



(51) International Patent Classification:

G01N 33/50 (2006.01) C12Q 1/689 (2018.01)

G01N 33/53 (2006.01) C12Q 1/06 (2006.01)

C12Q 1/6813 (2018.01) C07K 16/12 (2006.01)

LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(21) International Application Number:

PCT/US2024/049954

Published:

— with international search report (Art. 21(3))

— with sequence listing part of description (Rule 5.2(a))

(22) International Filing Date:

04 October 2024 (04.10.2024)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

63/588,491 06 October 2023 (06.10.2023) US

(71) Applicant: UNIVERSITY OF PITTSBURGH-OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION [US/US]; 1st Floor Gardner Steel Conference Center, 130 Thackeray Avenue, Pittsburgh, PA 15260 (US).

(72) Inventors: HAND, Timothy, Wesley; 1st Floor Gardner Steel Conference Center, 130 Thackeray Avenue, Pittsburgh, PA 15260 (US). ABBOTT, Darryl, A.; 1st Floor Gardner Steel Conference Center, 130 Thackeray Avenue, Pittsburgh, PA 15260 (US). DEPAS, William, H.; 1st Floor Gardner Steel Conference Center, 130 Thackeray Avenue, Pittsburgh, PA 15260 (US). TIN, Christine; 1st Floor Gardner Steel Conference Center, 130 Thackeray Avenue, Pittsburgh, PA 15260 (US).

(74) Agent: PAVENTO, Lisa, C. et al.; Meunier Carlin & Curfman LLC, 999 Peachtree Street, NE, Suite 1300, Atlanta, GA 30309 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT,

(54) Title: COMPOSITIONS AND METHODS FOR IDENTIFYING BACTERIAL CHARACTERISTICS

(57) Abstract: The present disclosure relates to a method of identifying two or more characteristics of bacterium in a testing sample comprising use of fluorescent labels and hybridization chain reaction.

WO 2025/076345 A1

COMPOSITIONS AND METHODS FOR IDENTIFYING BACTERIAL CHARACTERISTICS

CROSS REFERENCE TO RELATED APPLICATIONS

5 This application claims priority to, and the benefit of, U.S. Provisional Patent Application No. 63/588,491, filed October 6, 2023, which is incorporated by reference herein in its entirety.

STATEMENT OF GOVERNMENT INTEREST

This invention was made with Government support under grant number DK120697 awarded by the National Institutes of Health. The government has certain rights in the invention.

10 REFERENCE TO SEQUENCE LISTING

The sequence listing submitted on October 4, 2024 as an .XML file entitled "10504-091WO1_ST26" created on October 4, 2024 and having a file size of 69,679 bytes is hereby incorporated by reference pursuant to 37 C.F.R. § 1.52(e)(5).

BACKGROUND OF THE INVENTION

15 The intestine is colonized by a large and diverse microbiota that is important in the development of various intestinal infections and conditions ranging from inflammatory bowel disease to necrotizing enterocolitis. For example, both Crohn's Disease and ulcerative colitis are associated with shifts in the intestinal microbiota. Current methods for measuring the microbiota, based upon sequencing, provide data too slowly and of too high a complexity for most clinical use. Thus, there is
20 a critical need for a faster, more quantitative, and more accurate method for measuring both the structure of the intestinal microbiota and the presence of specific bacteria at the single cell level that may contribute to disease.

SUMMARY

In one aspect disclosed herein is a method for identifying two or more characteristics of a
25 bacterium in a testing sample. In some embodiments the method further includes an absolute quantification. In some embodiments the method further includes a relative quantification. In some embodiments, the method comprises the following steps 1) obtaining the testing sample comprising the bacterium and a first ligand; 2) contacting the bacterium and the first ligand with a second ligand that is specific for the first ligand and comprising a first fluorescent label, wherein binding of the
30 second ligand to the first ligand creates a labeled ligand conjugate comprising the first fluorescent label; 3) cross linking the labeled ligand conjugate that is bound to the bacterium to obtain a

fluorescently labeled bacterium; 4) fixing the bacterium; 5) permeabilizing the bacterium; 6) obtaining an identification sample by performing a hybridization chain reaction (HCR) using the permeabilized bacterium, wherein the identification sample comprises the fluorescently labeled permeabilized bacterium comprising the first fluorescent label and one or more fluorescently labeled bacterial polynucleotide HCR products comprising at least a second fluorescent label, and wherein the first and second fluorescent labels are different; and 7) isolating the bacterium from any other bacteria in the identification sample and identifying a first ligand binding characteristic represented by the first fluorescent label and second characteristic represented by the second fluorescent label. In some embodiments, the ligand is an antibody.

In some embodiments, the method further comprises determining the total number of bacteria in the identification sample. In some embodiments, the first antibody is an IgA antibody, and the first characteristic of the bacteria is an ability to bind IgA. In some embodiments, the first antibody is an IgM antibody, and the first characteristic of the bacteria is an ability to bind IgM. In some embodiments, the first antibody is an IgG antibody, and the first characteristic of the bacteria is an ability to bind IgG.

In some embodiments, the second bacterial characteristic is a bacterial domain. In some embodiments, the second bacterial characteristic is a bacterial phylum. In some embodiments, the second bacterial characteristic is a bacterial class. In some embodiments, the second bacterial characteristic is a bacterial order. In some embodiments, the second bacterial characteristic is a bacterial family. In some embodiments, the second bacterial characteristic is a bacterial genus. In some embodiments, the second bacterial characteristic is a bacterial species.

In some embodiments, prior to contacting the bacterium in the testing sample with the first ligand, one or more contaminants are removed from the testing sample using a PERCOLL solution. In some embodiments, the fluorescently labeled ligand comprises a red fluorescent label. In some embodiments, the labeled ligand conjugate is crosslinked to the bacterium using a crosslinking agent comprising bis-(sulfosuccinimidyl)-suberate.

In some embodiments, the bacterium is fixed using a fixing agent comprising paraformaldehyde. In some embodiments, the bacterium is permeabilized using a permeabilizing agent comprising lysozyme and lysostaphin. isolating the fluorescently labeled permeabilized bacterium and the one or more fluorescently labeled bacterial polynucleotide HCR products in the identification sample can be performed using fluorescence activated sorting. In some embodiments, the hybridization chain reaction (HCR) comprises use of an initiator probe selected from the group

consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40 and SEQ ID NO:41.

5 BRIEF DESCRIPTION OF FIGURES

Figure 1(A-C) shows a schematic of the hybridization chain reaction. Figure 1A shows the probes and initiator, Figure 1B shows the probes' alignment with the target mRNA, and Figure 1C shows the polymerization reactions.

Figure 2 shows a schematic of a method of determining the identity of the absolute amount of
10 and antibody binding to bacteria in a sample using hybridization chain reaction and flow cytometry.

Figure 3(A-B) shows that the present method can distinguish a defined bacteria community of 50% *S. aureus* and 50% *E. coli* grown and combined in cell culture. Figure 3A shows a flow cytometry analysis of the mixed sample, and Figure 3B shows confocal imaging of the mixed sample.

Figure 4 shows flow cytometry analyses demonstrating identification and quantification of
15 *Clostridium difficile* and *C. difficile* toxin expression in human patient samples that are either infected with *C. difficile* (left two panels) or an uninfected control (right panel).

Figure 5 shows that the present method discriminates IgA-bound bacteria in stool from a maternal milk-fed preterm infant. Flow cytometric analysis shows the method detects bacteria-specific IgA to subpopulations of different taxa in the same preterm stool sample. Isotype antibody control is
20 for mixed longitudinal samples from the same patient. Enterobacteriaceae and Bifidobacterium gated on Eubacteria+ cells.

Figure 6 shows that the present method quantifies total population changes (*Enterobacteriaceae* and *Bifidobacterium*) in a human microbiota sample. Longitudinal samples compared between a formula-fed (left) and a breast milk-fed (right) patient across days of life 10 to
25 17. Total bacterial cells normalized to mg of stool.

Figure 7(A-B) shows changes in relative abundances do not reflect true population changes in longitudinal stool. Figure 7A shows longitudinal analysis of stool from an infant at days of life 10 to 16. Absolute abundance shows stabilization of Bacilli and Gammaproteobacteria communities as Bifidobacterium increases. Figure 7B shows longitudinal analysis of stool from an infant at days of
30 life 15 to 17. IgA-bound bacteria were measured. Dashed line shows three distinct trends of absolute population changes despite similar changes in relative abundance. Relative & absolute abundances were measured with the present method.

Figure 8 shows that the present method retains IgA antibodies bound to bacteria. IgA was purified from breast milk and incubated with pure culture *E. coli*. Flow cytometric analysis of samples run before and after the present method shows that anti-bacteria antibodies remain on bacteria surface throughout the protocol.

Figure 9(A-C) shows that the present method quantifies all members of a pediatric microbial consortia (PedsCom: *Staphylococcus xylosus*, *Staphylococcus sciuri*, *Enterococcus faecalis*, *Lactobacillus johnsonii*, *Ligolactobacillus murinus*, *Anaerostipes sp.*, *Clostridium intestinale*, *Kosakonia cowanii*, *Parabacteroides distastonis*) in gnotobiotic mice. Figure 9A shows flow cytometric plots showing identification of all PedsCom members at the family level in fecal samples from gnotobiotic PedsCom mice. Figure 9B shows that the present method (MicFly) is comparable to qPCR to measure relative abundances. Figure 9C shows that the present method is comparable to qPCR for absolute abundance quantitation.

Figure 10(A-B) shows how the present method measures gene expression of arabinose-inducible GFP in *E. coli*. Figure 10A shows flow cytometric plots of GFP mRNA measured by the present method and GFP reporter protein expression during a time course. Figure 10B shows the mean fluorescence intensity of GFP reporter expression or GFP mRNA expression over time under different arabinose concentrations.

Figure 11 shows a sample absolute abundance calculation.

DETAILED DESCRIPTION

Provided herein is a method for identifying two or more characteristics of a bacterium in a testing sample. In some embodiments, the final identifications can be made simultaneously or near simultaneously. The present method is a significant improvement upon the prior art methods at least because, in some embodiments, it allows for a faster, more quantitative, and more accurate method for measuring both the structure of intestinal microbiota and the presence of specific bacteria that may contribute to disease at the single cell, or single bacterium, level. In some embodiments, it also allows for concurrent measurement of intestinal bacterial identities, combined with detection of surface protein expression, including mammalian proteins, allowing, for example, the identification of mammalian antibodies bound to the bacteria.

Terminology

Terms used throughout this application are to be construed with ordinary and typical meaning to those of ordinary skill in the art. However, Applicant desires that the following terms be given the particular definition as provided below.

As used in the specification and claims, the singular form "a," "an," and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

The term "antibody" is used in the broadest sense, and specifically covers monoclonal
5 antibodies (including full length monoclonal antibodies), polyclonal antibodies, and multi-specific antibodies (e.g., bispecific antibodies). Antibodies (Abs) and immunoglobulins (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific target, immunoglobulins include both antibodies and other antibody-like molecules which lack target specificity. Native antibodies and immunoglobulins are usually heterotetrameric glycoproteins of
10 about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end.

The term "antibody fragment" refers to a portion of a full-length antibody, generally the target binding or variable region. Examples of antibody fragments include Fab, Fab', $F(ab')_2$ and Fv
15 fragments. The phrase "functional fragment or analog" of an antibody is a compound having qualitative biological activity in common with a full-length antibody. For example, a functional fragment or analog of an anti-IgE antibody is one which can bind to an IgE immunoglobulin in such a manner so as to prevent or substantially reduce the ability of such molecule from having the ability to bind to the high affinity receptor, FcεRI. As used herein, "functional fragment" with respect to
20 antibodies, refers to Fv, F(ab) and $F(ab')_2$ fragments. An "Fv" fragment is the minimum antibody fragment which contains a complete target recognition and binding site. This region consists of a dimer of one heavy and one light chain variable domain in a tight, non-covalent association (V_H - V_L dimer). It is in this configuration that the three CDRs of each variable domain interact to define a target binding site on the surface of the V_H - V_L dimer. Collectively, the six CDRs confer target binding specificity to
25 the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for a target) has the ability to recognize and bind target, although at a lower affinity than the entire binding site. "Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of an antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the
30 sFv to form the desired structure for target binding. The Fab fragment contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxyl terminus of the heavy chain CH1

domain including one or more cysteines from the antibody hinge region. F(ab') fragments are produced by cleavage of the disulfide bond at the hinge cysteines of the F(ab')₂ pepsin digestion product. Additional chemical couplings of antibody fragments are known to those of ordinary skill in the art.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a substantially homogeneous population of antibodies, i.e., the individual antibodies within the population are identical except for possible naturally occurring mutations that may be present in a small subset of the antibody molecules.

A "control" is an alternative subject or sample used in an experiment for comparison purpose. A control can be "positive" or "negative."

The term "hybridization chain reaction" or "HCR" refers to a polynucleotide polymerization method that employs two labeled hairpin polynucleotides and an initiator polynucleotide. The initiator polynucleotide can also be referred to as an initiator probe. A sequence of the initiator probe is complementary to a sequence of the target polynucleotide. Here, the target polynucleotide is bacterial DNA or RNA. The single stranded initiator polynucleotide opens one of the hairpins, which exposes a single stranded region in that hairpin, which in turn opens the second hairpin. Therefore, HCR creates labeled polynucleotide products that correlate with the target polynucleotide. These HCR created polynucleotides are referred to herein as "labeled polynucleotide HCR products."

The term "identity" shall be construed to mean the percentage of nucleotide bases or amino acid residues in the candidate sequence that are identical with the bases or residues of a corresponding sequence to which it is compared, after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent identity for the entire sequence, and not considering any conservative substitutions as part of the sequence identity. Neither N- nor C-terminal extensions nor insertions shall be construed as reducing identity or homology. A polynucleotide or polynucleotide region (or a polypeptide or polypeptide region) that has a certain percentage (for example, 80%, 85%, 90%, or 95%) of "sequence identity" to another sequence means that, when aligned over their full lengths, that percentage of bases (or amino acids) are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art. In one embodiment, default parameters are used for alignment. In one embodiment a BLAST program is used with default parameters. In one embodiment, BLAST programs BLASTN and BLASTP are used with the following default parameters: Genetic code=standard; filter=none; strand=both; cutoff=60; expect=10; Matrix=BLOSUM62; Descriptions=50 sequences; sort by=HIGH SCORE; Databases=non-redundant, GenBank+EMBL+DDBJ+PDB+GenBank CDS

translations+SwissProtein+SPupdate+PIR.

As used herein the term “isolating” as in when “isolating the bacterium from any other bacteria in the identification sample,” refers to separation of the bacterium from other bacteria in the sample for a sufficient amount of time to detect one or more characteristics of the single bacterium. In some
5 embodiments, the isolating step is achieved using fluorescence activated cell sorting (FACS).

The word "label" when used herein refers to a detectable compound or composition which can be conjugated directly or indirectly to a molecule or protein, e.g., a ligand. The label may itself be detectable (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

10 “Ligand” refers to a molecule that binds to another molecule. In some embodiments, the ligand is a polypeptide. An antibody is one non-limiting example of a ligand. An antibody fragment is another non-limiting example of a ligand.

As used herein, the word “near” refers to close in time. In some embodiments, the phrase “near simultaneously” means within up to ten minutes. In other embodiments, the phrase “near
15 simultaneously” means within up to five minutes. In some embodiments, the phrase “near simultaneously” means within two minutes. In some embodiments, the phrase “near simultaneously” refers to within a single run of an assay, and in some embodiments, the assay is a flow cytometry assay.

The terms “specific binding,” “specifically binds,” “selective binding,” and “selectively binds”
20 mean that a ligand exhibits appreciable affinity for its binding partner. Appreciable binding affinity includes binding with an affinity of at least 10^6 M^{-1} , specifically at least 10^7 M^{-1} , more specifically at least 10^8 M^{-1} , yet more specifically at least 10^9 M^{-1} , or even yet more specifically at least 10^{10} M^{-1} . A binding affinity can also be indicated as a range of affinities, for example, 10^6 M^{-1} to 10^{10} M^{-1} , specifically 10^7 M^{-1} to 10^{10} M^{-1} , more specifically 10^8 M^{-1} to 10^{10} M^{-1} . Specific binding can be
25 determined according to any art-recognized means for determining such binding. In some embodiments, specific binding is determined according to Scatchard analysis and/or competitive binding assays.

The terms "polynucleotide" and "oligonucleotide" are used interchangeably and refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or
30 analogs thereof. Polynucleotides may have any three-dimensional structure, and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: a gene or gene fragment, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes,

cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, DNA of any sequence, RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components.

Compositions and Methods

Provided herein is a method for identifying two or more characteristics of a bacterium in a testing sample. In some embodiments the method further includes an absolute quantification. In some embodiments the method further includes a relative quantification. In some embodiments, the method comprises the following steps 1) obtaining the testing sample comprising the bacterium and a first ligand; 2) contacting the bacterium and the first ligand with a second ligand that is specific for the first ligand and comprising a first fluorescent label, wherein binding of the second ligand to the first ligand creates a labeled ligand conjugate comprising the first fluorescent label; 3) cross linking the labeled ligand conjugate that is bound to the bacterium to obtain a fluorescently labeled bacterium; 4) fixing the bacterium; 5) permeabilizing the bacterium; 6) obtaining an identification sample by performing a hybridization chain reaction (HCR) using the permeabilized bacterium, wherein the identification sample comprises the fluorescently labeled permeabilized bacterium comprising the first fluorescent label and one or more fluorescently labeled bacterial polynucleotide HCR products comprising at least a second fluorescent label, and wherein the first and second fluorescent labels are different; and 7) isolating the bacterium from any other bacteria in the identification sample and identifying a first ligand binding characteristic represented by the first fluorescent label and second characteristic represented by the second fluorescent label. Certain embodiments of the present method are sometimes referred to herein as the MICFLY or MicFly method or test.

As noted above, one of the benefits of the method is that it allows for a faster identification and/or quantification of multiple characteristics of a bacterium or a group of bacteria, and those identifications or quantifications can, in some embodiments, further allow for defining or characterizing subgroups of bacteria within the larger group of bacteria. That “faster identification” can be achieved by a step 8) identification of the two or more characteristics within a single assay run, and without the need for obtaining the identifications in two or more assays or performing a sequencing step. For example, in some embodiments, the two or more identifications can be obtained in the same flow cytometry assay.

The hybridization chain reaction (HCR) comprises contacting bacterial DNA and/or RNA with one or more initiator probes that are specific for a bacterial DNA or RNA sequence and two labeled hairpin sequences, that when used with the initiator probe, create polynucleotide HCR products that incorporate the label and that correspond to the bacterial DNA or RNA sequence. Accordingly, included herein are initiator probes, hairpin sequences, and labels for use in HCR. In some embodiments, the one or more initiator probes are selected from those listed in Table 1 and/or Table 2: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40 and SEQ ID NO:41. Accordingly, included herein are methods wherein an initiator probe comprises SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40 or SEQ ID NO:41. In some embodiments, the two labeled hairpin sequences are selected from those listed in Table 3: SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32 and SEQ ID NO:33. In some aspects, the two hairpin sequences comprise SEQ ID NO:12 and SEQ ID NO:13, respectively. In other aspects, the two hairpin sequences comprise SEQ ID NO:14 and SEQ ID NO:15, respectively. In other aspects, the two hairpin sequences comprise SEQ ID NO:16 and SEQ ID NO:17, respectively. In other aspects, the two hairpin sequences comprise SEQ ID NO:18 and SEQ ID NO:19, respectively. In other aspects, the two hairpin sequences comprise SEQ ID NO:20 and SEQ ID NO:21, respectively. In other aspects, the two hairpin sequences comprise SEQ ID NO:22 and SEQ ID NO:23, respectively. In other aspects, the two hairpin sequences comprise SEQ ID NO:24 and SEQ ID NO:25, respectively. In other aspects, the two hairpin sequences comprise SEQ ID NO:26 and SEQ ID NO:27, respectively. In other aspects, the two hairpin sequences comprise SEQ ID NO:28 and SEQ ID NO:29, respectively. In other aspects, the two hairpin sequences comprise SEQ ID NO:30 and SEQ ID NO:31, respectively. In other aspects, the two hairpin sequences comprise SEQ ID NO:32 and SEQ ID NO:33, respectively.

In some embodiments, the two hairpin sequences are labeled with a fluorophore. In some embodiments, the fluorophore used as a label for the two hairpin sequences is selected from the group consisting of Alexa Fluor 647, Alexa Fluor 594, Alexa Fluor 546, Alexa Fluor 488, Alexa Fluor 514,

Alexa Fluor 660, Alexa Fluor 405, ATTO 490LS, ATTO 647N, ATTO 620, and ATTO 565.

In some embodiments, the ligand is an antibody or antibody fragment. Accordingly, one of the characteristics that can be determined and quantified using the present method is antibody binding, or whether an antibody binds to a group or subgroup of bacteria. In some embodiments, the first characteristic of the bacteria is an ability to bind IgA, and accordingly, the first antibody is an IgA antibody. In other or further embodiments, a characteristic of the bacteria is an ability to bind IgM and in step 1), the bacteria is contacted with an IgM antibody and in step 2), a fluorescently labeled antibody is specific for the IgM antibody. In other or further embodiments, a characteristic of the bacteria is an ability to bind IgG and in step 1), the bacteria is contacted with an IgG antibody and in step 2), a fluorescently labeled antibody is specific for the IgG antibody. The present method can be used to quantify the bacterial subgroups within a sample on these bases.

In some embodiments, further characteristics that can be determined and quantified using the present method are bacterial phylum, bacterial class, bacterial order, bacterial family, bacterial genus and/or bacterial species. The present method can be used to quantify the bacterial subgroups within a sample on these bases. Quantification of the subgroups on these bases is achieved using phylum-, class-, order-, family-, genus- or species-specific initiator probes in the hybridization chain reaction. Tables 1 and 2 provide exemplary, and non-limiting, lists of initiator probes for use in the hybridization chain reaction.

Accordingly, in some embodiments, the one or more fluorescently labeled bacterial polynucleotide amplification products represent a second characteristic that is the bacterial domain, bacterial phylum, bacterial class, bacterial order, bacterial family, bacterial genus or bacterial species. In these embodiments, the fluorescently labeled bacterial polynucleotide amplification products depend on or correlate with the initiator probe. For example, an initiator probe that can identify an Actinobacteria phylum characteristic of a bacteria comprises SEQ ID NO:11 or a sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:11. In other embodiments, an initiator probe that can identify a Bifidobacterium species characteristic of a bacteria comprises SEQ ID NO:8, SEQ ID NO:9 or SEQ ID NO:10 or a sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:9 or SEQ ID NO:10. In other embodiments, an initiator probe that can identify a Bacilli class characteristic of a bacteria comprises SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7 or a sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7. In other embodiments, an initiator probe that can identify a Enterobacteriaceae family characteristic of a bacteria comprises SEQ ID NO:4 or SEQ ID NO:34 or a sequence having

about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:4 or SEQ ID NO:34. In some embodiments, the initiator probe that can identify a Enterobacteriaceae family characteristic of a bacteria comprises a sequence that binds to SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47 or SEQ ID NO:48. In some embodiments, an initiator probe

5 that can identify a Lactobacillaceae family characteristic of a bacteria comprises SEQ ID NO: 35 or a sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:35. In some embodiments, the initiator probe that can identify a Lactobacillaceae family characteristic of a bacteria comprises a sequence that binds to SEQ ID NO:49 or SEQ ID NO:50. In some embodiments, an initiator probe that can identify an Enterococaceae family characteristic of a bacteria comprises SEQ

10 ID NO:36 or a sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:36. In some embodiments, the initiator probe that can identify an Enterococaceae family characteristic of a bacteria comprises a sequence that binds to SEQ ID NO:52 or SEQ ID NO:53. In some embodiments, an initiator probe that can identify a Clostridiaceae family characteristic of a bacteria comprises SEQ ID NO:37 or a sequence having about at least 80%, 85%, 90% or 95% identity with

15 SEQ ID NO:37. In some embodiments, the initiator probe that can identify a Clostridiaceae family characteristic of a bacteria comprises a sequence that binds to SEQ ID NO:56 or SEQ ID NO:57. In some embodiments, an initiator probe that can identify a Bacteroidales family characteristic of a bacteria comprises SEQ ID NO:38 or a sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:38. In some embodiments, the initiator probe that can identify a Bacteroidales family

20 characteristic of a bacteria comprises a sequence that binds to SEQ ID NO:61 or SEQ ID NO:62. In some embodiments, an initiator probe that can identify a Staphylococaceae family characteristic of a bacteria comprises SEQ ID NO:39 or a sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:39. In some embodiments, the initiator probe that can identify a Staphylococaceae family characteristic of a bacteria comprises a sequence that binds to SEQ ID NO:63, SEQ ID NO:64

25 or SEQ ID NO:65. In some embodiments, an initiator probe that can identify a Lachnospiraceae family characteristic of a bacteria comprises SEQ ID NO:40 or a sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:40. In some embodiments, the initiator probe that can identify a Lachnospiraceae family characteristic of a bacteria comprises a sequence that binds to SEQ ID NO:67, SEQ ID NO:68 or SEQ ID NO:69. In some embodiments, an initiator probe that can identify a

30 Eubacteria domain characteristic of a bacteria comprises SEQ ID NO:41 or a sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:41. In some embodiments, the initiator probe that can identify a Eubacteria domain characteristic of a bacteria comprises a sequence that binds to

SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75 or SEQ ID NO:76.

Therefore, included herein are methods wherein the second bacterial characteristic is a bacterial domain. In some embodiments, the bacterial domain is Eubacteria and the HCR comprises use of an initiator probe comprising SEQ ID NO:41 or a polynucleotide sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:41.

Also included herein are methods wherein the second bacterial characteristic is a bacterial phylum. In some embodiments, the bacterial phylum is Actinobacteria. In some embodiments, the bacterial phylum is Actinobacteria and the HCR comprises use of an initiator probe comprising SEQ ID NO:11 or a polynucleotide sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:11. Also included herein are methods where the second bacterial characteristic is a bacterial genus or a bacterial order. In some embodiments, the second bacterial characteristic is a bacterial class. In some embodiments, the bacterial class is Bacilli and the HCR comprises use of an initiator probe comprising SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7 or a sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7.

In some embodiments, the second bacterial characteristic is a bacterial family, and in some embodiments, the bacterial family is an Enterobacteriaceae family, a Lactobacillaceae family, an Enterococacceae family, a Clostridiaceae family, a Bacteroidales family, a Staphylococacceae family or a Lachnospiraceae family. In some embodiments, the bacterial family is Enterobacteriaceae and the HCR comprises use of an initiator probe comprising SEQ ID NO:4, SEQ ID NO:34 or a polynucleotide sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:4 or SEQ ID NO:34. In some embodiments, the bacterial family is Lactobacillaceae and the HCR comprises use of an initiator probe comprising SEQ ID NO:35 or a polynucleotide sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:35. In some embodiments, the bacterial family is Enterococacceae and the HCR comprises use of an initiator probe comprising SEQ ID NO:36 or a polynucleotide sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:36. In some embodiments, the bacterial family is Clostridiaceae and the HCR comprises use of an initiator probe comprising SEQ ID NO:37 or a polynucleotide sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:37. In some embodiments, the bacterial family is Bacteroidales and the HCR comprises use of an initiator probe comprising SEQ ID NO:38 or a polynucleotide sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:38. In some embodiments, the bacterial family is Staphylococacceae and the HCR comprises use of an

initiator probe comprising SEQ ID NO:39 or a polynucleotide sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:39. In some embodiments, the bacterial family is Lachnospiraceae and the HCR comprises use of an initiator probe comprising SEQ ID NO:40 or a polynucleotide sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:40.

5 In some aspects, the second bacterial characteristic is a bacterial species, and in some embodiments the bacterial species is a Bifidobacterium species. Included herein are methods wherein the bacterial species is a Bifidobacterium species and the HCR comprises use of an initiator probe comprising SEQ ID NO:8, SEQ ID NO:9 or SEQ ID NO:10 or a sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:8, SEQ ID NO:9 or SEQ ID NO:10.

10 In some embodiments, the method further comprises determining a total, or absolute, amount of bacteria in the testing sample. This quantification can be made via any means known to one of ordinary skill in the art, and in some embodiments, absolute counting beads are added to the sample prior to step 7).

In certain aspects of the method, prior to contacting the group of bacteria in the testing sample
15 with the first ligand, one or more contaminants are removed from the testing sample using a PERCOLL solution. In some embodiments, the PERCOLL solution is an about 20% PERCOLL solution. In some embodiments, the PERCOLL solution is between an about 5% and about 25% PERCOLL solution. In some embodiments, the PERCOLL solution is about 5%, about 10%, about 15%, about 20% or about 25%. In some embodiments, the PERCOLL solution is about 1%, about
20 2%, about 3%, about 4%, about 5%, about 6%, about 7%, about 8%, about 9%, about 10%, about 11%, about 12%, about 13%, about 14%, about 15%, about 16%, about 17%, about 18%, about 19%, about 20%, about 21%, about 22%, about 23%, about 24% or about 25%. In some embodiments, the PERCOLL solution comprises PERCOLL and sodium chloride.

In certain aspects of the method, the fluorescently labeled second ligand comprises a red
25 fluorescent label. In some embodiments, the red fluorescent label is a CF633 label (Biotium).

In certain aspects of the method, the labeled ligand conjugate is crosslinked to the bacteria using a crosslinking agent comprising bis-(sulfosuccinimidyl)-suberate. In some embodiments the bis-(sulfosuccinimidyl)-suberate is at a concentration of about 1 mM.

In certain aspects of the method, the bacteria are fixed using a fixing agent comprising
30 paraformaldehyde. In some embodiments the fixing agent comprises about 4% paraformaldehyde. In some embodiments, the fixing agent comprises about 0.5% to about 8% paraformaldehyde. In some embodiments the fixing agent comprises about 0.5% paraformaldehyde, about 1% paraformaldehyde,

about 2% paraformaldehyde, about 3% paraformaldehyde, about 4% paraformaldehyde, about 5% paraformaldehyde, about 6% paraformaldehyde, about 7% paraformaldehyde, or about 8% paraformaldehyde.

In certain aspects of the method, the bacteria are permeabilized using a permeabilizing agent comprising lysozyme. In other or further embodiments, the permeabilizing agent comprises lysostaphin.

It should be understood that the foregoing relates to preferred embodiments of the present invention and that numerous changes may be made therein without departing from the scope of the invention. The invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof, which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims. All patents, patent applications, and publications referenced herein are incorporated by reference in their entirety for all purposes.

EXAMPLES

Example 1

Isolation of microbiota from human stool

Bacteria were isolated using a modified protocol for patient fecal microbiota transplantation. Approximately 50 to 400 mg of stool was weighed in a 50 mL Falcon tube, and the exact sample mass was recorded. Fecal material was homogenized by resuspending in 10 mL phosphate buffered saline (PBS) and hard vortexing for 1 minute. The fecal solution was centrifuged at low speed (50 x g) for 10 minutes at 4°C to remove larger cells and debris. Supernatant was collected and filtered through a 40 µm cell strainer and washed with an additional 10 mL PBS. To pellet bacteria, a high-speed spin (4700 x g) was performed on a benchtop centrifuge for 10 minutes at 4°C. Supernatant was discarded, and the pellet was resuspended in 2 mL of a 20% PERCOLL solution in 0.15 M NaCl. The Percoll resuspension was spun at 10,000 x g for 10 minutes at 4°C, allowing non-microbial intestinal matter to settle above the supernatant and bacterial cells to pellet at the bottom. Supernatant is discarded, and the pellet was washed in 2 mL PBS and centrifuged for 10 minutes at 9000 x g at 4°C. The final pellet was resuspended in exactly 2 mL PBS and filtered through a 20 µm cell strainer. This resuspension was the isolated fecal microbiota used in the present methods, and the final volume was recorded for absolute abundance calculations.

Example 2

Cross-linking and fixation of isolated fecal microbiota

All centrifugation steps were performed for 10 minutes at 9000 x g. The 2 mL isolated microbiota resuspension was split equally (500 µL each) into four separate 1.5 mL conical microcentrifuge tubes (allowing duplicate tests with matched isotype controls). Tubes were centrifuged, and supernatant was discarded. Two tubes were incubated with goat anti-human IgA CF633 (Biotium 20427) (1:10), and the other two tubes were incubated with goat anti-chicken IgY CF633 as an isotype control (Biotium 20126) (1:10) in 50 µL of PBS with 10% normal goat serum (ThermoFisher) in the dark for 1 hour on ice. CF633 was used because it was found that it is stable to both BS3 cross-linking and PFA fixation in downstream steps. All steps going forward using CF633-labeled bacteria were performed in the dark whenever possible. Bacteria were washed three times with PBS at pH 8.0, which is slightly basic to increase the cross-linking efficiency of bis-(sulfosuccinimidyl)-suberate (BS3) (ThermoFisher) to stabilize human IgA and the fluorophore-conjugated secondary antibody on the bacterial surface. After the last wash, the supernatant was removed, and each sample was resuspended with 100 µL of PBS at pH 8.0. To crosslink bacteria at a final concentration of 1 mM BS3, 100 µL of 2 mM BS3 (1:1 dilution) was added to 100 µL of bacteria in each tube then incubated for 1 hour on ice. Cross-linked bacteria were centrifuged, washed with 500 µL of PBS pH 7.4, then fixed with 4% PFA at room temperature for 1 hour to preserve the integrity of bacterial RNA for testing. Bacteria were centrifuged and washed twice with PBS pH 7.4, then stored in equal volume of PBS as initial sample volume prior to cross-linking/fixation (500 µL per tube) at 4°C in the dark for future testing.

Example 3

Hybridization chain reaction

All centrifugation steps were performed for 10 minutes at 9000 x g. 500 µL of cross-linked/fixed bacteria were used for hybridization chain reaction (HCR). Bacteria were permeabilized by incubating with 0.1 mg/mL lysozyme and 0.01 mg/mL lysostaphin in 10 mM Tris HCl at 37°C for 1 hour. Cells were centrifuged and washed with PBS with 0.1% Tween (PBST) once. Supernatant was discarded and the pellet was resuspended with 400 µL of probe hybridization buffer (Molecular Instruments Inc.) to pre-hybridize for 1 hour at 37°C. 100 µL of each initiator probe (Table 1 and Table 2) was pooled at a concentration of 16 nM and directly added to pre-hybridized samples. Samples were incubated overnight to hybridize at 37°C on a thermoshaker. After hybridization, cells were centrifuged and washed three times with probe wash buffer (Molecular Instruments, Inc.), with

10-minute incubation steps at 37°C during each wash. The supernatant was discarded, and the pellet was resuspended in 75 µL of amplification buffer (Molecular Instruments, Inc.). Fluorescent hairpin amplifiers (Table 3) were incubated on a dry heat block at 95°C for 90 seconds, then cooled for 30 minutes at room temperature. To tag the initiator-labeled bacteria, snap-cooled hairpins were added at a 80 nM concentration in 50 µL of amplification buffer to bacteria samples with 75 µL of amplification buffer (final volume 125 µL) then incubated overnight at room temperature. Samples were centrifuged and washed three times with 5X saline-sodium citrate buffer with 0.1% Tween (SSCT). Samples were stored in equal volume of 5X SSCT as initial sample volume used for HCR (500 µL) at 4°C in the dark until flow cytometric testing.

Example 4

Spectral Flow cytometry

Prior to flow cytometry, 500 µL HCR samples were filtered through a 20 µm cell strainer into a 5 mL round-bottom FACS tube. Pooled microbiota samples were used for single color spectral unmixing controls. To detect bacterial cells, a low threshold on the flow cytometer (Cytek Aurora) was set at 1500 for FSC-A and 1500 for SSC-A, with a gain of 400 for FSC-A, 300 for SSC-A, and 300 for SSC-B. To eliminate detection of debris and other particulate matter, a threshold of 500 was set for the channel that detects the Eubacteria probe. All parameters were acquired on a logarithmic scale. For all samples, the stopping gate was placed on Eubacteria-positive cells, with at least 20,000 events recorded. Data were analyzed using FlowJo v10.9. Absolute abundance was quantified using the volumetric function provided from the spectral cytometer. Absolute abundance can also be quantified using counting beads if the flow cytometer does not have a volumetric counting function.

Example 5

Absolute abundance calculation

Absolute count of bacteria cells per mg stool was calculated as follows:

$$\text{Bacteria cells per mg stool} = \frac{\text{Bacteria cells per } \mu\text{L of test sample} \times \text{Total volume of isolated fecal microbiota resuspension}}{\text{Total mass of extracted stool sample}}$$

$$\text{Where Bacteria cells per } \mu\text{L of test sample} = \frac{\text{Bacteria events}}{\text{Counting bead events}} \times \frac{\text{Number of counting beads added}}{\text{Volume of sample tested for flow cytometry}}$$

A sample calculation is provided in Figure 11.

Example 6

Use of MicFly to Determine Maternal IgA Modulates the Preterm Gut Microbiome

Microbiota research is primarily sequencing-based, subjected to PCR amplification bias, and restricted to relative abundances. Further, the lack of single cell methods obscures having a granular understanding of the microbiota that could be used for clinical and diagnostic purposes.

5 Secretory IgA regulates gut bacterial colonization, but its role in the assembly of the early life microbiome is unclear. Differential IgA binding of specific bacterial taxa has been implicated in several intestinal inflammatory and enteric diseases. Investigating how IgA alters the temporal development of specific bacterial populations is challenging with sequencing-based analyses of relative abundance. Disclosed herein is MicFly (Microbiome Flow Cytometry): a novel single-cell technology combining spectral flow cytometry, *in situ* RNA hybridization chain reaction, and surface
10 protein-targeted antibodies to quantitate taxa-specific, absolute population changes of IgA-bound bacteria. This approach allows for single cell analysis of the microbiota, which circumvents the biases associated with sequencing technologies that prevent accurate quantification. It was found that mIgA binding influences the growth and interactions of different bacterial communities in several breastfed preterm infants. Using an in-house curated 16s and 23s rRNA database, comprehensive probe sets
15 spanning all major taxa in the infant gut microbiome for use in a 14-color spectral panel, were designed. MicFly's ability to distinguish bacterial species bound by IgA was validated both *in vitro* culture using breast milk-derived IgA and *ex vivo* human stool. To investigate the effects of maternal IgA on bacterial population dynamics in the early life microbiome, longitudinal analyses were performed on a prospective cohort of preterm infants. MicFly was used to measure IgA-bound bacteria
20 for each major stage of early life microbiome development (Bacilli, Enterobacteriaceae, Bifidobacterium, Clostridiales, and Bacteroidota). Comparing breast milk-fed to formula-fed infants demonstrates that maternal IgA influences the growth of several gut bacterial communities. Lastly, detection of population fluctuations at the species-level (*K. pneumoniae* and *K. oxytoca*, strongly implicated in necrotizing enterocolitis development) reveals the clinical applicability of MicFly to
25 identify disease-associated bacteria with high granularity. For example, the clinical application of MicFly was tested on stool from *C. difficile* infected adult and pediatric patients who received fecal microbiota transplantation (FMT). MicFly is a tool that can be used in clinical settings (for example, during evaluation of infant gut ecosystem) that require precise information on both bacterial identity and function to predict or diagnose microbiome-related disease.

TABLES

Table 1. Oligonucleotides used as initiator probes for hybridization chain reaction.

Bolded nucleotides are specific to the target bacteria, underlined nucleotides are specific to the hairpin amplifier, and nucleotides that are neither bolded nor underlined are used as spacers.

Probe name	Target	Nucleotide sequence (5'-3')	Hairpin amplifier and fluorophore conjugate
B1-Eub338	16s rRNA Universal bacteria probe	GCT GCC TCC CGT AGG AGT ATA TAG CAT TCT <u>TTC TTG AGG AGG GCA GCA AAC GGG AAG AG</u> (SEQ ID NO:1)	B1 – Alexa Fluor 647
B1-Eub338-II	16s rRNA Universal bacteria probe	GCA GCC ACC CGT AGG TGT ATA TAG CAT TCT <u>TTC TTG AGG AGG GCA GCA AAC GGG AAG AG</u> (SEQ ID NO:2)	B1 – Alexa Fluor 647
B1-Eub338-III	16s rRNA Universal bacteria probe	GCT GCC ACC CGT AGG TGT ATA TAG CAT TCT <u>TTC TTG AGG AGG GCA GCA AAC GGG AAG AG</u> (SEQ ID NO:3)	B1 – Alexa Fluor 647
B2-Enterbact D	16s rRNA Enterobacteriaceae family	TGC TCT CGC GAG GTC GCT TCT CTT AAA AAG <u>CTC AGT CCA TCC TCG TAA ATC CTC ATC AAT CAT C</u> (SEQ ID NO:4)	B2 – Alexa Fluor 594
B3-LGC354A	16s rRNA Bacilli class	TGG AAG ATT CCC TAC TGC TAA AAA AAG CTA <u>ATC CGT CCC TGC CTC TAT ATC TCC ACT C</u> (SEQ ID NO:5)	B3 – Alexa Fluor 546
B3-LGC354B	16s rRNA Bacilli class	CGG AAG ATT CCC TAC TGC TAA AAA AAG CTA <u>ATC CGT CCC TGC CTC TAT ATC TCC ACT C</u> (SEQ ID NO:6)	B3 – Alexa Fluor 546
B3-LGC354C	16s rRNA Bacilli class	CCG AAG ATT CCC TAC TGC TAA AAA AAG CTA <u>ATC CGT CCC TGC CTC TAT ATC TCC ACT C</u> (SEQ ID NO:7)	B3 – Alexa Fluor 546
B4-Bif164	16s rRNA Bifidobacterium spp.	CAT CCG GCA TTA CCA CCC ATT TTC ACA TTT <u>ACA GAC CTC AAC CTA CCT CCA ACT CTC AC</u> (SEQ ID NO:8)	B4 – Alexa Fluor 488
B4-Bif228	16s rRNA Bifidobacterium spp.	GAT AGG ACG CGA CCC CAT ATT TTC ACA TTT <u>ACA GAC CTC AAC CTA CCT CCA ACT CTC AC</u> (SEQ ID NO:9)	B4 – Alexa Fluor 488
B4-Bif662	16s rRNA Bifidobacterium spp.	CCA CCG TTA CAC CGG GAA ATT TTC ACA TTT <u>ACA GAC CTC AAC CTA CCT CCA ACT CTC AC</u> (SEQ ID NO:10)	B4 – Alexa Fluor 488
B5-HGC69A	23s rRNA Actinobacteria phylum	TAT AGT TAC CAC CGC CGT ATT TTC ACT TCA <u>TAT CAC TCA CTC CCA ATC TCT ATC TAC CC</u> (SEQ ID NO:11)	B5 – Alexa Fluor 514

Table 2.

Target Group	Target Sequence	Hairpin Initiator Sequence	Hairpin	Fluorophore
Enterobacteriaceae	TGCTCTCGCGAGGTCG CTTCTCTT (SEQ ID NO:42)	AAAAAGCTCAGT CCATCCTCGTAAA TCCTCATCAATCATC (SEQ ID NO:34)	B2	Alexa Fluor 594
	CCCCCTCTTTGGTCTTG			

	C (SEQ ID NO:43)			
	CCCCCACTTTGGTCTTG C (SEQ ID NO:44)			
	GAGCCCCTGCTTTGGT CCGTA (SEQ ID NO:45)			
	GACGCACTTTATGAGG TC (SEQ ID NO:46)			
	GACATACTTTATGAGGT C (SEQ ID NO:47)			
	GACAGACTTTATGAGT TC (SEQ ID NO:48)			
Lactobacillaceae	ACATGGAGTTCCACT (SEQ ID NO:49)	TAAAAAAGTCTAATCCG TCCCTGCCTC	B3	Alexa Fluor 546
	GACTCGTTGTACCGTC CATT (SEQ ID NO:50)	TATATCTCCACTC (SEQ ID NO:35)		
	ACATGGAATTCCACT (SEQ ID NO:51)	(none; competitor probe for non-specific binding sites)		
Enterococacceae	GACTCGTTGTAATCTCCATT (SEQ ID NO:52)	ATTTTCACATTACAGACCTCA ACCTACCTCCAACCTCTCAC	B4	Alexa Fluor 488
	AACGCTTGTACTTCCATT (SEQ ID NO:53)	(SEQ ID NO:36)		
	GACTCGTTGTAATGTCATT (SEQ ID NO:54)	(none; competitor probe for non-specific binding sites)		
	GACTCGTTGTAATGCCATT (SEQ ID NO:55)			
Clostridiaceae	TTATGCGGTATTAATCTTCCTT T (SEQ ID NO:56)	ATTTTCATTCATATCACTCAC TCCCAATCTCTATCTACCC	B5	Alexa Fluor 514
	TTATGCGGTATTAATCTCCCTT T (SEQ ID NO:57)	(SEQ ID NO:37)		
	TTATGGGGTATTAATCTTCCT TT (SEQ ID NO:58)	(none; competitor probe for non-specific binding sites)		
	TTATGAGGTATTAATCTTCCTT T (SEQ ID NO:59)			
	TTATGGGGTATTAATCTCCCT TT (SEQ ID NO:60)			
Bacteroidales	ATGTTCTCCTCCGCTTGTG C (SEQ ID NO:61)	ATATTCCTCAAGATACTC CTCTACCTACTCGACTA	B10	Alexa Fluor 660
	TGGCACTTAAGCCGAC AC (SEQ ID NO:62)	CCCTAG (SEQ ID NO:38)		
Staphylococacceae	TGCCCTTTGTATTGTCC ATT (SEQ ID NO:63)	AAAAACGCTTAGATATC ACTCCTACGTCGACCAC	B11	ATTO647N
	TACCCCTTTGTATTGTCC ATT (SEQ ID NO:64)	ACTCATC (SEQ ID NO:39)		
	TGCCCTTTGTATTATCC ATT (SEQ ID NO:65)			
	TGCCCTTTGTATCGTCC ATT (SEQ ID NO:66)	(none; competitor probe for non-specific binding sites)		
Lachnospiraceae	ATAGAAGTTTACATACC GA (SEQ ID NO:67)	ATTTTCAGATTAAACACA CCACAAGGTATCTCGA	B15	ATTO565
	ATAGAGCTTTACATACC GA (SEQ ID NO:68)	ACACTCTC (SEQ ID NO:40)		

	GCTTCTTAGTCAGGTAC CG (SEQ ID NO:69)			
Eubacteria	GCT GCC TCC CGT AGG AGT (SEQ ID NO:70)	TGCTCTCGCGAGGTCG CTTCTCTT (SEQ ID NO:41)	B17	Alexa Fluor 405
	GCA GCC ACC CGT AGG TGT (SEQ ID NO:71)			
	GCT GCC ACC CGT AGG TGT (SEQ ID NO:72)			
	TTACCGCGGCTGCTGGC AC (SEQ ID NO:73)			
	CTACCAGGGTATCTAAT CC (SEQ ID NO:74)			
	CTACCGGGTATCTAAT CC (SEQ ID NO:75)			
	GACGGGCGGTGTGTAC AAG (SEQ ID NO:76)			

Table 3. Oligonucleotides and associated fluorophore-conjugates, used as hairpin amplifiers hybridization chain reaction.

Hairpin name	5'-conjugated fluorophore	Nucleotide sequence (5'-3')
B1h1	Alexa Fluor 647	GAGGAGGGCAGCAAACGGGAAGAGTCTTCCTTTACGCTCTTC CCGTTTGCTGCCCTCCTCAAGAAAGAATGC (SEQ ID NO:12)
B1h2	Alexa Fluor 647	CGTAAAGGAAGACTCTTCCCCTTGCTGCCCTCCTCGCATTCTT TCTTGAGGAGGGCAGCAAACGGGAAGAG (SEQ ID NO:13)
B2h1	Alexa Fluor 594	CCTCGTAAATCCTCATCAATCATCCAGTAAACCGCCGATGATTG ATGAGGATTTACGAGGATGGACTGAGCT (SEQ ID NO:14)
B2h2	Alexa Fluor 594	GGCGGTTTACTGGATGATTGATGAGGATTTACGAGGAGCTCAG TCCATCCTCGTAAATCCTCATCAATCATC (SEQ ID NO:15)
B3h1	Alexa Fluor 546	GTCCCTGCCTCTATATCTCCACTCAACTTTAACCCGGAGTGGAG ATATAGAGGCAGGGACGGATTAGACTTT (SEQ ID NO:16)
B3h2	Alexa Fluor 546	CGGGTTAAAGTTGAGTGGAGATATAGAGGCAGGGACAAAGTC TAATCCGTCCTGCCTCTATATCTCCACTC (SEQ ID NO:17)
B4h1	Alexa Fluor 488	CCTCAACCTACCTCCAACCTCTCACCATATTCGTTCTGTGAGAGT TGGAGGTAGGTTGAGGTCTGTAAATGTG (SEQ ID NO:18)
B4h2	Alexa Fluor 488	GAAGCGAATATGGTGAGAGTTGGAGGTAGGTTGAGGCACATTT ACAGACCTCAACCTACCTCCAACCTCTCAC (SEQ ID NO:19)
B5h1	Alexa Fluor 514	ATTGGATTTGTAGGGTAGATAGAGATTGGGAGTGAGCACTTCAT ATCACTCACTCC CAATCTCTATCTACCC (SEQ ID NO:20)
B5h2	Alexa Fluor 514	CTCACTCCCAATCTCTATCTACCCTACAAATCCAATGGGTAGAT AGAGATTGGGAGTGAGTGATATGAAGTG (SEQ ID NO:21)
B9h1	ATTO 490LS	CCACTCTCAGCACACTCCCAACCTACTACAAGCTCGGGTTGG GAGTGTGCTGAGAGTGGAGTAGATACGTG (SEQ ID NO:22)
B9h2	ATTO 490LS	GAGCTTGTAGTAGGGTTGGGAGTGTGCTGAGAGTGGCACGTAT CTACTCCACTCTCAGCACACTCCCAACCC (SEQ ID NO:23)
B10h1	Alexa Fluor 660	CCTCTACCTACTCGACTACCCTAGCCGTAACCTCACCTAGGGTA GTCGAGTAGGTA GAGGAGTATCTTGAGG (SEQ ID NO:24)
B10h2	Alexa Fluor 660	GTGAAGTTACGGCTAGGGTAGTCGAGTAGGTAGAGGCCTCAA GATACTCCTCTACCTACTCGACTACCCTAG (SEQ ID NO:25)

B11h1	ATTO 647N	ACTCCTACGTCGACCACACTCATCCTGCATGTTCCCGATGAGTG TGGTCGACGTAGGAGTGATATCTAAGCG (SEQ ID NO:26)
B11h2	ATTO 647N	GGGAACATGCAGGATGAGTGTGGTCGACGTAGGAGTCGCTTA GATATCACTCCTA CGTCGACCACACTCATC (SEQ ID NO:27)
B13h1	ATTO 620	CCTGCTTTATGCTCAACATACAACCAGAAATGCGGCGTTGTATG TTGAGCATAAAGCAGGAAGGCGTTACCT (SEQ ID NO:28)
B13h2	ATTO 620	GCCGCATTTCTGGTTGTATGTTGAGCATAAAGCAGGAGGTAAC GCCTTCCTGCTTTATGCTCAACATAACAAC (SEQ ID NO:29)
B15h1	ATTO 565	CCACAAGGTATCTCGAACACTCTCCAAATTGGCTACGAGAGTG TTCGAGATACCTTGTGGTGTGTTAATCTG (SEQ ID NO:30)
B15h2	ATTO 565	GTAGCCAATTTGGAGAGTGTTTCGAGATACCTTGTGGCAGATTA ACACACCACAAGGTATCTCGAACACTCTC (SEQ ID NO:31)
B17h1	Alexa Fluor 405	GTGGACACCTGCTAATCGGATGAGTGTTTCGTTATCGCTCATCCG ATTAGCAGGTGTCCACAACAAACAATCG (SEQ ID NO:32)
B17h2	Alexa Fluor 405	CGATAACGAACACTCATCCGATTAGCAGGTGTCCACCGATTGTT TGTTGTGGACACCTGCTAATCGGATGAG (SEQ ID NO:33)

CLAIMS

What is claimed is:

1. A method of identifying two or more characteristics of a bacterium in a testing sample comprising:
 - a. Obtaining the testing sample comprising the bacterium and a first ligand;
 - b. Contacting the bacterium and the first ligand with a second ligand that is specific for the first ligand and comprising a first fluorescent label, wherein binding of the second ligand to the first ligand creates a labeled ligand conjugate comprising the first fluorescent label;
 - c. Cross linking the labeled ligand conjugate that is bound to the bacterium to obtain a fluorescently labeled bacterium;
 - d. Fixing the bacterium;
 - e. Permeabilizing the bacterium;
 - f. Obtaining an identification sample by performing a hybridization chain reaction (HCR) using the permeabilized bacterium, wherein the identification sample comprises the fluorescently labeled permeabilized bacterium comprising the first fluorescent label and one or more fluorescently labeled bacterial polynucleotide HCR products comprising at least a second fluorescent label, and wherein the first and second fluorescent labels are different; and
 - g. Isolating the bacterium from any other bacteria in the identification sample and identifying a first ligand binding characteristic represented by the first fluorescent label and second characteristic represented by the second fluorescent label.
2. The method of claim 1, further comprising determining a total number of bacteria in the quantification sample.
3. The method of claim 1 or claim 2, wherein the first ligand is an antibody.
4. The method of any one of claims 1-3, wherein the first ligand is an IgA antibody, and the first characteristic of the bacteria is an ability to bind IgA.

5. The method of any one of claims 1-3, wherein the first ligand is an IgM antibody, and the first characteristic of the bacteria is an ability to bind IgM.
6. The method of any one of claims 1-3, wherein the first ligand is an IgG antibody, and the first characteristic of the bacteria is an ability to bind IgG.
7. The method of any one of claims 1-6, wherein the second characteristic is a bacterial phylum.
8. The method of claim 7, wherein the bacterial phylum is an Actinobacteria phylum.
9. The method of claim 8, wherein the HCR comprises use of an initiator probe comprising SEQ ID NO:11 or a polynucleotide sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:11.
10. The method of any one of claims 1-6, wherein the second characteristic is a bacterial class.
11. The method of claim 10, wherein the bacterial class is a Bacilli class.
12. The method of claim 10, wherein the HCR comprises use of an initiator probe comprising SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7 or a sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:5, SEQ ID NO:6 or SEQ ID NO:7.
13. The method of any one of claims 1-6, wherein the second characteristic is a bacterial order.
14. The method of any one of claims 1-6, wherein the second characteristic is a bacterial family.
15. The method of claim 14, wherein the bacterial family is an Enterobacteriaceae family, a Lactobacillaceae family, an Enterococacceae family, a Clostridiaceae family, a Bacteroidales family, a Staphylococacceae family or a Lachnospiraceae family.
16. The method of claim 15, wherein the bacterial family is the Enterobacteriaceae family and the HCR comprises use of an initiator probe comprising SEQ ID NO:4 or SEQ ID NO:34 or a polynucleotide sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:4 or SEQ ID NO:34.

17. The method of claim 15, wherein the bacterial family is the Lactobacillaceae family and the HCR comprises use of an initiator probe comprising SEQ ID NO:35 or a polynucleotide sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:35.
18. The method of claim 15, wherein the bacterial family is the Enterococacceae family and the HCR comprises use of an initiator probe comprising SEQ ID NO:36 or a polynucleotide sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:36.
19. The method of claim 15, wherein the bacterial family is the Clostridiaceae family and the HCR comprises use of an initiator probe comprising SEQ ID NO:37 or a polynucleotide sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:37.
20. The method of claim 15, wherein the bacterial family is the Bacteroidales family and the HCR comprises use of an initiator probe comprising SEQ ID NO:38 or a polynucleotide sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:38.
21. The method of claim 15, wherein the bacterial family is the Staphylococacceae family and the HCR comprises use of an initiator probe comprising SEQ ID NO:39 or a polynucleotide sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:39.
22. The method of claim 15, wherein the bacterial family is the Lachnospiraceae family and the HCR comprises use of an initiator probe comprising SEQ ID NO:40 or a polynucleotide sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:40.
23. The method of any one of claims 1-6, wherein the second characteristic is a bacterial genus.
24. The method of any one of claims 1-6, wherein the second characteristic is a bacterial species.
25. The method of claim 24, wherein the bacterial species is a Bifidobacterium species.
26. The method of claim 25, wherein the HCR comprises use of an initiator probe comprising SEQ ID NO:8, SEQ ID NO:9 or SEQ ID NO:10 or a sequence having about at least 80%, 85%, 90% or 95% identity with SEQ ID NO:8, SEQ ID NO:9 or SEQ ID NO:10.

27. The method of any one of claims 1-26, wherein prior to contacting the group of bacteria in the testing sample with the first antibody, one or more contaminants are removed from the testing sample using a PERCOLL solution.
28. The method of any one of claims 1-27, wherein the fluorescently labeled second ligand comprises a red fluorescent label.
29. The method of any one of claims 1-28, wherein the labeled antibody conjugate is crosslinked to the bacteria using a crosslinking agent comprising bis-(sulfosuccinimidyl)-suberate.
30. The method of any one of claims 1-29, wherein the bacteria are fixed using a fixing agent comprising paraformaldehyde.
31. The method of any one of claims 1-30, wherein the bacteria are permeabilized using a permeabilizing agent comprising lysozyme and lysostaphin.
32. The method of any one of claims 1-31, wherein step (g) is performed using fluorescence activated sorting.
33. The method of any one of claims 1-6, wherein the hybridization chain reaction (HCR) comprises use of an initiator probe selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40 and SEQ ID NO:41.
34. The method of any one of claims 1-33, wherein the hybridization chain reaction (HCR) comprises use of two hairpin sequences selected from a group consisting of SEQ ID NO:12 and SEQ ID NO:13, SEQ ID NO:14 and SEQ ID NO:15, SEQ ID NO:16 and SEQ ID NO:17, SEQ ID NO:18 and SEQ ID NO:19, SEQ ID NO:20 and SEQ ID NO:21, SEQ ID NO:22 and SEQ ID NO:23, SEQ ID NO:24 and SEQ ID NO:25, SEQ ID NO:26 and SEQ ID NO:27, SEQ ID NO:28 and SEQ ID NO:29, SEQ ID NO:30 and SEQ ID NO:31, and SEQ ID NO:32 and SEQ ID NO:23.

35. The method of claim 34, wherein the two hairpin sequences are labeled with a fluorophore selected from the group consisting of Alexa Fluor 647, Alexa Fluor 594, Alexa Fluor 546, Alexa Fluor 488, Alexa Fluor 514, Alexa Fluor 660, Alexa Fluor 405, ATTO 490LS, ATTO 647N, ATTO 620, and ATTO 565.
36. A composition comprising SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40 or SEQ ID NO:41.

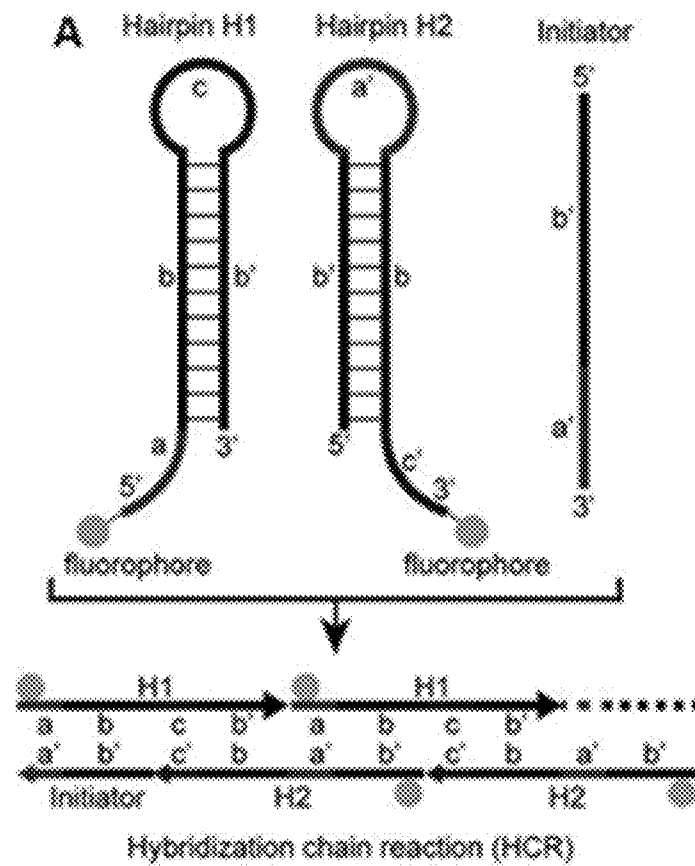


FIG. 1A

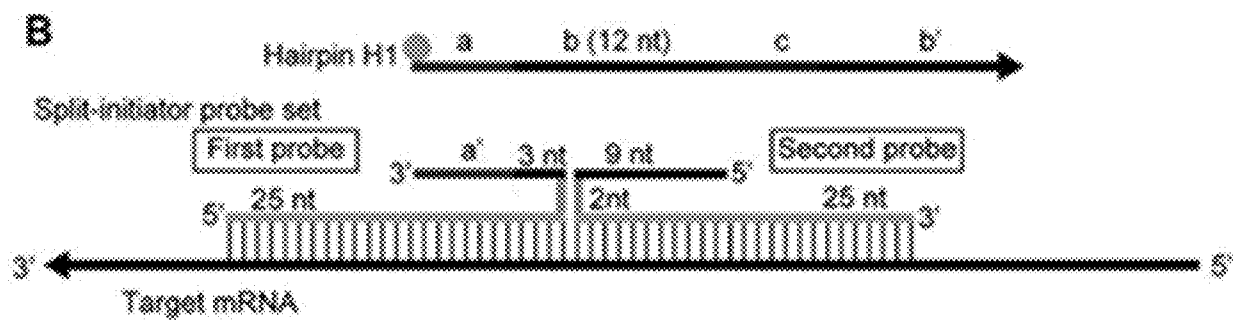
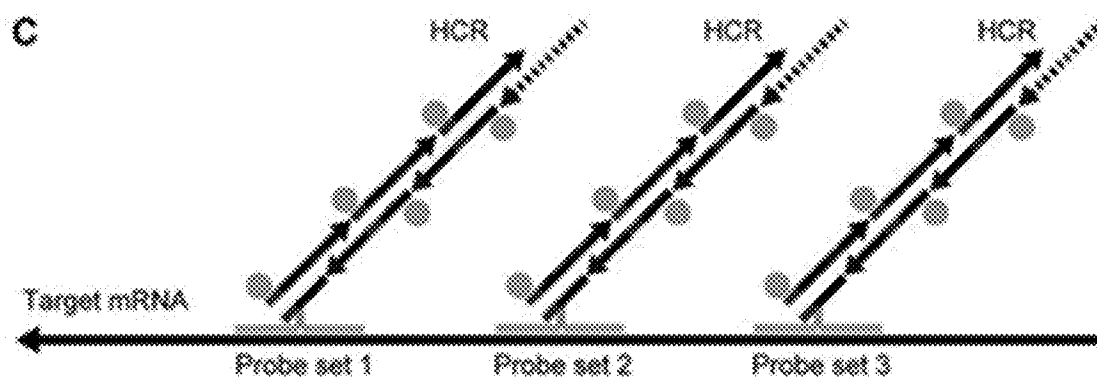


FIG. 1B

**FIG. 1C**

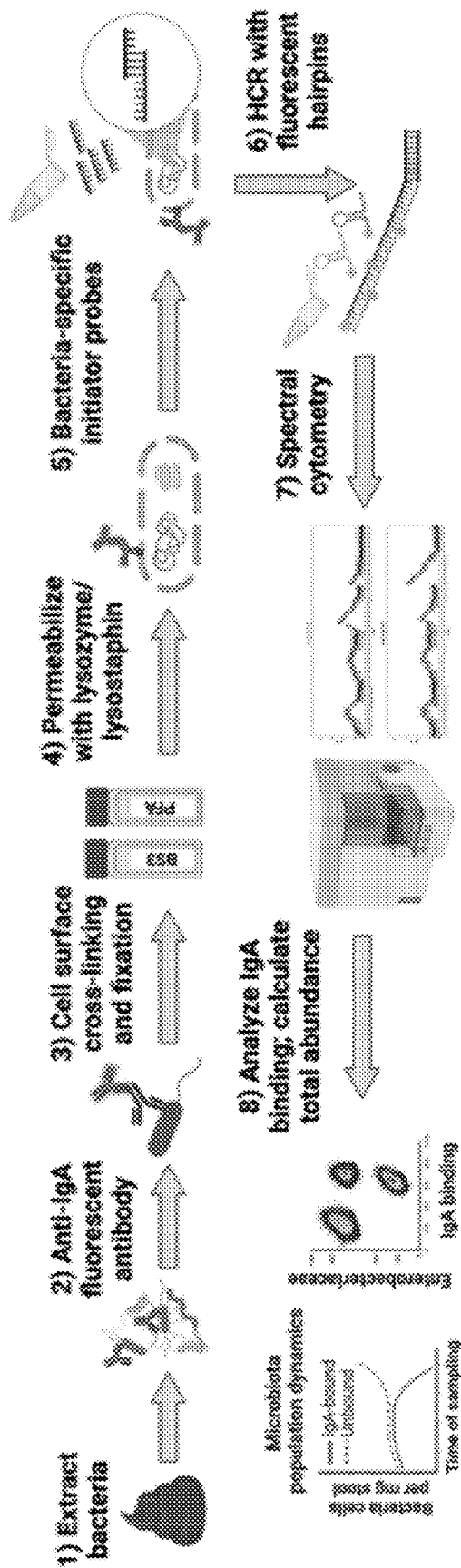


FIG. 2

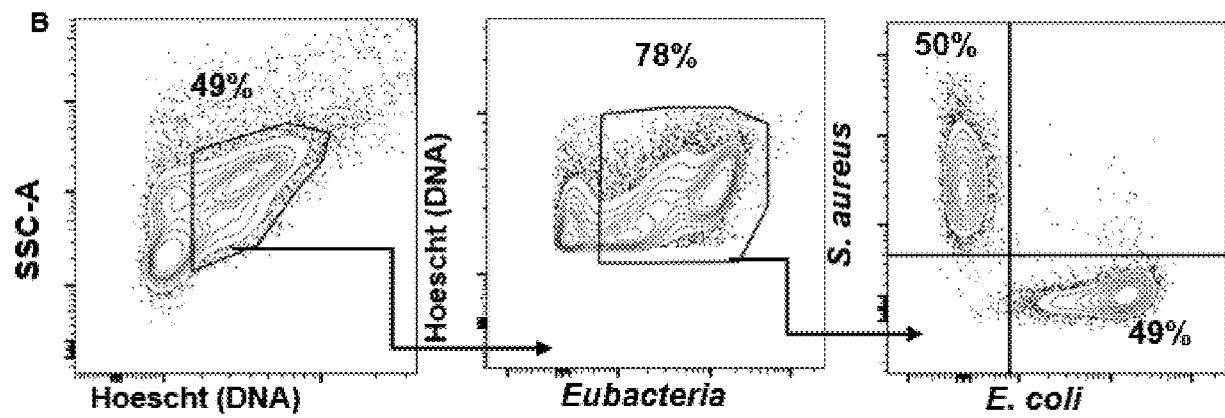


FIG.3A

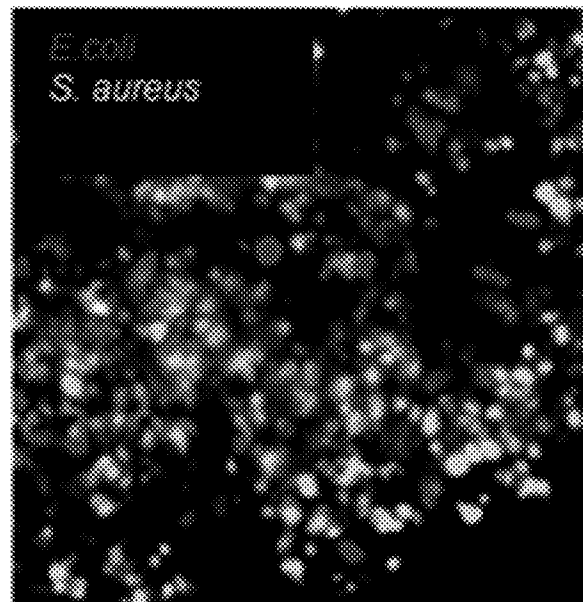
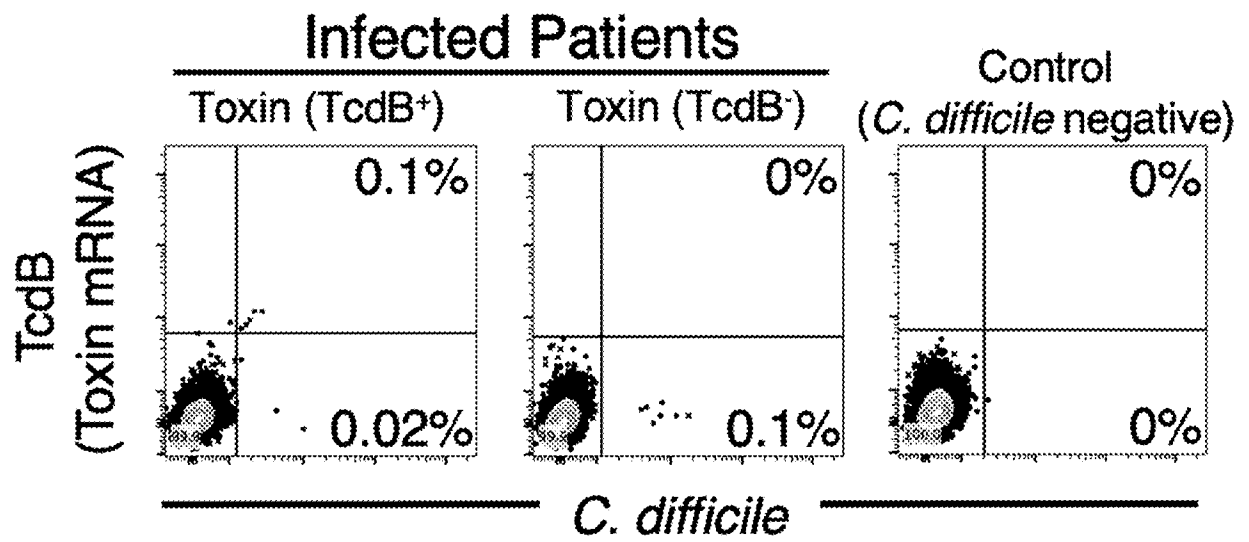


FIG. 3B

**FIG. 4**

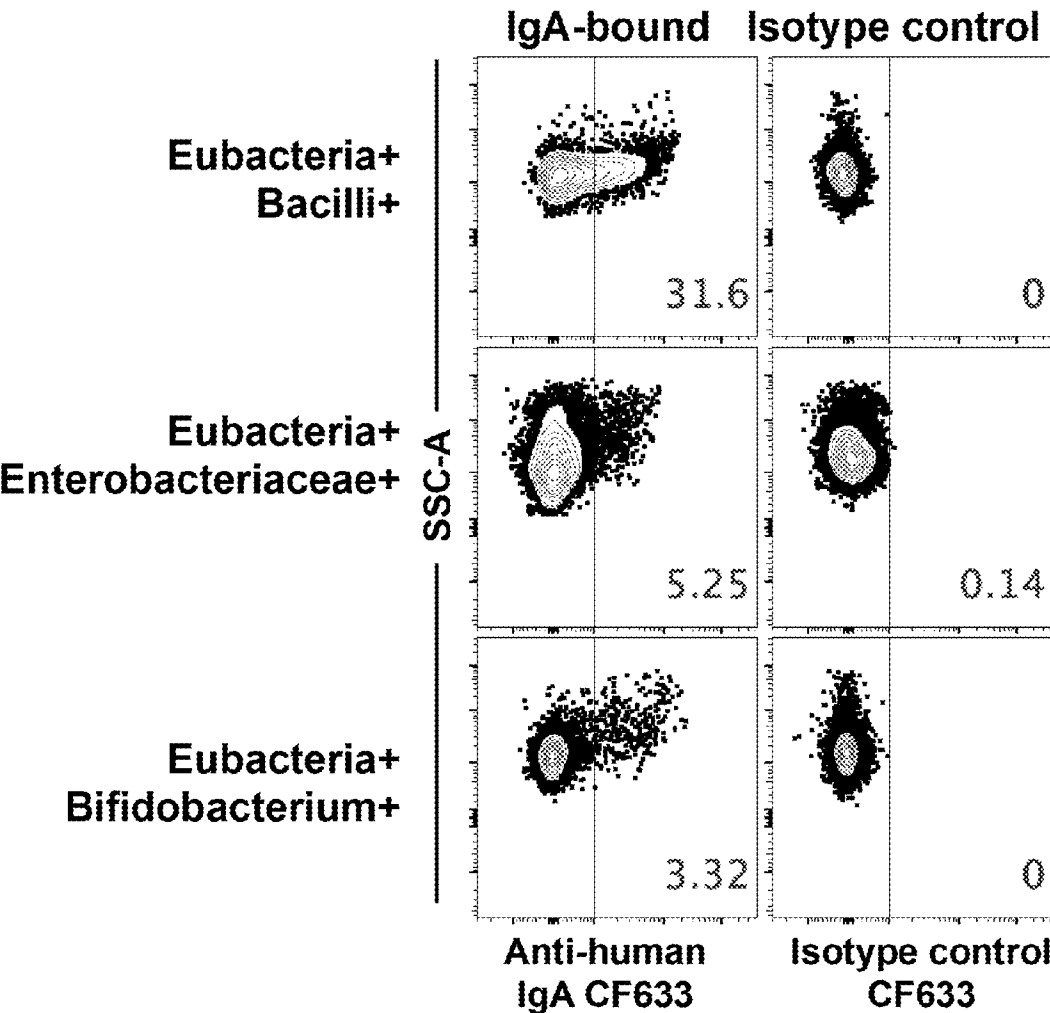


FIG. 5

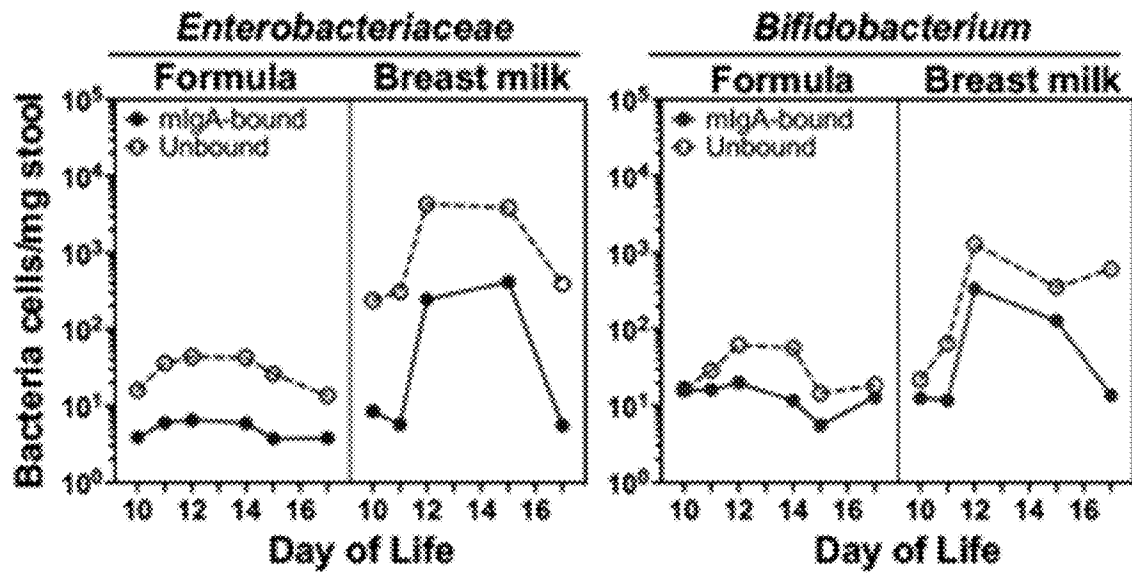


FIG. 6

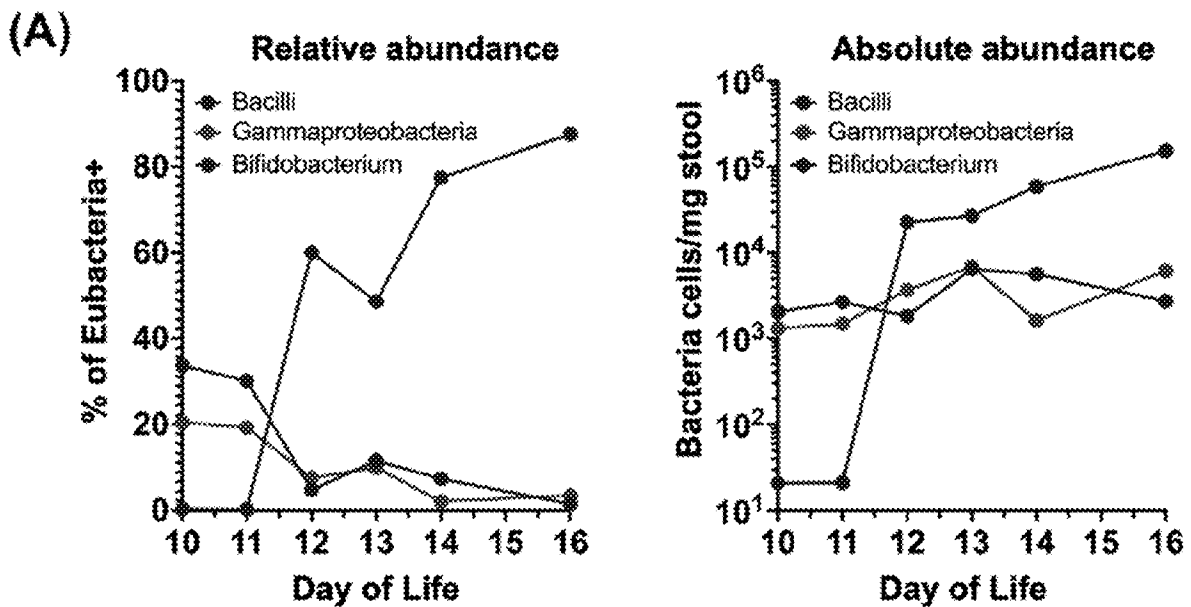


FIG. 7A

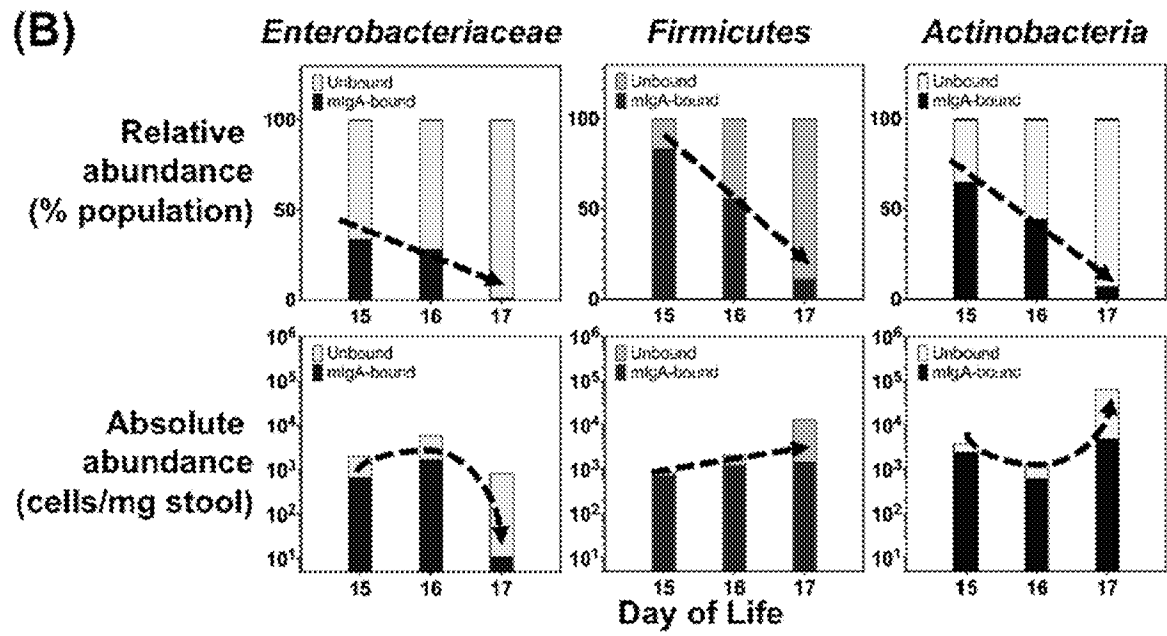


FIG. 7B

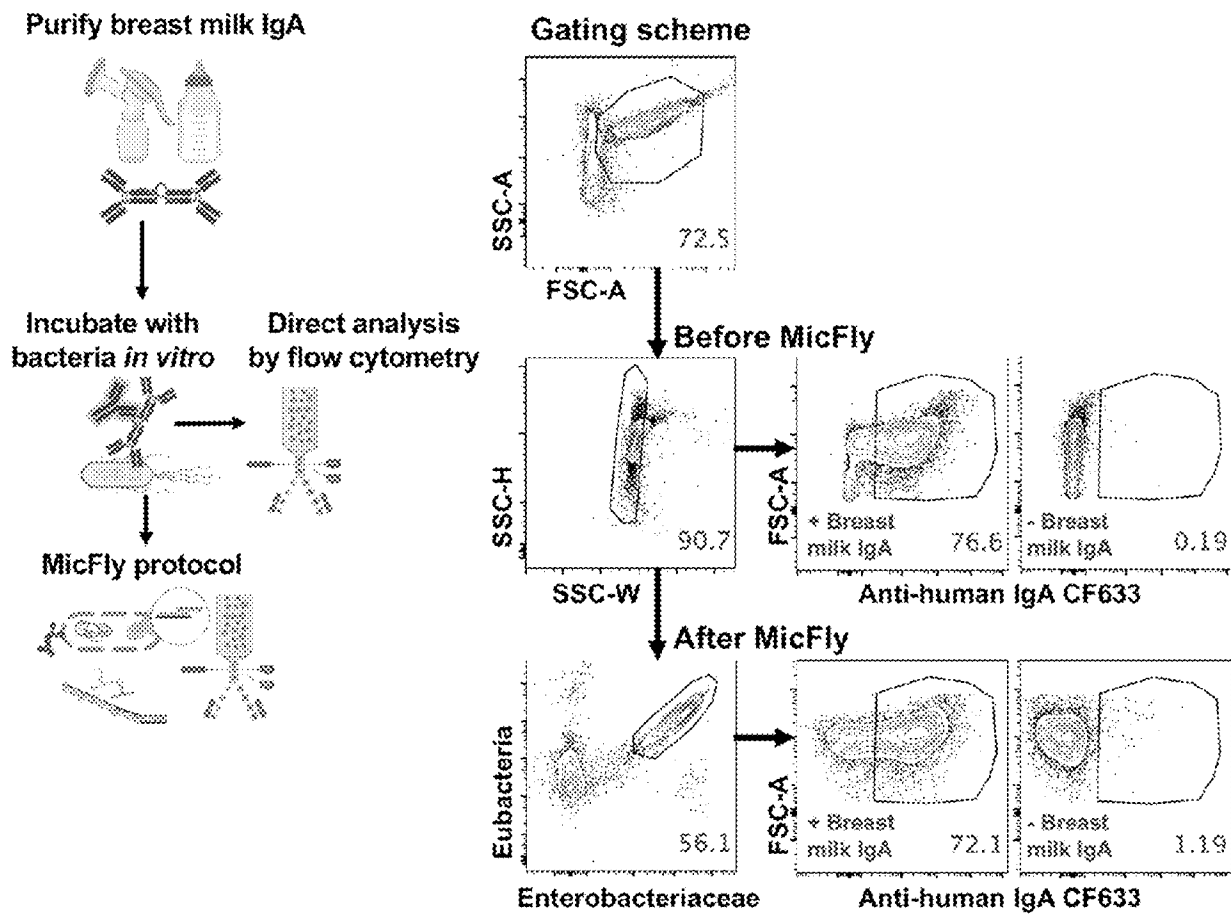


FIG. 8

10/16

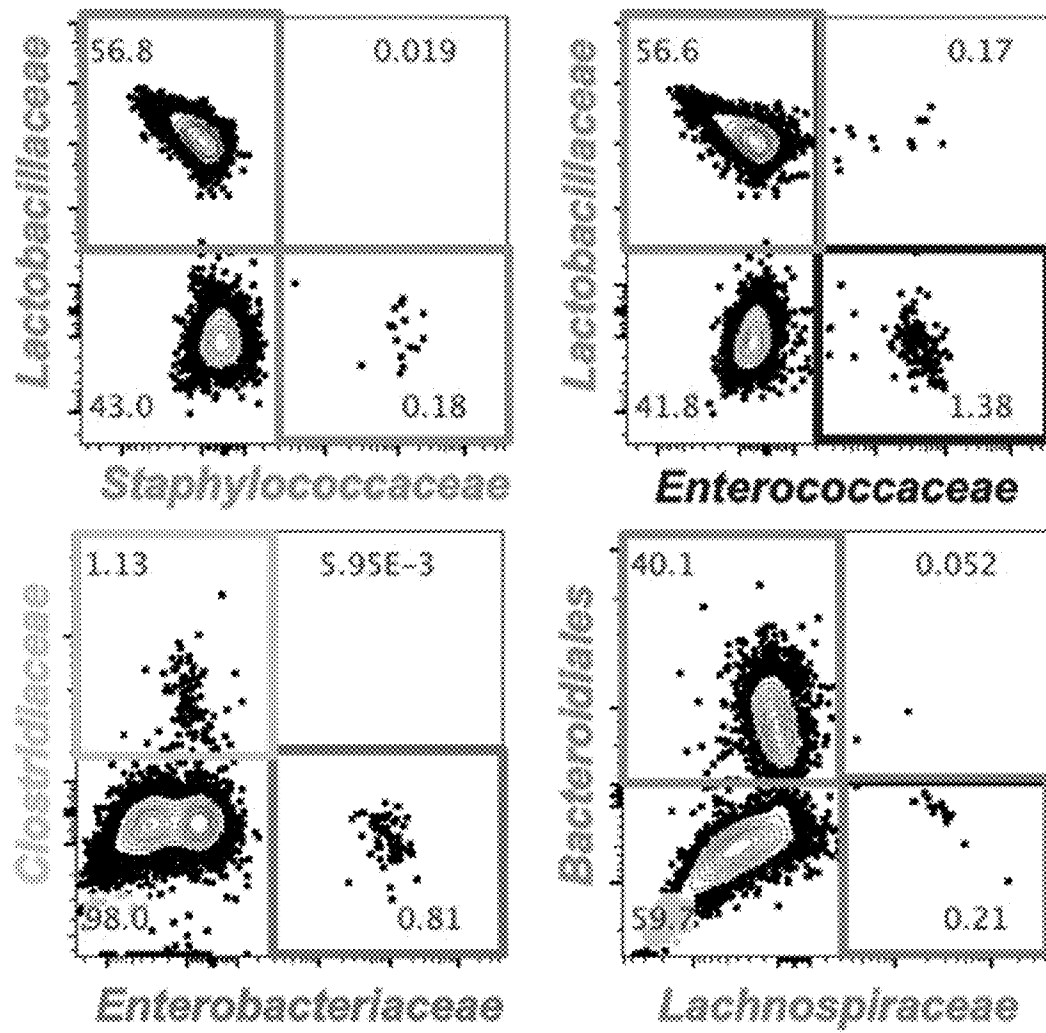


FIG. 9A

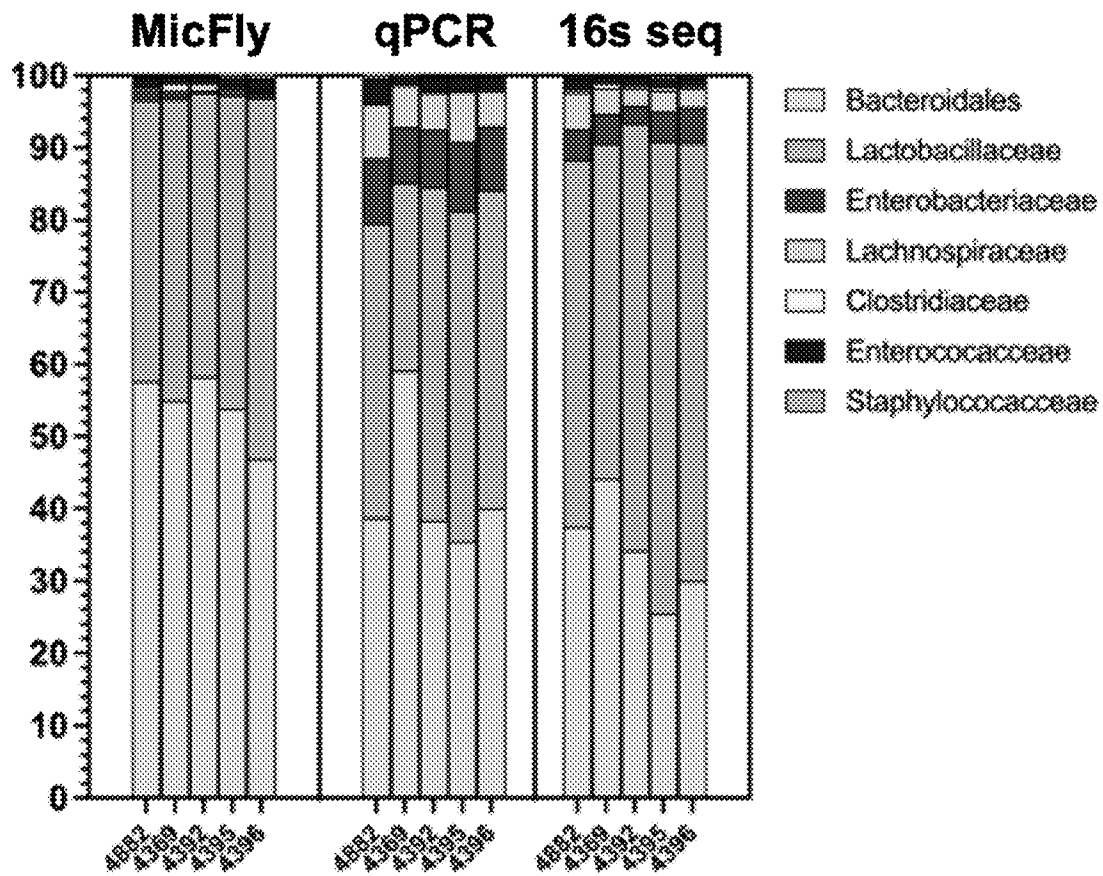


FIG. 9B

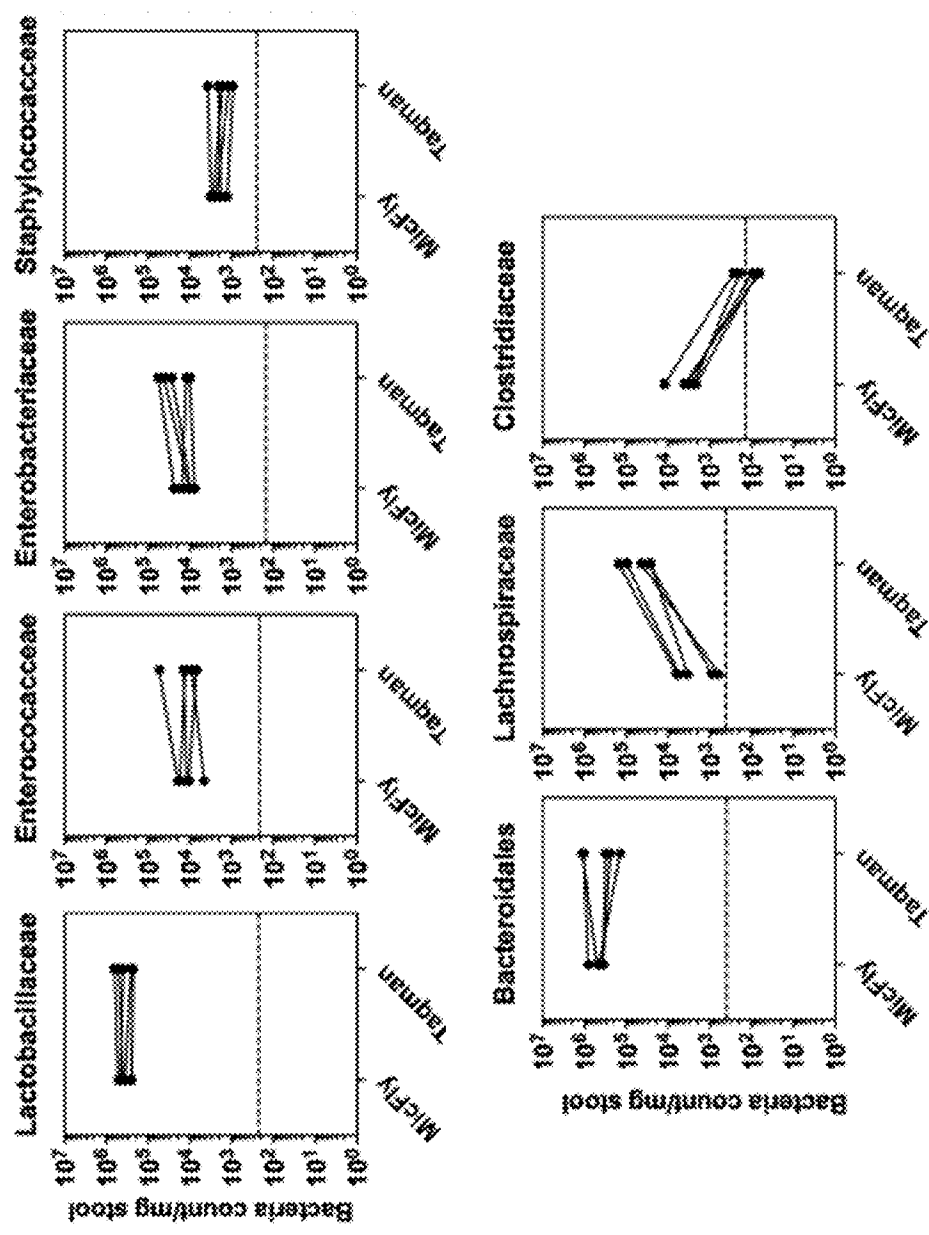


FIG. 9C

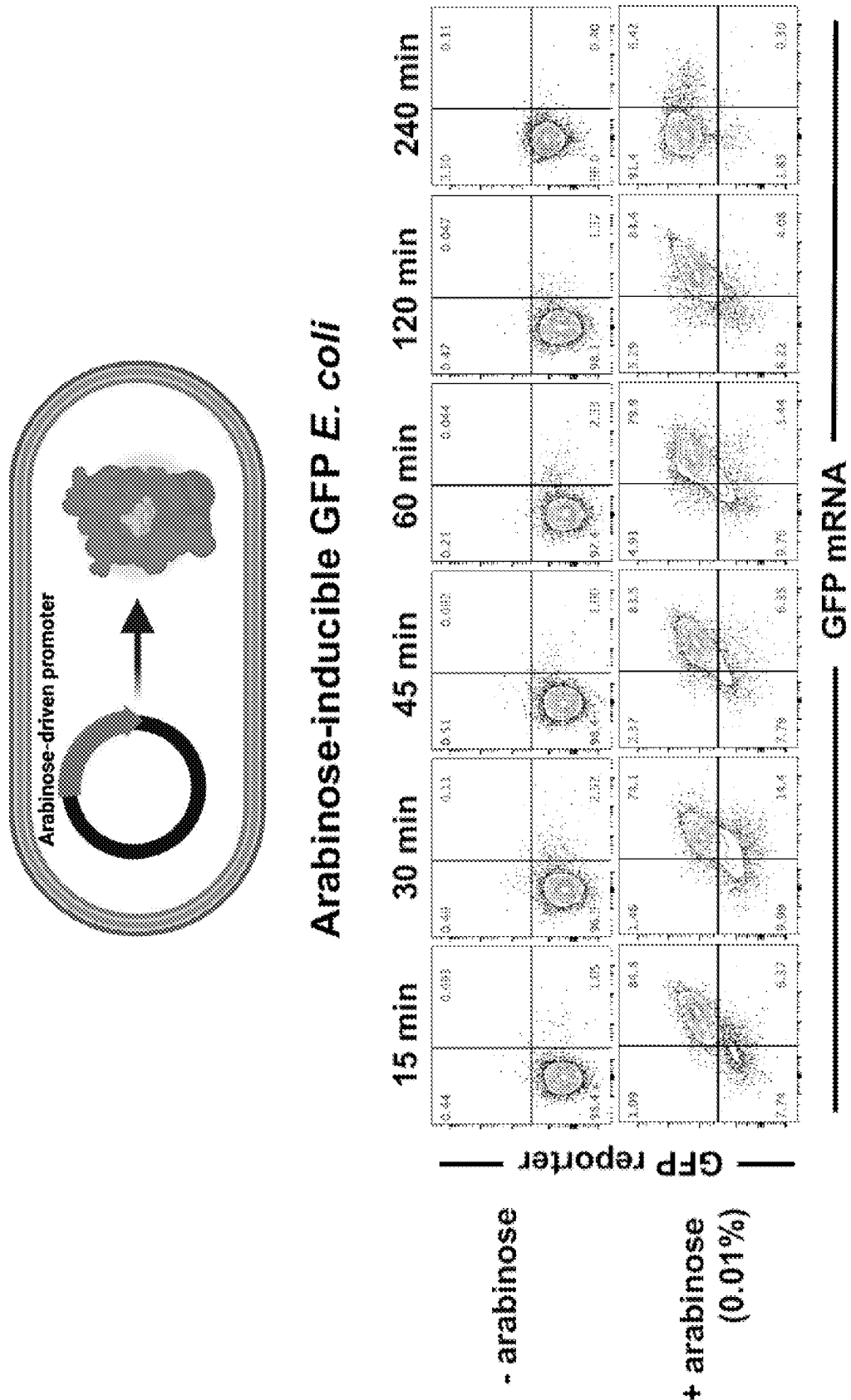
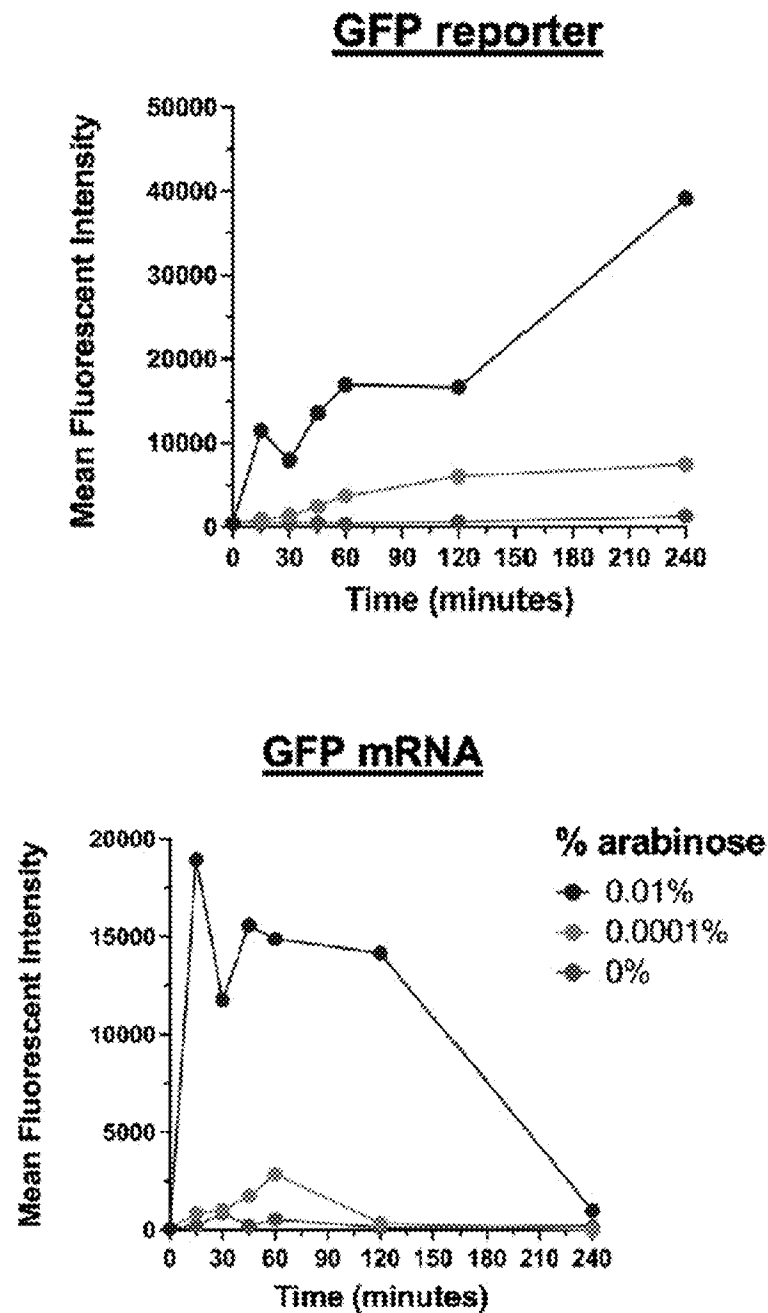


FIG. 10A

**FIG. 10B**

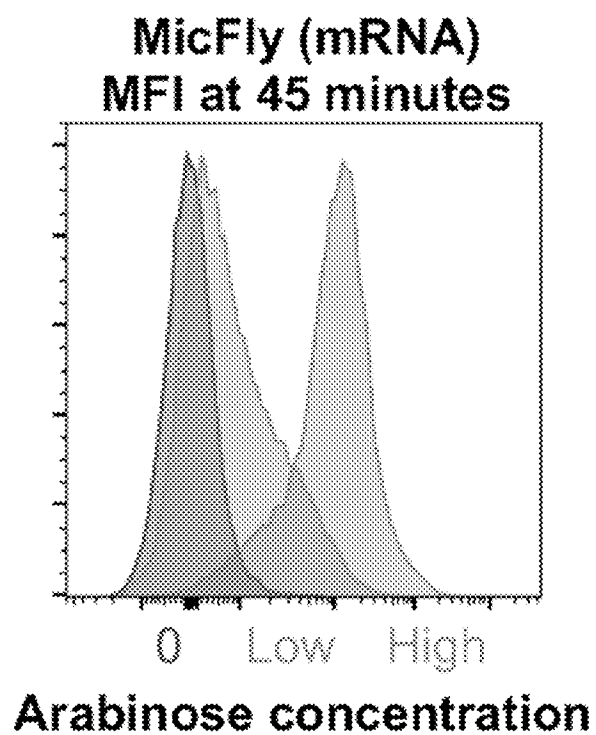
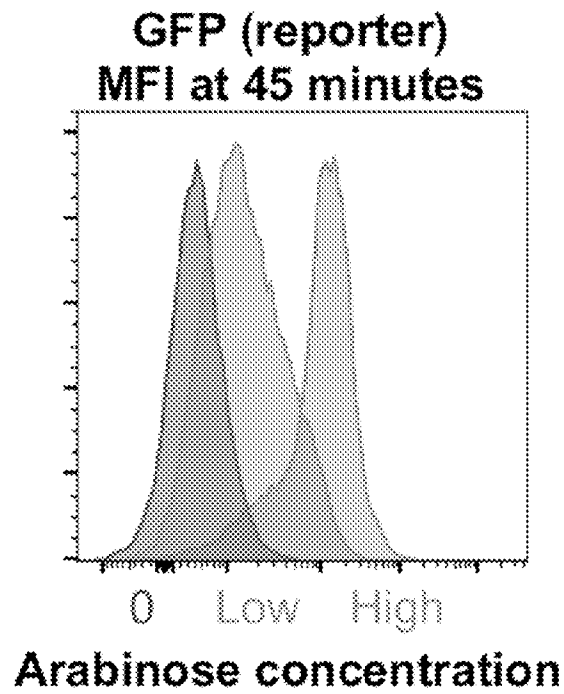


FIG. 10B (continued)

10,000 IgA bound Enterobacteriaceae per mg stool =

500 IgA bound Enterobacteriaceae per μ l of test sample + 2000 μ l of isolated fecal microbiota resuspension

100 mg of extracted stool sample

Where 500 IgA bound Enterobacteriaceae per μ l of test sample =

8000 IgA positive Enterobacteriaceae events

2000 counting bead events

50,000 counting beads added

400 μ l of sample tested for flow cytometry

*

FIG. 11

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/049954

A. CLASSIFICATION OF SUBJECT MATTERIPC: *G01N 33/50* (2024.01); *G01N 33/53* (2024.01); *C12Q 1/6813* (2024.01); *C12Q 1/689* (2024.01); *C12Q 1/06* (2024.01); *C07K 16/12* (2024.01)CPC: *G01N 33/50*; *G01N 33/53*; *C12Q 1/6813*; *C12Q 1/689*; *C12Q 1/06*; *C07K 16/12*

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	US 2023/0193362 A1 (CALIFORNIA INSTITUTE OF TECHNOLOGY et al.) 22 June 2023 (22.06.2023) abstract; para [0115], [0129], [0259]-[0260], [0263], [0679], [0683], [0929]	1-3
Y	MOOR et al., Analysis of bacterial-surface-specific antibodies in body fluids using bacterial flow cytometry, Nature Protocols, 28 July 2016, Vol. 11, No. 8, pages 1531-1553 abstract; pg 1531, col 1, para 1; pg 1535, col 2, para 3; pg 1551, col 1, para 2; Figure 1; Figure 1 Legend; Figure 5; Figure 5 Legend; Figure 7; Figure 7 Legend; Figure 13; Figure 13 Legend	1-3



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

09 January 2025 (09.01.2025)

Date of mailing of the international search report

21 January 2025 (21.01.2025)

Name and mailing address of the ISA/US

COMMISSIONER FOR PATENTS
MAIL STOP PCT, ATTN: ISA/US
P.O. Box 1450
Alexandria, VA 22313-1450
UNITED STATES OF AMERICA

Authorized officer

KARI RODRIQUEZ

Facsimile No. 571-273-8300

Telephone No. PCT Help Desk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/049954

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed.
 - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13ter.1(a)),
☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

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.: **4-35**
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.

Group I: Claims 1-3, directed to a method of identifying two or more characteristics of a bacterium in a testing sample.

Group II+: Claim 36, directed to a composition. Group II+ will be searched upon payment of additional fees. The composition may be searched for an additional fee and election as such, for example, to the extent that the composition encompasses SEQ ID NO: 1 (claim 36). Additional compositions will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected compositions. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. Another exemplary election would be a composition comprising SEQ ID NO: 2 (claim 36).

The inventions listed as Groups I and II+ 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:

Special Technical Features

Group I has the special technical feature of a method of identifying two or more characteristics of a bacterium in a testing sample, not required by Group II+.

Group II+ has the special technical feature of a composition, that is not required by Group I.

The inventions of Group II+ share the technical feature of an initiator probe.

However, these shared technical features do not represent a contribution over prior art, because the shared technical features are anticipated by the publication entitled "Exposing the Three-Dimensional Biogeography and Metabolic States of Pathogens in Cystic Fibrosis Sputum via Hydrogel Embedding, Clearing, and rRNA Labeling" by DePas et al. (hereinafter "DePas").

DePas discloses an initiator probe (pg 2, col 2, para 2 - "FISH with a dilabeled AlexaFluor 594 EUB338 probe and HCR with an initiator EUB338 probe and AlexaFluor 594 hairpins were performed separately on stationary-phase cultured cells embedded in (29A:1B)4P0 and cleared for 5 days").

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/049954

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

Groups I and II+ therefore lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

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.: **1-3**

- 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.