEs)?' by Harink et al (hereinafter Harink).

Harink discloses a method comprising a plurality of microbeads, wherein each of the microbeads has a lanthanide spectral signature and a peptide binding motif immobilized on the microbead (pg 1 abstract .. We demonstrate the utility of this package by applying it towards analyzing data generated via multiplexed measurement of calcineurin protein binding to MRBLEs (Microspheres with Ratiometric Barcode Lanthanide Encoding) containing known and mutant binding peptide motifs.. calcineurin binds to microspheres via the analyte, which is the known and mutant binding motifs, pg 2 para 2 .. Multiplexing bead-based assays requires the ability to reliably identify all bead-bound analytes. This can be accomplished via a 'one-code-one-compound' approach in which beads are encoded and each analyte is tethered to or synthesized on beads bearing a different code.. pg 2 para 3 .. We recently developed a novel technology for producing spectrally encoded beads, which we term MRBLEs (Microspheres with Ratiometric Barcode Lanthanide Encoding). In this approach, microspheres are encoded via the ratiometric incorporation of lanthanide nanophosphors (LNP) particles..) wherein each predetermined peptide binding motifs is paired with a specific lanthanide spectral signature, such that the motif immobilized on a microbead can be identified based on the lanthanide spectral signature of the microbead (pg 2 para 3 .. In this approach, microspheres are encoded via the ratiometric incorporation of lanthanide nanophosphors (LNP) particles.. pg 9 para 2 .. Ratiometric encoding, in which one coding species is incorporated into all encoded particles, can enhance resolution of intensity levels by normalizing for small spatial differences in excitation intensity or photon collection efficiency.. pg 4 para 1 .. Identifying analytes and measuring binding therefore requires the development of image processing software capable of identifying individual beads from microscopy images, quantifying fluorescent material bound to each bead, and calculating ratios of embedded LNPs to identify the bead code and thus, the identity of the bound analyte (Fig 1B).. a code is encoded into a particular particle type, see pg 12 para 3 .. The ultimate goal of multiplexed binding measurements is to quantify interactions between an experimental probe of interest and a variety of different analytes. This, in turn, requires aggregating per-bead information to calculate binding statistics at the per-analyte (per-code) level.. code is the identity of the immobilized analyte). Harink does not specifically disclose wherein the target is a polynucleotide and the immobilized motif which binds the target is an oligonucleotide with a predetermined polynucleotide sequence. It would have been obvious to one skilled to adapt the method disclosed by Harink with to an analogous system using DNA, which would be a predetermined target DNA sequence polynucleotide captured by a capture oligonucleotide which is complementary because this is how target DNA is typically captured (see the article titled 'Highly Rapid Amplification-Free and Quantitative DNA Imaging Assay' by Klamp et al in citations pg 2 Figure 1a and caption .. (a) Target DNA fragments are captured by beads functionalized with specific complementary oligonucleotides (capture probe) and labeled by reporter oligonucleotides with a fluorophore attached (detection probe).. oligonucleotide is a short polynucleotide).

As the technical features were known in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups. Groups I-II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Claims 6-7, 10-16, 18-30, 35-41, 49-67 were found to be unsearchable and were not included in any group because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).