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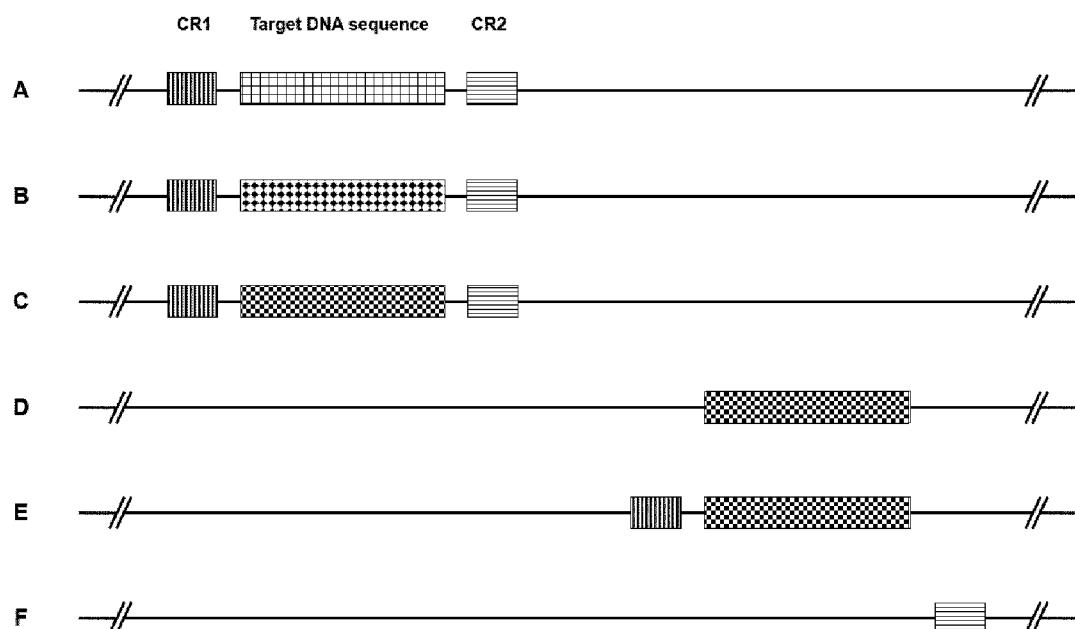
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(54) Title: MICROORGANISM DETECTION METHODS

Figure 1



(57) Abstract: The present invention relates to methods of determining the presence, absence or quantity of target microorganisms in a sample containing multiple microorganisms, wherein each target microorganism is a member of a group of related microorganisms and each target microorganism is a member of a pre-defined set of target microorganisms for the sample, and wherein each target microorganism in the set of target microorganisms can be distinguished from the other target microorganisms in the set. The present invention also relates to kits for performing the methods of the invention.

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MICROORGANISM DETECTION METHODS

FIELD OF THE INVENTION

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The present invention relates to methods of determining the presence, absence or quantity of target microorganisms in a sample containing multiple microorganisms, wherein each target microorganism is a member of a group of related microorganisms and each target microorganism is a member of a pre-defined set of target
10 microorganisms for the sample, and wherein each target microorganism in the set of target microorganisms can be distinguished from the other target microorganisms in the set. The present invention also relates to kits for performing the methods of the invention.

15 **BACKGROUND OF THE INVENTION**

The microbiome is defined as the sum total of all genetic material from microorganisms that inhabits a particular environment, for example the human gut. The role of the microbiome in health and disease is becoming increasingly recognized, and
20 affects many different disease states.

In various environments, many different species of microorganism often co-exist and work in concert in order to grow and perform various functions. Perturbations in a culture of microorganisms can affect the function of the combined collection or culture. For example, in the gastrointestinal tract (GI tract) or the gut, variations in the bacterial
25 culture (also called the microbiota) are associated with various health conditions. Identifying these variations and how they can affect the health of the individual is becoming more and more important in gaining a full understanding of the disease. This may give insights into the etiology of the disease, as well as determining the best treatment options, and options for monitoring the effect of the treatment. There is an
30 increased awareness of the importance of the gut microbiota in disease, as well as new treatment options (see Olle, B. (2013) Medicines from microbiota. Nature Biotechnology, 31, pp309–315).

Several methods exist for analysing the composition of the gut microbiome. The most commonly used methods include a variety of Next Generation DNA Sequencing (NGS) technologies. Standard NGS-based analysis of the gut microbiome consists of either 16S amplicon sequencing, or whole genome sequencing (WGS - also called
5 Metagenome or Shotgun sequencing). Each of these methods has certain benefits and drawbacks.

The method used in most studies is 16S amplicon sequencing, where variations in the 16S rRNA gene are exploited for identifying different bacteria in a sample. 16S amplicon sequencing is the preferred method to date because it is a relatively low cost
10 and quick technique and involves simpler processes than WGS. However, since there is a limited number of sequence variations within the 16S rRNA gene, it is, in most instances, not possible to determine bacterial specificity beyond genus level.

Several studies have highlighted that various bacteria within one genus may have very different properties. For example, different Bifidobacteria display different
15 distinct properties in addition to common properties (Hevia *et al.* (2016) PLoS ONE 11(2)). Also, Zeevi *et al.* (2015, Cell, 163, pp1079-1094) showed that with an optimal diet, the amount of bacteria of the family Ruminococcaceae and bacteria of the genus Ruminococcus decreased. However, while the amount of the species *Ruminococcus bromii* and *Ruminococcus obeum* decreased, the amount of some species of
20 Ruminococcus, *e.g. Ruminococcus lactaris*, increased. Likewise, whilst the amount of the species *Coprococcus catus* decreased, bacteria of the genus Coprococcus increased.

Thus, being able to quantify microorganisms such as bacteria present in a sample precisely at the species and strain level is important. Since this is not possible to achieve using 16S amplicon sequencing technology, one is therefore forced to use the
25 much more labour-intensive and costly WGS approach to NGS. In this approach, the complete bacterial genomes of all bacteria present in the sample are divided into short pieces, and each of the pieces are sequenced, followed by extensive bioinformatic processes to piece together all the different fragments in the right order. At the end of this labourious process it is possible to determine species and strain level identification
30 of the bacteria in the sample. However, due to the extensive processes needed to prepare, sequence and analyze the results, the use of WGS limits the ability to process samples comprising large numbers of different microorganisms and typically limits

processing of samples to those comprising a maximum of a few hundred different microorganisms.

The current NGS technologies are associated with some potential error hotspots (Walker *et al.* (2015) Microbiome 3:26, Avershina & Rudi (2015) Beneficial Microbes 6(5), pp1-4). Both 16S amplicon sequencing and WGS requires special software to assign the sequences to a specific bacterial genus, species and strain. Various software packages are available to do this. However, they all have their benefits and drawbacks, and none of them are able to correctly determine the presence of all the bacteria in a sample (Jovel *et al.* (2016) Front. Microbiol. 7:459).

DNA and RNA microarrays with overlapping sequences have also been used to detect various bacteria in culture (Rajilić-Stojanović *et al.* (2009), Environ Microbiol. Jul; 11(7): pp1736–1751). The use of such microarrays is laboursome as well as involving high cost and it can also be prone to errors.

Another approach, called GA-map, has been used to identify groups of bacteria, at the phylum, class, family, and in some instances genus level (Casen *et al.* (2015) Aliment Pharmacol Ther. Jul; 42(1): pp71-83). The GA-map method fails to give detailed information about the composition of the bacterial culture at the species and strain level. This limitation is due to biological constraints in identifying and defining DNA probes within the 16S rRNA amplicon that is used.

In summary, all of the current technologies available for analysing microbiomes, such as the gut microbiome, have some inherent challenges.

Being able to detect and quantify the individual microorganisms, such as bacteria, in a culture is important in understanding the function of the culture, for example in understanding associated disease and thus to determine treatment options. Particularly, being able to quantify microorganisms precisely at the species and strain level may be important for *e.g.* correct and optimal treatment. Since it is important to correctly identify the individual microorganisms in a complex mixture of many different microorganisms down to a species and strain level, and there is no method available to do this in a simple, low-cost and high throughput format, there is clearly a need for such a method.

SUMMARY OF THE INVENTION

The present invention provides a method of determining the presence, absence or quantity of target microorganisms in a sample containing multiple microorganisms, wherein each target microorganism is a member of a group of related microorganisms and each target microorganism is a member of a pre-defined set of target microorganisms for the sample, and wherein each target microorganism in the set of target microorganisms can be distinguished from the other target microorganisms in the set, the method comprising:

1. providing test DNA from the sample;
2. providing reaction conditions wherein amplification products from the DNA of each target microorganism in the pre-defined set can be generated;
3. generating a plurality of amplicons from the DNA, wherein each amplicon is specific for a different group of related target microorganisms of the pre-defined set; wherein amplification products within each amplicon comprise first and second conserved regions of DNA sequence interspersed with a target DNA sequence region, wherein both first and second conserved regions are conserved across members of the group of related microorganisms, and wherein a target DNA sequence region comprises a target DNA sequence that is a sequence within an amplicon which is unique to each target microorganism within the pre-defined set of microorganisms; and
4. determining the presence, absence or quantity of target DNA sequences of amplification products within each amplicon and thus determining the presence, absence or quantity of each target microorganism in the pre-defined set.

In any such method described above, the step of generating a plurality of amplicons comprises performing amplification reactions using forward and reverse primers designed to anneal to DNA sequence regions comprising respectively the sequences of first and second conserved regions.

In any such method described above, (a) amplification reactions may be performed by multiplex amplification in the same reaction vessel, preferably wherein

multiplex amplification is performed by PCR; or (b) DNA from the sample may be divided into aliquots and added to different reaction vessels, and wherein amplification reactions are performed by multiplex amplification in each reaction vessel, preferably wherein multiplex amplification is performed by PCR.

5 In any of the methods described above, for each amplicon generated the step of determining the presence, absence or quantity of target DNA sequences of amplification products (step 4) may comprise detecting the presence of target DNA sequences using probes, wherein each probe is specific for a target DNA sequence and wherein the sequence of each probe is designed to distinguish a given target DNA sequence from all
10 other target DNA sequences of target microorganisms in the pre-defined set.

 In such methods each probe may be a labelling oligonucleotide probe, as defined herein, conjugated to a labelling entity and the step of determining the presence, absence or quantity of target DNA sequences of amplification products (step 4) comprises measuring a signal from the labelling entity, for example the labelling entity
15 may be a fluorophore and the step of determining the presence, absence or quantity of target DNA sequences of amplification products (step 4) comprises measuring the energy (fluorescence) emitted by the fluorophore. Alternatively in such methods each probe may be a labelling oligonucleotide probe, as defined herein, conjugated to a labelling entity which is a quencher and the step of determining the presence, absence or
20 quantity of target DNA sequences of amplification products (step 4) comprises measuring a signal from a labelling entity which is a fluorophore by measuring the energy (fluorescence) emitted by the fluorophore, wherein the fluorophore is conjugated to a detection oligonucleotide probe, as defined herein, and wherein the energy (fluorescence) emitted by the fluorophore is measured when the nucleic acid strands of
25 a labelling oligonucleotide-detection oligonucleotide probe hybrid are separated.

 In the methods described above each labelling oligonucleotide probe comprises DNA and is designed to hybridize under stringent conditions to a target DNA sequence from the given target microorganism of the pre-defined set and not to hybridize under stringent conditions to target DNA sequences of all other target microorganisms in the
30 pre-defined set.

The probe-based methods described above may be implemented in a variety of different ways. Certain embodiments involve the use of probes in conjunction with the performance of single nucleotide extension (SNE) reactions.

In methods involving single nucleotide extension (SNE) reactions each probe is
5 designed to act as a primer in an enzymatic extension reaction and wherein the method further comprises performing a single nucleotide extension reaction (SNE) to add a single ddNTP to a terminal end of the probe, wherein each probe is designed to hybridise to the target DNA sequence in such a way that the next nucleotide to be added to the probe in the SNE is complementary to a nucleotide which is unique to the target
10 DNA sequence, and wherein the SNE is performed in a reaction mixture which comprises only ddNTPs which are complementary to said nucleotide which is unique to the target DNA sequence. In such methods the ddNTPs provided in the reaction mixture may be conjugated to a quencher of a signal, a labelling entity or a binding entity. In such methods, for amplification products the step of determining the
15 presence, absence or quantity of each target DNA sequence within each amplicon (step 4) may comprise the steps of:

- (a) providing at least one labelling oligonucleotide probe to form at least one labelling oligonucleotide probe-amplification product hybrid if an amplification product having target DNA sequence complementary with that of the probe is
20 present;
- (b) performing a single nucleotide extension reaction to add a ddNTP-conjugate to the at least one labelling oligonucleotide probe-amplification product hybrid to form at least one labelling oligonucleotide probe-ddNTP-conjugate;
- 25 (c) measuring the amount of the at least one labelling oligonucleotide probe-ddNTP-conjugate; and
- (d) determining the presence, absence or quantity of the target microorganism in the set (step 4) based on the amount of the at least one labelling oligonucleotide probe-ddNTP-conjugate.

30 In such methods a labelling oligonucleotide probe-ddNTP-conjugate purification step may be performed before step (c), preferably wherein step (b) comprises providing

ddNTPs conjugated to a binding entity and wherein the binding entity can bind to a capture entity.

In such methods which may involve the performance of step (c), step (c) may comprise hybridising the at least one labelling oligonucleotide probe-ddNTP-conjugate of step (b) to at least one detection oligonucleotide probe to form a reaction mixture comprising at least one labelling oligonucleotide probe-ddNTP-conjugate-detection oligonucleotide probe hybrid, and wherein in step (c) a signal can be generated when the nucleic acid strands of the at least one labelling oligonucleotide probe-ddNTP-conjugate-detection oligonucleotide probe hybrid are separated. In such methods the detection oligonucleotide probe may be coupled to at least one labelling entity and the step of measuring the amount of the at least one labelling oligonucleotide probe-ddNTP-conjugate comprises quantifying a signal generated via the labelling entity.

In probe-based methods involving SNE reactions the labelling entity may be a quencher and the signal is an increase in energy emitted by a fluorophore coupled to the detection oligonucleotide when the nucleic acid strands of a labelling oligonucleotide probe-ddNTP-conjugate-detection oligonucleotide probe hybrid are separated.

In probe-based methods involving SNE reactions: (a) the labelling entity coupled to a probe for the detection of a given target microorganism may differ from the the labelling entities coupled to probes for the detection of every other target microorganism in the pre-defined set; or (b) the labelling entity coupled to a probe for the detection of a given target microorganism may be the same as the labelling entities coupled to probes for the detection of every other target microorganism in the pre-defined set. Each labelling oligonucleotide probe-ddNTP-conjugate-detection oligonucleotide probe with the same labelling entity may have a different melting temperature. In such methods different detection oligonucleotide probes may have different labelling entities.

The step of measuring the amount of the at least one detection oligonucleotide coupled fluorophore may comprise:

- (i) measuring the fluorescence signal from the fluorophore;
- (ii) altering the conditions of the reaction mixture comprising the at least one hybridised labelling oligonucleotide probe-ddNTP-conjugate to favour melting

of the at least one hybridised labelling oligonucleotide probe-ddNTP-conjugate from the detection oligonucleotide containing the fluorophore;

(iii) measuring the fluorescence signal from the fluorophore; and

(iv) determining the amount of the least one hybridised labelling oligonucleotide probe-ddNTP-conjugate based on the difference between the fluorescence signal measured in (i) and the fluorescence signal measured in (iii).

A labelling oligonucleotide probe may have a labelling entity coupled to the 5' end of the probe.

A ddNTP may be conjugated to a binding moiety and the detection oligonucleotide probe may be coupled to at least one quencher, and the step of measuring the amount of the at least one labelling oligonucleotide probe-ddNTP-conjugate comprises quantifying a signal generated via the labelling entity.

In some embodiments each detection oligonucleotide probe may be conjugated to a labelling entity, and wherein each detection oligonucleotide probe corresponding to a specific target DNA sequence can be distinguished from the other detection oligonucleotide probes corresponding to all other specific target DNA sequences in the reaction vessel by different lengths. In such methods different labelling entities may be conjugated to different detection oligonucleotide probes. In such methods any combination of labelling entities and length of detection oligonucleotide probes may be used to uniquely determining the presence, absence or quantity of the target microorganism in the set (step 4) based on the amount of the at least one detection oligonucleotide probe.

In some embodiments the ddNTP may be conjugated to a labelling moiety and wherein each labelling oligonucleotide probe corresponding to a specific target DNA sequence can be distinguished from the other labelling oligonucleotide probes corresponding to all other specific target DNA sequences in the reaction vessel by different lengths. In such embodiments different labelling entities may be conjugated to different labelling oligonucleotide probes. Any combination of labelling entities and length of labelling oligonucleotide probes may be used to uniquely determine the presence, absence or quantity of the target microorganism in the set (step 4) based on the amount of the at least one labelling oligonucleotide probes.

In any of the embodiments involving SNE reactions the labelling moiety may be a fluorophore.

In any of the embodiments involving SNE reactions a binding moiety/entity may be able to be captured by an antibody or other capture entity.

- 5 In any of the embodiments involving SNE reactions a binding moiety/entity may be biotin and a capture entity may be streptavidin.

In any of the embodiments of the invention involving a labelling entity, a labelling entity may be a quencher. A quencher may be selected from the group consisting of BHQ0, BHQ1, BHQ2, BHQ3, BHQ10, TAMRA, QXL520, EDQ,
 10 QXL570, EDQ1, QXL610, DDQ-II, QXL670, QXL, DDQ-1, Dabcyl, Eclipse, Iowa Black FQ, QSY-7, QS-9, QSY-21, QS-35, Iowa Black RQ, malachite green, blackberry quencher 650, ElleQuencher and QSY-21.

In any of the embodiments of the invention involving the use of a ddNTP, a ddNTP is selected from the group consisting of ddC, ddT, ddA, ddG, dIdI or ddU.

- 15 As mentioned previously the methods of the invention may be implemented in a variety of different ways. As an alternative to the embodiments which involve the use of probes in conjunction with the performance of single nucleotide extension (SNE) reactions, the methods of the invention may be implemented by sequencing amplicons. Thus the invention provides methods wherein the step of determining the presence,
 20 absence or quantity of target DNA sequences of amplification products within each amplicon and thus determining the presence, absence or quantity of each target microorganism in the pre-defined set (step 4) comprises:

- A. for each target microorganism in the set, sequencing the target DNA sequence in the target DNA sequence region to identify the sequence and
 25 quantifying the amount thereof;
- B. comparing the sequence results of step A against known DNA sequences of all of the target microorganisms in the set; and
- C. determining the presence, absence or quantity of each target microorganism in the set.

- 30 Amplicons may be sequenced by next generation sequencing (NGS) methods. In such methods adaptor sequences may be attached to amplicons as part of the amplification steps to facilitate DNA sequencing. To facilitate DNA sequencing index

sequences may be attached to amplicons. To facilitate DNA sequencing index sequences may be attached to amplicons in the same amplification reaction performed to attach adaptor sequences, or wherein index sequences may be attached to amplicons in an amplification reaction which is separate from the reaction performed to attach adaptor sequences.

In any of the methods of the invention the presence, absence or quantity of at least 2, at least 4, at least 6, at least 8, at least 10 or at least 20 different target microorganisms in the sample may be determined, optionally wherein at least 50-200 different target microorganisms in the sample is determined.

In any of the methods of the invention the target DNA sequence of any or all target microorganisms has a length of between about 50 to about 1500 bases.

Any of the methods of the invention further comprise quantifying the target microorganisms in the sample. Such methods may comprise determining the proportions of target microorganisms present within the sample.

In any of the methods of the invention the target microorganisms may include bacteria, including gram-negative and gram-positive bacteria; and/or wherein target microorganisms may include fungi; and/or wherein target microorganisms include algae; and/or wherein target microorganisms may include viruses, including eukaryotic viruses and prokaryotic viruses.

In any of the methods of the invention the set of target microorganisms may be a set of microorganisms which inhabit a body region of an individual. The body region may be a region of the gastrointestinal tract. The sample may be from a mucosal layer of a region of the gastrointestinal tract or wherein the sample may be from the lumen of a region of the gastrointestinal tract. The region of the gastrointestinal tract may be the mouth, tongue, throat, oesophagus, stomach, small intestine, large intestine, colon or rectum. The body region may be the eye, ear, nasal cavity, skin, vagina or urethra. The body region may be a region of biofilm on a surface of the individual. The sample may be nasal mucous, saliva, sputum, oesophageal mucus, vomit, faeces, urine, vaginal mucous or skin.

In any of the methods of the invention the sample may be a sample from an individual, and wherein the individual is an animal, preferably a mammal such as an equine animal, a bovine animal, a porcine animal, a canine animal, a feline animal, an

ovine animal, a rodent animal such as a murine animal including species of the genus mus and species of the genus rattus, preferably the individual is human.

In any of the methods of the invention the set of target microorganisms may be a set of microorganisms which inhabit an environment or medium, and wherein the
5 sample is a sample from the environment or medium. The medium may be soil.

In any of the methods of the invention the group of microorganisms may be a microorganism genus and the target microorganism may be a microorganism species or strain.

In any of the methods of the invention the target microorganisms may be
10 bacteria. In such methods the target DNA sequence region may be outside of a ribosomal 16S sequence region. The first and second DNA sequence conserved regions may be outside of a ribosomal 16S sequence region. The target microorganisms may comprise bifidobacteria, enterobacteria, archebacteria, lactobacilli or chlostridia. The target microorganisms may comprise are *bifidobacterium adolescentis*, *bifidobacterium*
15 *angulatum*, *bifidobacterium animalis*, *bifidobacterium bifidum*, *bifidobacterium breve*, *bifidobacterium catenulatum*, *bifidobacterium dentium*, *bifidobacterium gallicum*, *bifidobacterium kashiwanohense*, *bifidobacterium longum subsp. longum*, *bifidobacterium longum subsp. infantis*, *bifidobacterium pseudocatenulatum* and/or *bifidobacterium stercoris*.

20 Also provided within the scope of the invention is a kit adapted for use in the method of any one of the methods of the invention.

A kit of the invention may comprise:

- (i) at least one labelling oligonucleotide probe;
- (ii) at least one detection oligonucleotide probe;
- 25 (iii) a ddNTP conjugated to at least one labelling moiety; and
- (iv) primers;

wherein the at least one labelling oligonucleotide probe is complementary to a target DNA sequence in at least one genomic region and the at least one labelling oligonucleotide probe is complementary to the at least one detection
30 oligonucleotide probe.

The kit may comprise at least two, at least three, at least four, at least five, at least ten or at least twenty labelling oligonucleotide probes.

In the kits of the invention the at least one labelling entity may be a quencher or a molecule to which a quencher may be attached.

In any of the kits of the invention the at least one labelling moiety may be a molecule to which a quencher may be attached which is a first binding moiety, and
5 wherein the method comprises attaching the ddNTP conjugated to the first binding moiety to a quencher by exposing the ddNTP conjugated to the first binding moiety to a quencher-second binding moiety conjugate, wherein the first binding moiety and the second binding moiety have affinity for one another.

In any of the kits of the invention the quencher may be selected from the group
10 consisting of BHQ0, BHQ1, BHQ2, BHQ3, BHQ10, TAMRA, QXL520, EDQ, QXL570, EDQ1, QXL610, DDQ-II, QXL670, QXL, DDQ-1, Dabcyl, Eclipse, Iowa Black FQ, QSY-7, QS-9, Iowa Black RQ, malachite green, blackberry quencher 650, ElleQuencher and QSY-21.

In any of the kits of the invention the kit may comprise at least 2, at least 4, at
15 least 6, at least 8, at least 10 or at least 20 different labelling oligonucleotide probes.

In any of the kits of the invention the at least one detection oligonucleotide probe may be conjugated to a fluorophore.

In any of the kits of the invention the kit comprises at least 2 at least 4, at least 6, at least 8, at least 10 or at least 20 different detection oligonucleotide probes. In such
20 kits each detection oligonucleotide probe may be unique in that it has either a different fluorophore or a different melting temperature compared to other detection oligonucleotide probes present.

Any of the kits of the invention may further comprise at least one affinity chromatography column.

25 Any of the kits of the invention may further comprise at least one buffer.

Any of the kits of the invention may further comprise enzymes for performing reactions required in methods of the invention. The enzymes may comprise Taq polymerase. The enzymes may comprise two different Taq polymerases. The enzymes may comprise Exonuclease I. The enzymes may comprise Shrimp Alkaline
30 Phosphatase.

A kit of the invention may also be a kit comprising forward and reverse primer pairs for generating a plurality of amplicons, wherein each amplicon is specific for a

different group of related target microorganisms of the pre-defined set as defined herein, and wherein forward and reverse primer pairs are designed to anneal to DNA sequence regions comprising respectively the sequences of first and second conserved regions as defined herein. Any of these kits of the invention may also further comprise at least one
5 buffer and may further comprise enzymes for performing reactions required in methods of the invention. The enzymes may comprise Taq polymerase. The enzymes may comprise two different Taq polymerases. The enzymes may comprise Exonuclease I. The enzymes may comprise Shrimp Alkaline Phosphatase.

10 **BRIEF DESCRIPTION OF THE FIGURES**

Figure 1 provides an example cartoon depiction of sections of the genomes of six different microorganisms. Microorganisms A, B and C are target microorganisms which are all members of a group of related microorganisms. Microorganisms D, E and
15 F are different microorganisms from different groups of microorganisms.

Figure 2 (A to C) provides example cartoon depictions of four example non-limiting alternative method schematics for detecting probes specific for target DNA sequences using methods described herein for the incorporation of ddNTPs into labelling oligonucleotide probes. Figure 2 (D) provides an example cartoon depiction
20 of an example non-limiting alternative method schematic for detecting probes specific for target DNA sequences using methods described herein using bead-based reagents. Figure 2 (E and F) provides example cartoon depictions of two example non-limiting alternative method schematics for detecting probes specific for target DNA sequences using methods described herein for the incorporation of ddNTPs into labelling
25 oligonucleotide probes and wherein probes may be separated and detectably resolved on the basis of their unique sizes.

Figure 3 shows genomic sequences of several exemplary Bifidobacteria species in an alignment. Species and strain specific sequence areas are shown in the boxed area together with example putative forward and reverse primer sequence areas. Each
30 Bifidobacterium species corresponds to a target microorganism. Forward and reverse primer binding sequence areas correspond to first and second conserved regions of DNA sequence, wherein conserved regions are regions which are common to the group

of Bifidobacteria microorganisms of which each target microorganism is a member and unique to that particular group. Species and strain specific sequence areas shown between first and second conserved regions correspond to DNA sequences of target DNA sequence regions, each target DNA sequence region comprising DNA sequence
 5 that is unique to each target microorganism of the group within the group-specific amplicon generated via the first and second conserved regions.

Figure 4 shows a gel containing the result of a 16S rRNA amplification of genomic DNA. Lanes 1-12 indicate the 12 different bacteria amplified. The lanes headed BM1-BM6 designate the mock libraries. The lane headed Control contains a
 10 non-template control (containing no genomic DNA, but otherwise containing everything else for the PCR to occur).

Figure 5 provides a gel containing the result of a Bifidobacteria amplification of genomic DNA. Lanes 1-12 indicate the 12 different bacteria that were amplified. The lanes entitled BM1-7 designate the mock libraries. The Control is a non-template
 15 control (containing no genomic DNA, but otherwise containing everything else for the PCR to occur).

Figures 6 and 7 provide the results from LightCycler analysis. The lines marked with a star represent 1Ssang PCR product, 1SsangL (LP) and 1SsangD (DP). The lines identified with a square represent 2CramsD (DP).

Figure 8 provides 6 Tables. Table 1 describes the whole genome bacterial sequences that were used in Example 3. Tables 2 and 3 lists the specific PCR primers and labelling oligonucleotide probes (LP) and detection oligonucleotide probes (DP) used in Example 4. Table 4 describes the set-up of analysis of specificity of labelling oligonucleotide probes (LP) and detection oligonucleotide probes (DP). Table 5
 20 describes the specificity of each of the LP and DP. Table 6 lists mock libraries of different compositions of the 12 genomic bacterial DNA listed in Table 1.

Figure 9 shows the separation of labelled probes on a Urea-PAGE gel on the basis of size.

DETAILED DESCRIPTION OF THE INVENTION

The current invention applies a unique combination of steps which result in the desired outcome, *i.e.*, a method to determine the presence, absence or quantity of target
5 microorganisms in a sample containing multiple microorganisms at species and strain level in a convenient, fast, high-throughput and low-cost manner.

A first step in this process is to pre-define the target microorganisms of interest. By pre-defining the target microorganisms of interest, the detection of those microorganisms becomes much faster and more accurate, and eliminates the occurrence
10 of false positives and false negatives. This is because only the target microorganisms of interest in a sample will be analysed, and the analysis will involve the use of sequences which are unique to each target microorganism of interest.

A further step is to identify sequences in the DNA of groups of the pre-defined target microorganisms that can give rise to common amplicons (*i.e.* a part of the DNA
15 sequence that is amenable to amplification, *e.g.* by PCR) wherein each amplicon is unique to a given group. This is a critical step in order to detect only those target microorganisms within the selected groups, and leads to the high specificity of the methods of the invention.

By contrast, all other current amplicon-based approaches, including 16S
20 amplicon sequencing and those disclosed, for example, in patent application publications WO2016/156251A1, WO2012/080754A1 and WO2016/120494A1 focus on using broad-range amplicons particularly within the 16S rRNA gene that covers as many different bacteria as possible and may not therefore be suitable to uniquely identify specific target microorganisms of interest. This leads to challenges in
25 identifying microorganisms in a complex mixture down to species level, since there is not enough resolution in the DNA sequence of the 16S rRNA gene specifically to distinguish bacteria down to species and/or strain solely based on variations within this sequence region.

The methods of the current invention instead limit the number of microbial
30 sequences that are amplified in an amplicon. This leads to simpler and more specific detection, as only the target microorganisms that are amplified in the amplicon will need to be considered when determining the specific target microorganisms present in a

complex mixture. A unique sequence within a narrow amplicon can unequivocally be assigned to a specific microbial species or strain. Other microorganisms from a different group of microorganisms which might harbour closely related (or identical) DNA sequences relative to a given target DNA sequence will not be represented in the same amplicon, and will therefore not be detected and will therefore not interfere with subsequent analysis. This is depicted visually in Figure 1. Thus the methods of the invention provide a high degree of specificity and selectivity.

Since the methods of the invention will require a plurality of several specific amplicons in order to detect a wide range of microorganisms, multiplex amplification of the specific amplicons may be performed. These amplicons can later optionally be combined, since it is possible to identify each amplicon in a multiplex sequencing reaction, for example by using specific probes.

A further option is to divide the test DNA into different aliquots/reaction volumes, and perform single or multiplex amplification in each aliquot. This leads to higher flexibility in obtaining species and strain level identification of the pre-selected microorganisms.

This could for example lead to several different amplicons within a single gene, *e.g.* the 16S rRNA gene, each of the amplicons targeting a different group of related microorganisms. However, in most instances, amplicons outside the 16S rRNA gene will be used to form these amplicons.

By using a combination of different amplicons and several aliquots, it is thus possible to obtain unprecedented specificity of microorganisms down to species and strain level.

Therefore the process of pre-defining the target microorganisms, using amplicons that are specific to groups of microorganisms and optionally using several aliquots during amplification steps, all combine to achieve the specific detection of microorganisms down to species and strain level.

Taken together, these steps of the invention are new and different from the common methods used to identify microorganisms in a sample, and facilitate in determining the presence, absence or quantity of target microorganisms down to species and strain level in a sample containing multiple microorganisms.

The present methods, therefore, result in detailed and accurate information about the composition of a microbiome, such as the gut microbiome, in a simplified and high-throughput format.

5 **General Definitions**

The term “comprises” (comprise, comprising) should be understood to have its normal meaning in the art, *i.e.* that the stated feature or group of features is included, but that the term does not exclude any other stated feature or group of features from also
10 being present.

The term “consists” (consist, consisting) should be understood to have its normal meaning in the art, *i.e.* that the stated feature or a group of features is included, and that the addition of other features is excluded.

For the purposes of the present invention, a target DNA sequence, as defined
15 herein, derived from a target microorganism is considered to be “present” in a sample, if it is detected using a method of the invention. Similarly, a target microorganism is considered to be “absent” from a sample if the target DNA sequence, as defined herein, derived from the target microorganism is not detected using the methods of the invention.

20 The term “quantify” (quantifying) should be understood to have its normal meaning in the art, *i.e.* that the stated feature or group of features is quantified, either absolutely or relatively to other microorganism or to previous analysis of similar samples, but that the term does not exclude any other stated feature or group of features from also being present.

25 A nucleic acid sequence, such as an oligonucleotide sequence, is “complementary” to another oligonucleotide sequence if it is exactly complementary with the other oligonucleotide sequence (for example TAGG is exactly complementary to ATCC) or if it has a sequence that is similar to a sequence that is exactly complementary with the other oligonucleotide sequence. For example, a nucleic acid
30 sequence, such as a labelling oligonucleotide probe as described and defined herein, may be 80% or more, 90% or more, 95% or more, 99% or 100% identical to a sequence that is exactly complementary to another nucleic acid sequence, such as a target DNA

sequence as described and defined herein. In an embodiment, the term “complementary” refers to a sequence that is exactly complementary (100% identical).

A moderately stringent hybridisation condition uses a prewashing solution containing 5x sodium chloride/sodium citrate (SSC), 0.5% SDS, 1.0 mM EDTA (pH 8.0), hybridisation buffer of about 50% formamide, 6xSSC, and a hybridisation temperature of 55° C (or other similar hybridisation solutions, such as one containing about 50% formamide, with a hybridisation temperature of 42° C), and washing conditions of 60° C, in 0.5xSSC, 0.1% SDS. A stringent hybridisation condition hybridises in 6xSSC at 45° C, followed by one or more washes in 0.1xSSC, 0.2% SDS at 68° C.

Use of Pre-Selected Amplicons and Pre-Selected Target DNA Sequences

The methods of the invention involve determining the presence, absence or quantity of target DNA sequences from a sample which can uniquely identify a target microorganism and distinguish that target microorganism from all other target microorganisms in a pre-defined set of target microorganisms for the sample.

To perform the methods of the invention, first and second DNA sequence conserved regions are identified which flank a target DNA sequence region. A target DNA sequence region comprises a target DNA sequence.

The first and second conserved DNA sequence regions are selected such that they are conserved across a group of microorganisms of which the target microorganism is a member, but not across members of any other group. Thus, in combination, a given first and second conserved DNA sequence region pair is unique to a particular group of microorganisms.

The first and second conserved DNA sequence regions are selected such that they may give rise to an amplicon under appropriate amplification reaction conditions. Thus, in combination, a first and second conserved DNA sequence region pair can define an amplicon which is unique to that particular group of microorganisms.

The target DNA sequences interspersed between first and second conserved DNA sequence regions are selected such that within the context of the group-specific

amplicon each target DNA sequence is unique to a species of microorganism of the group or to a strain of microorganism of the group.

Since the first and second conserved DNA sequence regions are selected such that a given pair of first and second conserved DNA sequence regions is conserved
5 across a group of microorganisms and is unique to that particular group of microorganisms, an amplicon arising from that particular first and second conserved DNA sequence region pair will define an amplicon unique to a particular group of microorganisms and which amplicon comprises amplified products comprising target DNA sequences which can uniquely identify all species or strains of microorganism of
10 the group which have been selected for analysis in the pre-defined set of microorganisms.

A target DNA sequence of a given target microorganism to be detected may not necessarily be a sequence that is unique to the genome of that given target microorganism compared to all other microorganisms. However, if that specific target
15 DNA sequence is represented in the genome of any other different species or any other different strain of microorganism (*i.e.* a non-target microorganism, or a target microorganism from a different group) it will not be present in amplified products arising from such other microorganisms because it will not contain the two conserved DNA regions of the target DNA sequence. Consequently, it will not interfere with
20 detection of the target DNA sequence from the given target microorganism. This is because the relevant sequence will not be amplified from such other microorganisms by virtue of the fact that a given target DNA sequence is pre-selected such that it is a sequence which is unique to a given target microorganism of a group of target microorganisms within a pre-selected amplicon which is unique for the group of target
25 microorganisms to which the given target microorganism belongs (see Figure 1).

Thus by applying the concept outlined above, the methods of the invention provide improvements over pre-existing methods by reducing or eliminating false positives, since the same or similar target DNA sequences which might be represented in the genomes of such other microorganisms will in contrast not be represented in
30 amplicons generated for detection.

Sample Containing Target Microorganisms

The present methods involve determining the presence, absence or quantity of target microorganisms in a sample containing multiple microorganisms.

- 5 The sample may be any suitable sample which will contain multiple microorganisms and wherein the precise number, composition and proportion of microorganisms within the sample may vary depending upon the origin of the sample.

 The sample may for example be a specific environment or medium such as soil. Soil is known to contain many different microorganisms. The present methods enable
10 the establishment of a set of target microorganisms, *i.e.* the set of target microorganisms is pre-defined. The set of target microorganisms represents groups of microorganisms, wherein each target microorganism in the set is known to be potentially present in any given test sample. Thus in any soil sample the precise number, composition and proportion of known target microorganisms within the sample may vary. The methods
15 allow the identity of target microorganisms of the set of potential target microorganisms to be determined for any given sample. The proportion of target microorganisms within the set of potential target microorganisms can also be determined for any given sample, thus allowing for quantification of target microorganisms within the set of potential target microorganisms.

- 20 The sample is typically a sample from an individual, such as a sample from a body region of an individual. The individual is typically an animal, preferably a mammal such as an equine animal, a bovine animal, a porcine animal, a canine animal, a feline animal, an ovine animal, a rodent animal such as a murine animal including species of the genus *mus* and species of the genus *rattus*, preferably the individual is
25 human.

 The sample may for example be a sputum sample, skin swab or a fecal or stool sample.

- Identification of the target microorganisms of a set of target microorganisms known to be potentially present in such samples will assist in the diagnosis of
30 conditions relevant to the gastrointestinal tract, such as irritable bowel syndrome. Identification of the target microorganisms present in such samples may also help in

determining the most appropriate treatment options for individuals suffering from particular disorders.

The methods of the present invention also allow the determination of target microorganisms which might be lacking in a body region of an individual. For example, the identification of the absence or low abundance of a particular group, species or strain of target microorganisms in a body region of an individual, *e.g.* bifidobacteria in the stomach or intestine, may allow the individual to supplement that body region with the target microorganisms that are absent or present in low abundance.

Thus in any of the present methods the sample may be from a body region and/or may represent target microorganisms which inhabit a body region. The body region may be a region of the gastrointestinal tract such as the mouth, tongue, throat, oesophagus, stomach, small intestine, large intestine, colon or rectum. The body region may be the eye, ear, nasal cavity, skin, vagina or urethra. The body region may be a region of biofilm on a surface of the individual.

In any of the present methods the sample may be a sample of for example nasal mucous, saliva, sputum, oesophageal mucus, vomit, faeces, urine, vaginal mucous or skin.

Target Microorganisms

In principle any suitable target microorganism may be detected using the methods of the present invention. Since a set of target microorganisms to be detected is pre-defined, the methods allow a determination to be made as the presence, absence or quantity of a given target microorganism of the set.

Bacteria

Target microorganisms which may be detected using the methods of the invention include bacteria.

Both gram-negative and gram-positive bacteria may be detected. For example, bacteria that can be detected include but are not limited to *Staphylococcus aureus*, *Streptococcus pyogenes* (group A), *Streptococcus sp.* (viridans group), *Streptococcus*

agalactiae (group B), *S. bovis*, *Streptococcus* (anaerobic species), *Streptococcus pneumoniae*, and *Enterococcus* sp.; gram-negative cocci such as, for example, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and *Branhamella catarrhalis*; gram-positive bacilli such as *Bacillus anthracis*, *Bacillus subtilis*, *Propionibacterium acnes*,
 5 *Corynebacterium diphtheriae* and *Corynebacterium* species which are diptheroids (aerobic and anerobic), *Listeria monocytogenes*, *Clostridium tetani*, *Clostridium difficile*, *Escherichia coli*, *Enterobacter* species, *Proteus mirabilis* and other sp., *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella*, *Shigella*, *Serratia*, and *Campylobacter jejuni*. Infection with one or more of these bacteria can result in diseases
 10 such as bacteremia, pneumonia, meningitis, osteomyelitis, endocarditis, sinusitis, arthritis, urinary tract infections, tetanus, gangrene, colitis, acute gastroenteritis, impetigo, acne, acne posacue, wound infections, born infections, fascitis, bronchitis, and a variety of abscesses, nosocomial infections, and opportunistic infections.

Other target bacteria include but are not limited to *Achrombacter* sp.,
 15 *Acinetobacter* sp. including *Aerobacter aerogeus*; *Alcaligenes* sp.; *Bacillus* sp. including *Bacillus cerius*, *Bacillus subtilis*; *Beggiatoa* sp.; *Brevibacterium* sp.; *Burkholderia cepacia*, *Citrobacter* sp.; *Clostridium* sp.; *Corynebacterium* sp.; *Crenothrix* sp.; *Desulfobacter* sp.; *Desulfovibrio* sp.; *Enterobacter* sp. including *Enterobacter aerogeus*; *Escherichia* sp. including *Escherichia coli*; *Flavobacterium*
 20 sp.; *Gallionella* sp.; *Klebsiella* sp.; *Leptothrix* sp.; *Pseudomonas* sp. including *Pseudomonas aeruginosa*, *Pseudomonas alcaligenes*, *Pseudomonas cepacia*, *Pseudomonas fluorescens*, *Pseudomonas oleoverans*, *Pseudomonas paucimobilis*, *Pseudomonas putida*; *Proteus* sp. including *Proteus morganella*; *Proteus-Prov* sp.; *Salmonella* sp.; *Sarcina* sp.; *Serratia* sp. including *Serratia marscens*; *Shigella* sp.;
 25 *Sphaerotilus* sp.; *Staphylococcus* sp. including *Staphylococcus aureus*; *Streptococcus* sp.; *Thiobacillus* sp.; *Xanthomonas* sp.

Fungi

30 Target fungal microorganisms which may detected using the methods described herein include but are not limited to dermatophytes (e.g., *Microsporum canis* and other *Microsporum* sp.; and *Trichophyton* sp. such as *T. rubrum*, and *T. mentagrophytes*),

yeasts (e.g., *Candida albicans*, *C. tropicalis*, or other *Candida* species), *Saccharomyces cerevisiae*, *Torulopsis glabrata*, *Epidermophyton floccosum*, *Malassezia furfur* (*Pityrosporon orbiculare*, or *P. ovale*), *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Aspergillus nidulans*, and other *Aspergillus* sp., Zygomycetes (e.g.,
 5 *Rhizopus*, *Mucor*), *Paracoccidioides brasiliensis*, *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Coccidioides immitis*, and *Sporothrix schenckii*.

Other target fungi include but are not limited to *Alternaria* sp.; *Amorphotheca* sp.; *Aspergillus niger*, *Aureobasidium* sp.; *Cephalosporium* sp.; *Chaetomium globosum*, *Cladosporium* sp.; *Fungi imperfecti*; *Fusarium* sp.; *Geotricum* sp.; *Gloeophyllum* sp.;
 10 *Lentinus* sp.; *Mucro* sp.; *Penicillium* sp.; *Phoma* sp.; *Rhizopus* sp.; *Saccharomyces* sp.; *Trichoderma* sp.; *Tricophyton* sp.; *Trichosporon* sp.

Algae

15 Target algal microorganisms which may be detected using the methods described herein include but are not limited to *Anabaena* sp.; *Anacystis* sp.; *Ankistrodesmus* sp.; *Ascomycetes*; *Basidiomycetes*; *Chlorella* sp.; *Calothrix* sp.; *Chlorococcum* sp.; *Coccomyxa* sp.; *Microcystis* sp.; *Nostoc* sp.; *Oscillatoria* sp.; *Pleurococcus*; *Phormidium* sp.; *Phordium luridum*; *Scenedesmus* sp.; *Schizothrix* sp.; *Selenastrum* sp.;
 20 *Spirogyra* sp.; *Ulothrix* sp.

Viruses

25 Target microorganisms which may be detected using any of the methods of the invention described herein include viruses.

Viruses which may be detected using any of the methods described herein include eukaryotic viruses and prokaryotic viruses.

Eukaryotic viruses

30

Target eukaryotic viruses which may be detected using any of the methods described herein include, but are not limited to, the following.

Viruses of the *Reoviridae* family, such as rotavirus and reovirus; viruses of the *Orthomyxoviridae* family, such as influenza virus; viruses of the *Arenaviridae* family, such as Lymphocytic Choriomeningitis Virus (LCMV); viruses of the *Flaviviridae* family, such as dengue virus; viruses of the *Picornaviridae* family, such as Theiler's

5 Murine Encephalomyelitis Virus (TMEV), poliovirus and Coxsackievirus B3 (CVB3); viruses of the *Retroviridae* family, such as Mouse Mammary Tumor Virus (MMTV), Murine Leukemia Virus (MLV) and Human Immunodeficiency Virus (HIV); viruses of the *Adenoviridae* family; viruses of the *Caliciviridae* family, such as norovirus; viruses of the *Papovaviridae* family, such as Human Papillomavirus (HPV); viruses of the

10 *Parvoviridae* family, such as Kilham Rat Virus (KRV); plant viruses, such as Tobacco mosaic virus (TMV) and Cauliflower mosaic virus (CaMV).

Prokaryotic viruses

15 Target prokaryotic viruses which may be detected using any of the methods described herein include, but are not limited to, archaeal viruses and bacterial viruses (bacteriophage).

Target prokaryotic viruses which may be detected using any of the methods described herein include lysogenic bacteriophages and lytic bacteriophages.

20 Target prokaryotic viruses which may be detected using any of the methods described herein include viruses of the *Microviridae* family.

Target prokaryotic viruses which may be detected using any of the methods described herein include viruses of the *Caudovirales* order, such as viruses of the *Podoviridae* family, viruses of the *Siphoviridae* family and viruses of the *Myoviridae*

25 family.

Target microorganisms which inhabit specific body regions

Non-limiting examples of target microorganisms which can colonise specific

30 body regions are described as follow.

Target microorganisms which can colonise the nasopharynx and which can be detected using the methods described herein include but are not limited to the following.

Haemophilus, Neisseria, Staph. aureus, Staph. epidermidis, Strep. viridans and Strep. pneumoniae.

Target microorganisms which can colonise the outer ear and which can be detected using the methods described herein include but are not limited to the following.

- 5 *Enterobacteriaceae, Pseudomonas, Staph. epidermidis and Strep. pneumoniae.*

Target microorganisms which can colonise the eye and which can be detected using the methods described herein include but are not limited to the following.

Haemophilus and Staph. epidermidis.

- 10 Target microorganisms which can colonise the stomach and which can be detected using the methods described herein include but are not limited to the following.

Helicobacter pylori, Lactobacillus, Bifidobacteria and Streptococcus.

Target microorganisms which can colonise the small intestine and which can be detected using the methods described herein include but are not limited to the following.

- 15 *Bacteroides, Candida, Clostridium, Enterobacteriaceae, Enterococcus, Fusobacterium, Lactobacillus, Peptostreptococcus, Staphylococcus, and Streptococcus.*

Target microorganisms which can colonise the large intestine and which can be detected using the methods described herein include but are not limited to the following.

- 20 *Bacteroides, Candida, Clostridium, Corynebacterium, Enterobacteriaceae, Enterococcus, Fusobacterium, Lactobacillus, Mycobacterium, Peptostreptococcus, Pseudomonas, Staphylococcus and Streptococcus.*

Target microorganisms which can colonise the anterior urethra and which can be detected using the methods described herein include but are not limited to the following.

- 25 *Candida, Corynebacterium, Enterobacteriaceae, Enterococcus, Gardnerella vaginalis, Lactobacillus, Mycoplasma, Staph. epidermidis, Streptococcus and Ureaplasma.*

Target microorganisms which can colonise the vagina and which can be detected using the methods described herein include but are not limited to the following.

- 30 *Actinomyces, Bacteroides, Candida, Clostridium, Enterobacteriaceae, Enterococcus, Fusobacterium, Gardnerella vaginalis, Lactobacillus, Mobiluncus, Mycoplasma, Staphylococcus, Streptococcus, Torulopsis and Ureaplasma.*

Target microorganisms which can colonise the skin and which can be detected using the methods described herein include but are not limited to the following.

Candida, Clostridium, Corynebacterium, Propionibacterium, Staph. aureus, Staph. epidermidis and Strep. pyogenes.

Pre-Defined Set of Target Microorganisms

5

As described in detail herein, the present methods allow for determining the presence, absence or quantity of target microorganisms in a sample containing multiple microorganisms, wherein each target microorganism is a member of a pre-defined set of target microorganisms for the sample.

10

The step of pre-defining the set of target microorganisms which may be present within a given sample is performed before the procedural steps of analysing DNA in a given sample to determine the presence or absence of specific target microorganisms within that sample.

15

Pre-defining a set of target microorganisms for a sample, each of which may potentially be present in any given subsequent test sample may be achieved by suitable techniques such as described in the Examples herein.

20

For example, the likelihood of one or more given target microorganisms to be present in a given sample from a given environment or medium may be known, *e.g.* from the literature. It is therefore possible to arrive at a pre-defined set of microorganisms for a given environment, medium or sample therefrom, wherein the set represents a set of microorganisms any of which may be present in a test sample. By applying the analysis methods of the present invention it is possible to determine whether any given target microorganism is indeed present or absent in a test sample.

25

Groups of Target Microorganisms Within the Set of Target Microorganisms

In the present methods, the “set” of target microorganisms is the sum total of all “target microorganisms” which are pre-defined to be potentially present in a given sample.

30

Any given target microorganism in the “set” is a member of a “group” of related microorganisms within the set. By “related” it is meant that any given target

microorganism may be for example a species or strain belonging to a particular group, *e.g.* a genus, of microorganisms.

Purely by way of illustration, the set of target microorganisms may comprise several species and/or strains from the group of bifidobacteria, several species and/or strains from the group of enterobacteria, several species and/or strains from the group of archaeobacteria, several species and/or strains from the group of lactobacilli and several species and/or strains from the group of chlostridia. In this illustration, each species/strain whose presence, absence or quantity is to be determined is a “target microorganism”. Each species/strain is a target microorganism belonging to a particular “group” of related target microorganisms, the groups being bifidobacteria, enterobacteria, archaeobacteria, lactobacilli and chlostridia. The pre-defined set comprises the sum total of all target microorganisms of all groups of related microorganisms which may be potentially present in a given sample.

15 **Target Microorganism Detection Using DNA Sequence Regions**

Use of conserved regions of DNA sequence

In methods of the invention the target DNA sequence regions of the target microorganism to be detected are interspersed between two conserved regions of DNA sequence, wherein the conserved regions are conserved across the group of microorganisms of which the target microorganism is a member.

The two conserved regions of DNA sequence may be referred to herein as first and second conserved regions of DNA sequence. These sequences are selected such that they are capable of promoting the formation of an amplicon under appropriate amplification conditions. Thus the first and second conserved regions of DNA sequence are selected such that they may promote the formation of a group-specific amplicon under appropriate amplification conditions wherein the group-specific amplicon comprises amplification products which are unique to a given group of target microorganisms, and wherein the amplification products of that group-specific amplicon do not comprise amplification products derived from any other group of target microorganisms. As such, the methods of the invention involve the generation of a

plurality of group-specific amplicons wherein each group-specific amplicon may comprise amplification products derived from multiple different individual members of that particular group of microorganisms.

By first and second “conserved” regions of DNA sequence it is meant regions of
5 sequence both of which are represented in each member of a particular group of microorganisms and in a positional configuration in the genome which may allow the formation of an amplicon under appropriate amplification reaction conditions; and wherein one or both sequences are not represented in any member of another group of microorganisms, or cannot give rise to an amplicon derived from any member of
10 another group of microorganisms under appropriate amplification reaction conditions.

For example, to analyse the composition of all desired target microorganisms in a mixture of microorganisms consisting of multiple members of the *Lactobacillus* genus, multiple members of the *Clostridia* genus and multiple members of the *Enterobacteria* genus it will be necessary to pre-select first and second conserved
15 regions of DNA sequence which are represented in all members of the *Lactobacillus* genus whose detection is desired, but absent from all members of the *Clostridia* and *Enterobacteria* genres or which cannot promote the formation of amplicons from members of the *Clostridia* and *Enterobacteria* genres. Likewise it will be necessary to pre-select first and second conserved regions of DNA sequence which are represented
20 in all members of the *Clostridia* genus whose detection is desired, but absent from all members of the *Lactobacillus* and *Enterobacteria* genres or which cannot promote the formation of amplicons from members of the *Lactobacillus* and *Enterobacteria* genres. Likewise it will be necessary to pre-select first and second conserved regions of DNA sequence which are represented in all members of the *Enterobacteria* genus whose
25 detection is desired, but absent from all members of the *Clostridia* and *Lactobacillus* genres or which cannot promote the formation of amplicons from members of the *Clostridia* and *Lactobacillus* genres.

It is not critical that the sequences within a pre-selected first conserved region of DNA sequence are 100% identical across all members of a given group whose detection
30 is desired. Similarly, it is not critical that the sequences within the associated pre-selected second conserved region of DNA sequence are 100% identical across all members of that given group whose detection is desired. Degeneracy in both sequence

regions may be permitted, provided that the pre-selected sequences, when used together under appropriate amplification reaction conditions, are capable of promoting the formation of an amplicon from the desired group of target microorganisms but are not capable of promoting the formation of an amplicon from any other different group of target microorganisms. Amplicons can be generated using degenerate sequence regions which are conserved across members of a group of target microorganisms using techniques known in the art, such as by using the same forward and reverse primers which are designed to take into account such degeneracy and which can thus specifically anneal to first and second conserved regions notwithstanding any sequence degeneracy that may exist between different members of the same group.

Thus a “conserved” region, may be a region of DNA having a sequence which has *e.g.* 85% or more, 90% or more, 95% or more, 98% or more, 99% or 100% sequence identity to the sequence in a corresponding region in a different microorganism within the same group of microorganisms. For example, if a method is used to determine whether different species of *Ruminococcus* are present, a conserved region could be one where the sequence was 90% identical between at least two sequences of *Ruminococcus*.

As noted, the methods of the invention involve the generation of a plurality of group-specific amplicons. By “plurality”, the methods may allow the generation of two or more amplicons. This would mean the method would allow the identification of target microorganism members of two different groups of target microorganism. The methods of the invention are not limited by the number of amplicons that can be generated for any given sample, particularly since multiplex amplification of DNA from the same sample in multiple aliquots allows versatility in the number of primers that can be used in separate parallel reactions. Thus the methods may allow the generation of 5 or more amplicons, 10 or more, 15 or more, 20 or more, 25 or more, 30 or more, 35 or more, 40 or more, 45 or more, 50 or more amplicons from a given sample. Preferably DNA from a given sample is split into aliquots and added to separate reactions for amplicon generation. Multiple different amplicons may be generated in each aliquot. Typically 5 or more amplicons may be generated in each aliquot, 10 or more amplicons may be generated in each aliquot, 15 or more amplicons may be generated in each

aliquot, 20 or more amplicons may be generated in each aliquot, 25 or more amplicons may be generated in each aliquot, 30 amplicons may be generated in each aliquot.

First and second conserved DNA regions may comprise any appropriate length, provided that the above-noted principles are applied. Typically first and second conserved DNA regions will comprise binding sites for primers to be used in amplification reactions, and therefore the length of these regions may be dictated by primer binding requirements, and further to take into account any possible sequence degeneracy that may exist. Typically, first and second conserved DNA regions may be from about 15 to about 50 bases in length, preferably about 25 bases in length.

Use of target DNA sequence regions

As noted above, in methods of the invention target DNA sequence regions of target microorganisms to be detected in any group-specific amplicon are interspersed between the two conserved regions of DNA sequence.

A target DNA sequence region comprises a target DNA sequence. A target DNA sequence is a variable sequence region within amplification products of the amplicon, wherein the target DNA sequence comprises DNA sequence that is unique sequence to a specific target microorganism within the amplicon. Exemplary methods for determining the presence, absence or quantity of such target DNA sequence regions in test samples are described in more detail herein, including in the Examples herein.

A target DNA sequence may have less than 90%, less than 95%, less than 98% or less than 100% identity compared to corresponding sequence areas in other bacteria. As noted further herein, a target DNA sequence of a given target microorganism to be detected may not necessarily be a sequence that is unique to the genome of that given target microorganism compared to all other microorganisms. However, if that specific target DNA sequence is represented in the genome of any other different species or any other different strain of microorganism (*i.e.* a non-target microorganism, or a target microorganism from a different group) it will not be present in amplified products arising from such other microorganisms. This is because the first and second conserved regions noted above will have been pre-selected in such a way that any such DNA sequence which might be represented in the genome of any other different species or

any other different strain of microorganism will not form part of any amplicon. Consequently, any such sequence will not interfere with detection of the target DNA sequence from the given target microorganism to be detected (see Figure 1).

Target DNA sequences may comprise any appropriate length, provided that the
5 above-noted principles are applied. Typically target DNA sequences will be about 50 to about 1500 bases in length.

Core methodology

10 The core methodology of the methods of the invention may be outlined by reference to Figure 1.

Figure 1 provides an example cartoon depiction of sections of the genomes of six different microorganisms. Microorganisms A, B and C are target microorganisms which are all members of a group of related microorganisms. For example,
15 microorganism A could be *bifidobacterium bifidum*, microorganism B could be *bifidobacterium breve* and microorganism C could be *bifidobacterium catenulatum*.

The boxes depicted with vertical line shade represent first conserved DNA regions (CR1) which are conserved across all members of the group of related target microorganisms. The boxes depicted with horizontal line shade represent second
20 conserved DNA regions (CR2) which are also conserved across all members of the group of related target microorganisms. CR1 and CR2 are pre-selected so that they can promote the formation of an amplicon under appropriate amplification reaction conditions, e.g. by acting as binding sites for amplification primers such as forward and reverse PCR primers. The sequences of CR1 and CR2 are deliberately pre-selected
25 such that they are in an appropriate positional configuration in the genomes of all members of a specific group of target microorganisms so that they will promote the generation of an amplicon under appropriate amplification conditions, and such that they will not promote the generation of amplicons derived from microorganisms which are members of a different group of microorganisms. As such, the amplicon derived
30 from CR1 and CR2 will be a group-specific amplicon.

CR1 and CR2 of each of target microorganisms A, B and C are interspersed with target DNA sequences, each of which are, within the group-specific amplicon, unique to

the target microorganism. Therefore, specific detection of each target DNA sequence within the group-specific amplicon provides a means to identify a target microorganism and distinguish it from all other target microorganisms in a pre-defined set of target microorganisms.

5 Detection of a given target DNA sequence in an amplicon generated from DNA from a sample indicates that the given target microorganism is present in the sample because the target DNA sequence was amplified. Conversely, if a given target DNA sequence is not detected in an amplicon generated from DNA from a sample this indicates that the given target microorganism is absent from the sample as the target
10 DNA sequence was not amplified.

 Even if the exact same target DNA sequence of a specific relevant target microorganism is present in the genome of a different microorganism, this will still not interfere with the ability to specifically detect the relevant target microorganism and distinguish it from all other target microorganisms in a pre-defined set. This is because
15 CR1 and CR2 are pre-selected such that only unique target DNA sequences within group-specific amplicons will be available for the purposes of subsequent detection. For example, microorganism D of Figure 1 harbours a sequence within its genome which is identical to the target DNA sequence of microorganism C. However, this sequence of microorganism D will not be amplified because it is not interspersed
20 between sequences CR1 and CR2. Sequences CR1 and CR2 are deliberately pre-selected such that they promote the formation of amplicons only from the group of microorganisms of which microorganism C is a member.

 Even if the exact same target DNA sequence of a specific relevant target microorganism is present in the genome of a different microorganism and juxtaposed
25 next to another sequence which is identical to the sequence of a CR1 or a CR2, this will still not interfere with the ability to specifically detect the relevant target microorganism and distinguish it from all other target microorganisms in a pre-defined set. For example, microorganism E of Figure 1 harbours a sequence within its genome which is identical to the target DNA sequence of microorganism C and thus sequence is
30 juxtaposed next to a DNA sequence which is identical to CR1. However, this sequence of microorganism E will not be amplified because it is not interspersed between sequences CR1 and CR2.

In an analogous manner, it is not necessarily critical that CR1 and CR2 comprise sequences that are 100% unique to microorganism members of the specific group of microorganisms. For example, microorganism F depicted in Figure 1 may be a different microorganism from a different group of microorganisms compared to microorganisms A, B and C. Microorganism F harbours a sequence which is 100% identical to the sequence of CR2. However, in microorganism F CR2 is not positioned next to a sequence corresponding to CR1, and consequently no amplicon will be generated from microorganism F.

Thus it is possible to form a group-specific amplicon of appropriate length by pre-selecting sequences for CR1 and CR2 that are both represented in and conserved across the genomes of all target microorganisms in a group of related microorganisms, and such that their relative positioning in the genomes of microorganisms of that group allows the formation of an amplicon of appropriate length; and conversely by also ensuring that such sequences are not both represented in the genomes of different microorganism members of different groups, or that the positioning of sequences in the genomes of such other microorganisms is such that no amplicon can form.

By generating a plurality of group-specific amplicons each comprising target DNA sequences which within the relevant amplicon are unique for each target microorganism it is possible to uniquely identify individual target microorganisms in a complex mixture of microorganisms and to distinguish individual target microorganisms from all other target microorganisms in a pre-defined set of target microorganisms. At the same time the occurrence of false positive results and false negative results is significantly reduced or eliminated completely. Furthermore, by generating a plurality of group-specific amplicons the amount of sequence information that needs to be detected and processed is dramatically reduced.

Thus the core methodology of the invention provides for exquisite sensitivity, specificity and efficiency in the detection of target microorganisms within complex mixtures.

Amplifying DNA

Amplicons can be generated from pre-selected first and second conserved regions of DNA sequence using any standard amplification technique known in the art.

- 5 Typically, amplification may be performed by PCR using forward and reverse primers wherein a forward primer may bind to a first conserved region and a reverse primer may bind to a second conserved region.

- 10 Amplification may be performed by any suitable method, such as polymerase chain reaction (PCR), polymerase spiral reaction (PSR), loop mediated isothermal amplification (LAMP), nucleic acid sequence based amplification (NASBA), self-sustained sequence replication (3SR), rolling circle amplification (RCA), strand displacement amplification (SDA), multiple displacement amplification (MDA), ligase chain reaction (LCR), helicase dependant amplification (HDA), ramification amplification method (RAM) etc. Preferably, amplification is performed by polymerase chain reaction (PCR). A variation of PCR may be used, for instance Real Time PCR (also known as quantitative PCR, qPCR), hot-start PCR, competitive PCR, or any other.

Exemplary amplification techniques are set out in Molecular Cloning: A Laboratory Manual (Fourth Edition), Green, M. R. and Sambrook, J., Cold Spring Harbor Press (15 Jun. 2012).

- 20 In an non-limiting example, the PCR reaction used may comprise 20-40 repeats of the following steps:

- (i) a denaturation step which may comprise subjecting the oligonucleotides in the aliquot or in the sample to a temperature between 90-100°C for 10-40 seconds;
- 25 (ii) an annealing step which may comprise subjecting the oligonucleotides in the aliquot or in the sample to a temperature between 50-65°C for 10-40 seconds; and
- (iii) an extension step which may comprise subjecting the oligonucleotides in the aliquot or in the sample to a temperature in which the DNA
- 30 polymerase has optimum activity.

A suitable DNA polymerase is Taq polymerase, which has its optimum activity between 70-85 °C.

Since the methods of the invention may require multiple parallel amplification reactions, the reactions may be performed separately in parallel using multiplex approaches. For these purposes the sample may be divided into one or more aliquots. In such embodiments, the method may comprise a step of preparing one or more
5 aliquots from the sample. In the context of the present invention, an “*aliquot*” is a portion of the sample. It is possible to use any number of aliquots in the method of the invention.

Quantification

10

As discussed the presence or absence of a target DNA sequence is detected using detection methods described herein. Such detection methods may be used to quantify the amount of target DNA sequence that is present.

For example, if multiple identical probes are applied, the number of these which
15 hybridise will be proportional to the amount of the target DNA sequence that is present in the original sample. Thus, if a sample is probed with multiple copies of a first probe that hybridises to a first target DNA sequence and multiple copies of a second probe that hybridises to a second oligonucleotide, it will be possible to determine the amount of the first target DNA sequence relative to the amount of the second target DNA
20 sequence.

For the purposes of the invention, therefore, the term “quantity” may be used interchangeably with “relative amount” and both terms may refer to the amount of one target DNA sequence in a sample compared to the amount of a second target DNA sequence in a sample. The second target DNA sequence may be a reference
25 oligonucleotide. For example, a known quantity of a known oligonucleotide may be added to the sample. The user can then compare the relative amount of a target DNA sequence to the amount of the known oligonucleotide to estimate the absolute concentration of the target DNA sequence in the sample. The term “quantity” may also be used interchangeably with “absolute amount”. The methods of the invention provide
30 a means to determine the absolute amount of a given microorganism in a sample. A skilled person will appreciate that such determination may be achieved *e.g.* relative to a calibrated control.

Preparing Target DNA Sequences for Detection

Any of the methods described and defined herein may comprise a step of
5 preparing the target DNA sequences for the step of detecting the presence, absence or
quantity of the target DNA sequences. The step of preparing the target DNA sequences
occurs prior to the step of detecting the presence, absence or quantity of the target DNA
sequences. Preferably the step of preparing the target DNA sequences removes or
10 inactivates any DNA/RNA polymerase and/or PCR primers and/or free nucleotides that
may be present. For example, the step of preparing the target DNA sequences may be
used to remove DNA/RNA polymerase and/or primers and/or free nucleotides that were
used in a step of amplifying the target DNA sequences.

The step of preparing the target DNA sequences may comprise a step of
purifying the target DNA sequences. For example, the step of preparing the target DNA
15 sequences may comprise a chromatography step, such as an affinity chromatography
step. The target DNA sequences may be purified by application to a column comprising
glass beads under conditions in which the target DNA sequences bind to the glass
beads. Suitable conditions include the addition of a chaotropic agent. Suitable
chaotropic agents include sodium iodide or sodium perchlorate. Alternatively or in
20 addition, the purification step may comprise a filtration step, such as filtration using a
filter having a pore size of 0.2 μm . Purification may be used to remove primers and free
nucleotides. For example primers may be removed using one or more washing steps
(*e.g.* with water or a buffered solution which may contain formamide and/or a
detergent), or by electrophoresis, centrifugation, capture onto solid supports,
25 chromatography or any combination thereof.

The step of preparing the target DNA sequences may also comprise an
enzymatic treatment step. For example, it may be desirable to add an enzyme that
inactivates DNA/RNA polymerase. Suitable enzymes include proteinase K.

Similarly, it may be desirable to add enzymes that inactivate primers. For
30 example, surplus primers can be inactivated with an exonuclease that digests any free
single stranded oligonucleotides in solution. Suitable enzymes that can be used for this
purpose, include for example, an enzyme with a 3' $\rightarrow$ 5' single strand exonuclease

activity such as Exonuclease I and an enzyme that catalyzes the dephosphorylation of 5' and 3' ends of deoxyribonucleoside triphosphates and inactivates free nucleotides, for example Shrimp Alkaline Phosphatase.

The combination of Exonuclease I and Shrimp Alkaline Phosphatase reagents is
5 useful, as they can be heat inactivated after use, which eliminates the need for any further purification steps. Thus, the preparation step may further comprising a heat exposure step, such as exposure of the target DNA sequences to a temperature greater than 40°C, 50°C, 60°C, 70°C or 80°C. For example the target DNA sequences could be exposed to a temperature between 60°C and 120°C, between 70°C and 100°C, or around
10 80°C for a period of time of greater than 30 minutes, greater than 40 minutes, between 30 minutes and 2 hours, between 20 minutes and 1 hour, or around 50 minutes. Such a heat treatment step can be used to inactivate enzymes and avoids the need for a further purification step.

Methods of the invention may also comprise a step of measuring the amount of
15 total target DNA sequences before the step of detecting the presence, absence or relative amount of the target DNA sequences, *i.e.* if three target DNA sequences are present in the sample, the amount of all three target DNA sequences can be measured using a technique such as gel electrophoresis or spectrophotometry. For example, the absorbance at 260 nm can provide a measure of total oligonucleotides present in a
20 sample, and so measuring the absorbance at 260 nm after the step of preparing the target DNA sequences will provide a measure of the total amount of target DNA sequences in the sample. Other suitable methods includes quantitative PCR methods such as using the Fento™ bacterial DNA quantification kit (Zymo Research). The amount of target DNA sequences may be compared to a reference, for example a sample having a known
25 amount of DNA present.

Labelling Oligonucleotide Probes

The methods of the invention may use at least one labelling oligonucleotide
30 probe, and the kits of the invention may comprise at least one labelling oligonucleotide probe. The at least one labelling oligonucleotide probe is an oligonucleotide that is complementary to at least one of the target DNA sequences. Thus, if it is desired to

detect the presence, absence or quantity of three different target DNA sequences, three different labelling oligonucleotide probes will be used and each of the three different labelling oligonucleotide probes will hybridise with one of the three different target DNA sequences and each of the three different labelling oligonucleotide probes will
5 hybridise with a different target DNA sequence. For example, at least 2, at least 4, at least 6, at least 8, at least 10, at least 20, at least 30, at least 50, between 2 and 75, between 4 and 50, between 6 and 30, between 8 and 25 or between 10 and 20 different labelling oligonucleotide probes can be used, and each of the labelling oligonucleotide probes will hybridise with a different target DNA sequence. A kit of the invention may
10 comprise at least 2, at least 4, at least 6, at least 8, at least 10, at least 20, at least 30, at least 50, between 2 and 75, between 4 and 50, between 6 and 30, between 8 and 25, between 10 and 20 and between 50-200 different labelling oligonucleotide probes.

Preferably the at least one labelling oligonucleotide probe hybridises to at least one of the target DNA sequences under a moderately stringent hybridisation condition.
15 Optionally the at least one labelling oligonucleotide probe hybridises to at least one of the target DNA sequences under a stringent hybridisation condition.

Hybridising Target DNA Sequences to Labelling Oligonucleotide Probes to Form Hybridised Labelling Oligonucleotide Probes

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Methods of the invention may comprise a step of hybridising the target DNA sequences to at least one labelling oligonucleotide probe to form hybridised labelling oligonucleotide probes.

In the context of the invention, the term “hybridising” the target DNA sequences
25 to at least one labelling oligonucleotide probe, is intended to refer to exposing the target DNA sequences to at least one labelling oligonucleotide probe under conditions in which hybridisation of the target DNA sequence to the at least one labelling oligonucleotide probe can occur. If the target DNA sequence is present in the sample, then the at least one labelling oligonucleotide probe will anneal to the target DNA
30 sequence under suitable conditions. For example, conditions suitable for hybridisation to occur include cool temperatures and high salt conditions.

Preferably the at least one labelling oligonucleotide probe and the target DNA sequences are mixed together and exposed to temperatures between 40°C and 70°C or between 50°C and 65°C for at least 10 seconds (for example at least 20 seconds, at least 30 seconds, between 10 seconds and 5 minutes, between 20 seconds and 2 minutes, or
5 between 20 seconds and 1 minute).

Incorporating a Dideoxynucleotide (ddNTP) Into Labelling Oligonucleotide Probes Using SNE

10 As described in more detail herein, methods of the invention may comprise a step of performing a single nucleotide extension (SNE) reaction to add a ddNTP to the at least one labelling oligonucleotide probe-amplification product hybrid to form at least one ddNTP-labelling oligonucleotide probe-amplification product hybrid.

The ddNTP that is used may be selected from ddC (dideoxycytosine), ddT
15 (dideoxythymine), ddG (dideoxyguanine), ddA (dideoxyadenine), ddI (dideoxyinosine) or ddU (dideoxyuracil). ddC is more preferred because it will bind to guanine more strongly than ddA or ddT will bind to adenine or thymine (adenine binds to thymine with three hydrogen bonds whilst guanine binds to cytosine with two hydrogen bonds).

As described in more detail herein, the sample may be divided into multiple
20 aliquots. In such methods different ddNTPs may be used in different aliquots.

The labelling oligonucleotide probe is designed in such a way that it hybridises to the target DNA sequence in such a way that the next nucleotide to be added to the probe in an enzymatic nucleic acid extension reaction is the specific nucleotide of the ddNTP that is present in the reaction mixture.

25 As described further below, single nucleotide extension reaction is a reaction in which a single ddNTP is added to an oligonucleotide. The ddNTP is added to hybridised labelling oligonucleotide probes, as the hybridised labelling oligonucleotide probes will comprise an overhang region. The at least one labelling oligonucleotide probe is shorter than the target DNA sequence to which it hybridised, meaning that the
30 hybridised labelling oligonucleotide probes will comprise a double-stranded region and a single stranded (overhang) region. A ddNTP may be added that is complementary to the first nucleotide of the overhang region and this is a “single nucleotide extension

reaction” of the invention. Since ddNTPs lack the 3'-hydroxyl group of normal deoxynucleotides, only a single ddNTP can be added to the chain. This provides an additional degree of specificity to probe-based detection methods of the invention in addition to only hybridization.

5 The single nucleotide extension reaction may be performed by any suitable DNA polymerase and in any set of conditions that are suitable for the DNA polymerase to work. A suitable polymerase for use in the step of performing a single nucleotide extension reaction is a DNA polymerase that has enhanced efficiency for incorporating unconventional nucleotides. Preferably the DNA polymerase has 5' to 3' polymerase
10 activity. For example, the DNA polymerase used in the step of performing a single nucleotide extension reaction may be HotTERMIPol DNA Polymerase (Solis BioDyne).

 Suitably the single nucleotide extension reaction is carried out in a polymerase chain reaction (PCR) machine. For example, the PCR machine may be used to provide
15 suitable conditions to add the ddNTP to the hybridised labelling oligonucleotide probe. Suitable conditions include at least one cycle of high temperature (for example a temperature higher than 80°C) followed by at least one cycle at a lower temperature (for example a temperature lower than 70°C). Preferably the suitable conditions include at least one cycle at a temperature between 80°C and 100°C, between 80°C and 95°C or
20 between 85°C and 95°C, followed by at least one cycle at a temperature between 30°C and 70°C, between 40°C and 65°C, or between 50°C and 65°C. 2 or more cycles may be used. For example, 3, 4, 5, 6, 7, 8, 9 or 10 or more cycles may be used.

 By using several cycles in the linear PCR reaction, an excess of labelled oligonucleotide will develop in the solution, and these excess labelled oligonucleotide
25 can be used in subsequent detection steps as explained below.

Incorporating a Labelling Moiety-Conjugated ddNTP Into a Labelling Oligonucleotide Probe Using SNE

30 Methods of the invention may comprise a step of performing a single nucleotide extension reaction to add a ddNTP to a hybridised labelling oligonucleotide probe, as described above, optionally wherein the ddNTP is conjugated to at least one further

labelling moiety. Kits of the invention may comprise a ddNTP conjugated to at least one labelling moiety.

A method comprising a step of performing a single nucleotide extension reaction to add a ddNTP conjugated to at least one labelling moiety to the hybridised labelling
5 oligonucleotide probe allows a labelling moiety to be added to any labelling oligonucleotide probe that successfully hybridised to a target DNA sequence, since a ddNTP can only be added to a terminal end of the probe in an oligonucleotide overhang region wherein the ddNTP to be incorporated is complimentary to the nucleotide in the target DNA sequence at the corresponding position to the position which will be
10 occupied by the incorporated ddNTP.

The single nucleotide extension reaction may be performed by adding free ddNTP conjugated to at least one labelling moiety and a DNA polymerase in conditions that are suitable for the DNA polymerase to work. Since ddNTPs lack the 3'-hydroxyl group of normal deoxynucleotides, only a single ddNTP can be added to the chain. This
15 is useful as it means that only a single labelling moiety will be added to the hybridised labelling oligonucleotide probes.

As described above, suitably the single nucleotide extension reaction to add a ddNTP conjugated to at least one labelling moiety is carried out in a polymerase chain reaction (PCR) machine. For example, the PCR machine may be used to provide
20 suitable conditions to add the conjugated ddNTP to the hybridised labelling oligonucleotide probe. Suitable conditions are described above.

The method may comprise a step of removing unlabelled labelling oligonucleotide probe and free ddNTP conjugated to at least one labelling moiety. If the method does contain such a step, the step is preferably performed after the step of
25 performing a single nucleotide extension reaction and before the step of hybridising the ddNTP-labelling oligonucleotide probe to the at least one detection probe.

The method may comprise an affinity chromatography step. For example, if the labelling moiety is a molecule to which a labelling entity or quencher is to be attached, the method may comprise affinity chromatography using a column comprising beads
30 that have affinity for the molecule to which the labelling entity or quencher is to be attached. If the molecule to which the labelling entity or quencher is to be attached is a

binding entity such as biotin, the affinity chromatography column could comprise beads comprising avidin or streptavidin.

The methods may comprise a step of adding proteinase K, in order to inactivate DNA polymerase.

- 5 Optionally the step of removing unlabelled labelling oligonucleotide probe and free ddNTP is performed before the step of measuring the amount of the at least one hybridised ddNTP-labelling oligonucleotide probe.

 An advantage of incorporating a ddNTP conjugated to a specific labelling moiety is that it can allow a bias towards the detection of a labelling oligonucleotide probe only when the labelling oligonucleotide probe is hybridised to the correct specific target DNA sequence.

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 For example, the sequence of the labelling oligonucleotide probe can be designed so that it will hybridise to a specifically-selected target DNA sequence. The terminal 3' nucleobase of the labelling oligonucleotide probe will hybridise to a complementary nucleobase in the specific target DNA sequence region and such that the next nucleobase in the strand comprising the specific target DNA sequence region overhangs the terminal 3' nucleobase of the labelling oligonucleotide probe. The region of complementarity between target sequence and probe, and their specific sequences, are specifically selected so that this overhanging nucleobase is unique for the specific target DNA sequence (*e.g.* A), and such that nucleobases at this position relative to non-target sequence regions which may be very similar to target sequence regions will be different (*i.e.* in this example C, T or G). By performing single nucleotide extension reactions in separate aliquots where only a single type of ddNTP is provided (*i.e.* in this example ddTTP), the single nucleotide extension reaction will only proceed to successfully incorporate the ddTTP into the labelling oligonucleotide probe if the labelling oligonucleotide probe is hybridised to the specific target DNA sequence region (*i.e.* comprising the overhanging A nucleobase). In contrast, the single nucleotide extension reaction will not proceed to incorporate the ddTTP into the labelling oligonucleotide probe if the labelling oligonucleotide probe is hybridised to a non-specific non-target DNA sequence region. This is because in this situation the nucleobase overhanging the 3' terminal nucleobase of the labelling oligonucleotide probe will be either C, T or G, *i.e.* non-complimentary with respect to the ddTTP provided in that specific aliquot.

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Thus by selecting specific target DNA sequence regions and regions of complementarity between the target DNA sequence region and the labelling onlignonucleotide probe so as to provide a target DNA sequence region-specific overhang one can bias the single nucleotide extension reaction to incorporate a ddNTP only into labelling onlignonucleotide probes that have hybridised to the correct target DNA sequence region. In such methods, the further provision of a labelling moiety conjugated to the ddNTP means that only labelling onlignonucleotide probes that have hybridised to the correct target DNA sequence region will become labelled.

10 **Labelling Moieties**

For the purposes of the present invention, a “labelling moiety” is a generic term which may be used to describe a quencher or a molecule or conjugate which may be attached to a probe and which may act to tether a quencher to the probe. The term “labelling moiety” may also be used to describe a “labelling entity” or a molecule or conjugate which may be attached to a probe and which may act to tether a labelling entity to the probe.

For the purposes of the present invention, a “labelling entity” may be any compound/molecule or group of compounds/molecules which can give rise to a detectable signal. A labelling entity will typically be a fluorophore. A labelling entity may be a radioactive isotope or may be a molecule which may comprise a radioactive isotope. A labelling entity may be a colorimetric molecule. A labelling entity may be a luminescent molecule, for example luciferase.

A molecule or conjugate which may be attached to a probe and which may act to tether a quencher or a labelling entity to the probe may be any suitable molecule or conjugate. A conjugate may be two or more molecules or a pair of molecules which form an affinity interaction. For example, the molecule biotin may be attached to a probe and a quencher or labelling entity may be attached to streptavidin. When contacted together biotin will bind to streptavidin thus tethering the quencher or labelling entity to the probe. Thus a “conjugate” may be two or more molecules which can form an affinity interaction such as biotin and streptavidin so as to tether the

quencher or labelling entity to the probe. Other examples include an antibody and an antigen to which the antibody binds.

As will be described further herein, one way in which a quencher or a labelling entity such as a fluorophore may be tethered to a probe is by attaching the quencher or labelling entity to a ddNTP and incorporating the ddNTP into the probe via a single nucleotide extension reaction. In this situation a ddNTP may itself act as a labelling moiety which tethers the quencher or a labelling entity (*i.e.* further labelling moieties) to the probe. It is envisaged that a quencher or a labelling entity could be tethered to a probe via a conjugate *e.g.* comprising two or more molecules which can form an affinity interaction such as biotin and streptavidin. For example, biotin could be attached to a ddNTP and incorporated into the probe via a single nucleotide extension reaction and then the quencher or the labelling entity could be attached to streptavidin and subsequently tethered to the probe.

As described further herein, in some non-limiting embodiments all of the ddNTPs may be conjugated to the same labelling moiety. In some non-limiting embodiments, the sample may be separated into multiple aliquots, and in these embodiments all of the ddNTPs used in the same aliquot are conjugated to the same labelling moiety.

As described further herein, typically, the labelling moiety is attached at the 3' end of the at least one labelling oligonucleotide probe as part of the single nucleotide extension reaction.

Quenchers

The labelling entity may be a quencher. For the purposes of the invention, the term “quencher” refer to a substance that absorbs energy from a fluorophore and dissipates the energy as heat. Thus, if a quencher is placed in proximity to a fluorophore, it will reduce the fluorescence emitted by the fluorophore.

Preferably the labelling entity is a broad spectrum quencher, *i.e.* a quencher that is able to absorb fluorescence energy across a large range of the visible light spectrum. Preferably all of the labelling entities used are the same quencher. For example, in some non-limiting embodiments all of the ddNTPs may be conjugated to the same

quencher. Alternatively, the ddNTPs may be conjugated to first binding moieties and the first binding moieties may be associated with second binding moieties wherein all of the second binding moieties are conjugated to the same quencher.

The quencher may be selected from the group consisting of BHQ0 (Black Hole Quencher 0), BHQ1 (Black Hole Quencher 1), BHQ2 (Black Hole Quencher 2), BHQ3 (Black Hole Quencher 3), BHQ10 (Black Hole Quencher 10), TAMRA (carboxytetramethylrhodamine), QXL520, EDQ, EDQ1, QXL570, EDQ1, QXL610, DDQ-II (2, 3-dichloro-5, 6-dicyano-1, 4-benzoquinone II), QXL670, QXL, DDQ-1 (2, 3-dichloro-5, 6-dicyano-1, 4-benzoquinone I), Dabcyl, Eclipse, Iowa Black FQ (fluorescence quencher), QSY-7, QS-9, QS-21, QS-35, Iowa Black RQ (Iowa black red quencher), malachite green, blackberry quencher 650, ElleQuencher and QSY-21. Preferably the quencher is a black hole quencher (such as BHQ0, BHQ1, BHQ2 and BHQ3). Preferably the quencher is selected from the group consisting of BHQ0, BHQ1, BHQ2 and BHQ3.

15

Fluorophores

Labelling oligonucleotide probes or detection oligonucleotide probes may be attached to a fluorophore. Suitable fluorophores include the following, 7-AAD (7-Aminoactinomycin D), Acridine Orange (+DNA), Acridine Organe (+RNA), Alexa Fluor® 350, Alexa Fluor® 430, Alexa Fluor® 488, Alexa Fluor® 532, Alexa Fluor® 546, Alexa Fluor® 555, Alexa Fluor® 568, Alexa Fluor® 594, Alexa Fluor® 633, Alexa Fluor® 647, Alexa Fluor® 660, Alexa Fluor® 680, Alexa Fluor® 700, Alexa Fluor® 750, Allophycocyanin (APC), AMCA / AMCA-X, 7-Aminoactinomycin D (7-AAD), 7- Amino-4-methylcoumarin, 6-Aminoquinoline, Aniline Blue, ANS, APC-Cy7, ATTO-TAG™ CBQCA, ATTO-TAG™ FQ, Auramine O-Feulgen, BCECF (high pH), BFP (Blue Fluorescent Protein), BFP / GFP FRET, BOBO™-1 / BO-PRO™-1, BOBO™-3 / BO-PRO™-3, BODIPY® FL, BODIPY® TMR, BODIPY® TR-X, BODIPY® 530/550, BODIPY® 558/568, BODIPY® 564/570, BODIPY® 581/591, BODIPY® 630/650-X, BODIPY® 650-665-X, BTC, Calcein, Calcein Blue, Calcium Crimson™, Calcium Green-1™, Calcium Orange™, Calcofluor® White, 5-Carboxyfluorescein (5-FAM), 5-Carboxynaphthofluorescein, 6-Carboxyrhodamine 6G,

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5-Carboxytetramethylrhodamine (5-TAMRA), Carboxy-X-rhodamine (5-ROX),
 Cascade Blue®, Cascade Yellow™, CCF2 (GeneBLAzer™), CFP (Cyan Fluorescent
 Protein), CFP / YFP FRET, Chromomycin A3, Cl-NERF (low pH), CPM, 6-CR 6G,
 CTC Formazan, Cy2®, Cy3®, Cy3.5®, Cy5®, Cy5.5®, Cy7®, Cychrome (PE-Cy5),
 5 Dansylamine, Dansyl cadaverine, Dansylchloride, DAPI, Dapoxyl, DCFH, DHR, DiA
 (4-Di-16-ASP), DiD (DiIC18(5)), DIDS, Dil (DiIC18(3)), DiO (DiOC18(3)), DiR
 (DiIC18(7)), Di-4 ANEPPS, Di-8 ANEPPS, DM-NERF (4.5-6.5 pH), DsRed (Red
 Fluorescent Protein), EBFP, ECFP, EGFP, ELF® -97 alcohol, Eosin, Erythrosin,
 Ethidium bromide, Ethidium homodimer-1 (EthD-1), Europium (III) Chloride, 5-FAM
 10 (5-Carboxyfluorescein), Fast Blue, Fluorescein (FITC), Fluo-3, Fluo-4, FluorX®,
 Fluoro-Gold™ (high pH), Fluoro-Gold™ (low pH), Fluoro-Jade, FM® 1-43, Fura-2
 (high calcium), Fura-2 / BCECF, Fura Red™ (high calcium), Fura Red™ / Fluo-3,
 GeneBLAzer™ (CCF2), GFP Red Shifted (rsGFP), GFP Wild Type, UV Excitation,
 GFP Wild Type, non-UV Excitation, GFP / BFP FRET, GFP / DsRed FRET, Hoechst
 15 33342 & 33258, 7-Hydroxy-4-methylcoumarin (pH 9), 1,5 IAEDANS, Indo-1 (high
 calcium), Indo-1 (low calcium), Indodicarbocyanine, Indotricarbocyanine, JC-1, 6-JOE,
 JOJO™-1 / JO-PRO™-1, LDS 751 (+DNA), LDS 751 (+RNA), LOLO™-1 / LO-
 PRO™-1, Lucifer Yellow, LysoSensor™ Blue (pH 5), LysoSensor™ Green (pH 5),
 LysoSensor™ Yellow/Blue (pH 4.2), LysoTracker® Green, LysoTracker® Red,
 20 LysoTracker® Yellow, Mag-Fura-2, Mag-Indo-1, Magnesium Green™, Marina Blue®,
 4-Methylumbelliferone, Mithramycin, MitoTracker® Green, MitoTracker® Orange,
 MitoTracker® Red, NBD (amine), Nile Red, Oregon Green® 488, Oregon Green® 500,
 Oregon Green® 514, Pacific Blue, PBF1, PE (R-phycoerythrin), PE-Cy5, PE-Cy7, PE-
 Texas Red, PerCP (Peridinin chlorophyll protein), PerCP-Cy5.5 (TruRed), PharRed
 25 (APC-Cy7), C-phycoerythrin, R-phycoerythrin (PE), PI (Propidium
 Iodide), PKH26, PKH67, POPO™-1 / PO-PRO™-1, POPO™-3 / PO-PRO™-3,
 Propidium Iodide (PI), PyMPO, Pyrene, Pyronin Y, Quantam Red (PE-Cy5),
 Quinacrine Mustard, R670 (PE-Cy5), Red 613 (PE-Texas Red), Red Fluorescent
 Protein (DsRed), Resorufin, RH 414, Rhod-2, Rhodamine B, Rhodamine Green™,
 30 Rhodamine Red™, Rhodamine Phalloidin, Rhodamine 110, Rhodamine 123, 5-ROX
 (carboxy-X-rhodamine), S65A, S65C, S65L, S65T, SBFI, SITS,

SNAFL®-1 (high pH), SNAFL®-2, SNARF®-1 (high pH), SNARF®-1 (low pH), Sodium Green™, SpectrumAqua®, SpectrumGreen® #1, SpectrumGreen® #2, SpectrumOrange®, SpectrumRed®, SYTO® 11, SYTO® 13, SYTO® 17, SYTO® 45, SYTOX® Blue, SYTOX® Green, SYTOX® Orange, 5-TAMRA (5-Carboxytetramethylrhodamine), Tetramethylrhodamine (TRITC), Texas Red® / Texas Red®-X, Thiadicarbocyanine, Thiazole Orange, TOTO®-1 / TO-PRO®-1, TOTO®-3 / TO-PRO®-3, TO-PRO®-5, Tri-color (PE-Cy5), TRITC (Tetramethylrhodamine), TruRed (PerCP-Cy5.5), WW 781, X-Rhodamine (XRITC), Y66F, Y66H, Y66W, YFP (Yellow Fluorescent Protein), YOYO®-1 / YO-PRO®-1, and YOYO®-3 / YO-PRO®-3.

Attaching a quencher or a labelling entity to a labelling moiety

If a labelling moiety is a molecule to which a labelling entity or a quencher may be attached, the methods of the invention may comprise a step of attaching a labelling entity or a quencher to the at least one labelling moiety.

The molecule to which a labelling entity or a quencher may be attached can be a first binding moiety. In such cases, the method comprises attaching a ddNTP conjugated to a first binding moiety to a labelling entity or a quencher. For example, the method may comprise exposing the ddNTP conjugated to the first binding moiety to a labelling entity-second binding moiety conjugate or to a quencher-second binding moiety conjugate, wherein the first binding moiety and the second binding moiety have affinity for one another. Optionally the step of exposing the ddNTP conjugated to the first binding moiety to a labelling entity-second binding moiety conjugate or to a quencher-second binding moiety conjugate takes place under conditions suitable for the first binding moiety and the second binding moiety to associate with one another.

For example the labelling moiety may be biotin, and, if this is the case, a labelling entity or quencher may be attached to the labelling moiety by exposing a ddNTP-biotin conjugate to an avidin/streptavidin-labelling entity conjugate. Alternatively, the labelling moiety may be avidin or streptavidin and a labelling entity may be attached to the labelling moiety by exposing a ddNTP-avidin/streptavidin conjugate to a biotin-labelling entity conjugate.

If the method comprises a step of attaching a labelling entity or a quencher to the labelling moiety, the step preferably takes place before the step of hybridising the ddNTP-labelling oligonucleotide probes to the at least one detection oligonucleotide probe.

5

Use of Detection Oligonucleotide Probes

Methods of the invention may comprise a step of hybridising the ddNTP-labelling oligonucleotide probes to at least one detection oligonucleotide probe to form
10 a reaction mixture comprising at least one hybridised ddNTP-labelling oligonucleotide probe. A kit of the invention may comprise at least one detection oligonucleotide probe.

The at least one detection oligonucleotide probe is an oligonucleotide that is complementary to at least one of the ddNTP-labelling oligonucleotide probes. Thus, if
15 it is desired to detect the presence, absence or quantity of three different target DNA sequences, three different labelling oligonucleotide probes will be used and will detect the presence of these three different labelling oligonucleotide probes using three different detection oligonucleotide probes. Each of the three different labelling oligonucleotide probes will hybridise to one of the three different detection
20 oligonucleotide probes. Thus the same number of detection oligonucleotide probes as labelling oligonucleotide probes can be used, and each of the detection oligonucleotide probes will hybridise with a different labelling oligonucleotide probe.

Preferably the at least one detection oligonucleotide probe hybridises to at least one of the labelling oligonucleotide probes under a moderately stringent hybridisation
25 condition. Optionally the at least one detection oligonucleotide probe hybridises to at least one of the at least one labelling oligonucleotide probes under a stringent hybridisation condition.

Hybridising a ddNTP-labelling oligonucleotide probe to a detection oligonucleotide probe.

Methods of the invention may comprise a step of hybridising the ddNTP-
5 labelling oligonucleotide probes to at least one detection oligonucleotide probe to form a reaction mixture comprising at least one hybridised ddNTP-labelling oligonucleotide probe.

In the context of the invention, the term “hybridising” ddNTP-labelling oligonucleotide probes to detection oligonucleotide probes, is intended to refer to
10 exposing the ddNTP-labelling oligonucleotide probe to a detection oligonucleotide probe under conditions in which hybridisation of the ddNTP-labelling oligonucleotide probe to the detection oligonucleotide probe can occur. For example, conditions suitable for hybridisation to occur include cool temperatures and high salt conditions. Preferably the ddNTP-labelling oligonucleotide probe and the detection oligonucleotide
15 probe are mixed together and exposed to temperatures between 40°C and 70°C or between 50°C and 65°C for at least 10 seconds (for example at least 20 seconds, at least 30 seconds, between 10 seconds and 5 minutes, between 20 seconds and 2 minutes, or between 20 seconds and 1 minute).

The different detection oligonucleotide probes may have different melting
20 temperatures. Optionally the different detection oligonucleotide probes have at least 2, at least 4, at least 6, at least 8 or between 2 and 8 different melting temperatures. In such methods when the different detection oligonucleotides probes are hybridised to a ddNTP-labelling-oligonucleotide probe, the temperature at which the hybridised ddNTP-labelling-oligonucleotide probe melts will be different for one detection
25 oligonucleotide probe compared to another detection oligonucleotide probe. As will be discussed below, this can help with determining the amount of the at least one hybridised ddNTP-labelling oligonucleotide probe.

The method may further comprise a step of purifying the at least one hybridised ddNTP-labelling oligonucleotide probe. This step is preferably after the step of
30 hybridising the ddNTP-labelling oligonucleotide probe and before the step of measuring the amount of the at least one hybridised ddNTP-labelling oligonucleotide probe. Such a purification step will remove some of the excess detection oligonucleotide probe and

will ensure that the fluorescence signal that is detected will be more accurate. The purification step is optionally performed by affinity chromatography.

The method may comprise a step of melting the ddNTP-labelling oligonucleotide probes to form single stranded oligonucleotides. This melting step is preferably performed before the step of hybridising the ddNTP-labelling oligonucleotide probes and may comprise heating the ddNTP-labelling oligonucleotide probes to between 70°C and 120°C or around 95°C.

Purification of Probes

10

In any of the methods of the invention which involve a labelling oligonucleotide probe, the methods may comprise a labelling oligonucleotide probe purification step which is performed before the step of measuring the amount of the at least one ddNTP-labelling oligonucleotide probe. Such a method may comprise providing a labelling oligonucleotide probe with a binding moiety.

For the purposes of the present invention, a “binding moiety” is a generic term which may be used to describe a binding entity which may be attached to a probe or a molecule or conjugate which may be attached to a probe and which may act to tether a binding entity to the probe.

For the purposes of the present invention, a “binding entity” may be any compound/molecule or group of compounds/molecules which can be attached to a probe or tethered to a probe and which can bind to another compound/molecule or group of compounds/molecules to facilitate purification of the probe. A binding entity will naturally bind to another molecule when brought into close proximity. For example, binding entities can include biotin, avidin, streptavidin, digoxigenin and anti-digoxigenin.

A molecule or conjugate which may be attached to a probe and which may act to tether a binding entity to the probe may be any suitable molecule or conjugate.

A binding entity will bind to a “capture entity” to facilitate purification of the probe. For the purposes of the present invention, a “capture entity” is any entity which will bind to a binding entity to facilitate purification of the probe.

A binding entity/capture entity may be two or more molecules or a pair of molecules which form an affinity interaction. For example, the binding entity may be biotin which is attached to or tethered to the probe and the capture entity may be streptavidin attached to a substrate. When contacted together biotin will bind to
5 streptavidin thus tethering the probe to the substrate. Probes which are not tethered to the substrate can then be washed away. After a wash step an elution step may be performed to elute the tethered probes from the subbstrate.

Thus a binding entity and capture entity may be two or more molecules which can form an affinity interaction such as biotin and streptavidin so as to facilitate
10 purification of the probe. Other examples include an antibody and an antigen to which the antibody binds.

As will be described further herein, one way in which a binding entity such as biotin may be tethered to a probe is by attaching the binding entity to a ddNTP and incorporating the ddNTP into the probe via a single nucleotide extension reaction. In
15 this situation a ddNTP may itself act as a binding moiety which tethers the binding entity to the probe.

For example, the methods may involve the incorporation into a labelling oligonucleotide probe of a ddNTP if the labelling oligonucleotide probe is hybridised to a target DNA sequence. Such methods may further comprise providing the ddNTP with
20 a binding entity and wherein the binding entity allows the labelling oligonucleotide probe to be purified.

Such a method may comprise providing the ddNTP with a binding entity and wherein a labelling oligonucleotide probe purification step is performed before the step of measuring the amount of the labelling oligonucleotide probe, the purification step
25 comprising:

- (i) optionally, dissociating the at least one ddNTP-labelling oligonucleotide probe of step (b) from the amplification product;
- (ii) immobilizing the at least one ddNTP-labelling oligonucleotide probe on a substrate by allowing the binding entity to bind to a capture entity, wherein the
30 capture entity is coupled to the substrate; and
- (iii) washing the substrate. Such a method may further optionally comprise a dissociation step (step iv), wherein after step (iii) the dissociation step (step iv) is

performed comprising dissociating the binding entity from the capture entity to release the at least one ddNTP-labelling oligonucleotide probe from the substrate.

In one embodiment the methods may comprise methods wherein the labelling oligonucleotide probe for detection of amplicons generated from the DNA of a given target microorganism differ in length from the labelling oligonucleotide probes for detection of amplicons generated from the DNA of every other target microorganism in the pre-defined set, and wherein a step of measuring the amount of the labelling oligonucleotide probe comprises performing the dissociation step (step iv) and separating all labelling oligonucleotide probes for the pre-defined set of target microorganisms on the basis of their length before the step of quantifying a signal generated via the labelling entity, optionally wherein separation is performed by electrophoresis, *e.g.* capillary electrophoresis.

In embodiments involving detection oligonucleotide probes, the detection oligonucleotide probe for detection of amplicons generated from the DNA of a given target microorganism may differ in length from the detection oligonucleotide probes for detection of amplicons generated from the DNA of every other target microorganism in the pre-defined set, and wherein the step of measuring the amount of the at least one ddNTP-labelling oligonucleotide probe comprises separating all detection oligonucleotide probes for the pre-defined set of target microorganisms on the basis of their length before the step of quantifying a signal generated via the labelling entity, optionally wherein separation is performed by electrophoresis, *e.g.* capillary electrophoresis.

Use of Fluorophores to Label Labelling Oligonucleotide Probes and Detection

Oligonucleotide Probes

Since more than one labelling oligonucleotide probe or detection oligonucleotide probe will be used, more than one fluorophore may be used. Optionally at least 2, at least 4, at least 6, at least 8, at least 10, or between 2 and 20 different fluorophores may be used.

Each of the different labelling oligonucleotide probes or detection oligonucleotide probes may be conjugated to a different fluorophore. Alternatively,

more than one of the labelling oligonucleotide probes or detection oligonucleotide probes may be conjugated to the same fluorophore. For example, if five different detection oligonucleotide probes are used, each of the five different detection oligonucleotide probes may be conjugated to a different fluorophore, or two of the
5 detection oligonucleotide probes could be conjugated to the same fluorophore and three of the detection oligonucleotide probes could be conjugated to a different fluorophore.

If the sample is separated into more than one aliquot the same fluorophores may be used in different aliquots. For example, 5 different detection oligonucleotide probes conjugated to 5 different fluorophores may be used in a first aliquot and those same 5
10 fluorophores may be conjugated to 5 different detection oligonucleotide probes used in a second separate aliquot.

A fluorophore may be attached to either the 5' end or 3' end of a detection oligonucleotide probe or labelling oligonucleotide probe. Preferably, a labelling moiety is at the 3' end of a labelling oligonucleotide probe, and a fluorophore is attached to the
15 5' end of a detection oligonucleotide probe. However, a fluorophore could also be attached to the 3' end or somewhere along the length of the detection oligonucleotide probe or labelling oligonucleotide probe, provided that the distance from the labelling moiety to the fluorophore is such that quenching occurs when the two probes are hybridised, typically 10-100 Å. The at least one detection oligonucleotide probe may
20 optionally be shortened by 1 – 5, at least 2 – 3 nucleotides at the 5' end, in order for the fluorophore not to be too close to the labelling moiety, to avoid any steric hinderance.

Measuring the Amount of the at Least one Hybridised ddNTP-Labelling Oligonucleotide Probe

25

The methods comprise a step of measuring the amount of the at least one hybridised ddNTP-labelling oligonucleotide probe. As discussed above, ddNTPs will only be added to labelling oligonucleotide probes that successfully hybridised with a target DNA sequence. Thus, the amount of one of the different at least one hybridised
30 ddNTP-labelling oligonucleotide probes will be proportional to the amount of the target DNA sequence that was complementary to that at least one labelling oligonucleotide probe in the sample.

Each of the different detection oligonucleotide probes is conjugated to a detection moiety, for example a fluorophore. As discussed above, the ddNTP-labelling oligonucleotide probe can be conjugated to a quencher. Thus, when the ddNTP-labelling oligonucleotide probe is hybridised to the detection oligonucleotide probe the fluorescence from a fluorophore will be quenched. However, if the hybridised ddNTP-labelling oligonucleotide probes are melted, fluorescence from the fluorophore can be detected. Thus, the difference in fluorescence before and after a step of melting the hybridised ddNTP-labelling oligonucleotide probe provides the user with information on the amount of labelling oligonucleotide probe comprising a fluorophore that is present in the sample.

Preferably the user uses several detection oligonucleotide probes and these are unique in that they each have a different fluorophore or a different melting temperature compared to other detection oligonucleotide probes present, *i.e.* each detection oligonucleotide probe has a different combination of melting temperature and fluorophore to each other detection oligonucleotide. For example:

Probe 1	Melting temperature 1	Fluorophore 1
Probe 2	Melting temperature 1	Fluorophore 2
Probe 3	Melting temperature 2	Fluorophore 1

This allows the user to detect each different detection oligonucleotide probe and thereby determine the amount of the at least one hybridised ddNTP-labelling oligonucleotide probe that is present. For example, if three different detection oligonucleotide probes are present and two of these have the same melting temperature and the third has a different melting temperature, the user could firstly apply the first melting temperature and thereby melt two of the detection oligonucleotide probes. Assuming that the two detection oligonucleotide probes have fluorophores and melting temperatures to detect a wide variety of detection oligonucleotides and therefore target DNA sequences. This is typically done in a qPCR instrument that can measure and distinguish different fluorophores in a temperature-gradient, thus obtaining specific peaks of the different fluorophores at different time-points during the temperature-gradient.

If more than one fluorophore is used, that fluoresce at different wavelengths, the user could detect both of these two detection oligonucleotide probes simultaneously using a qPCR instrument. The user could then apply the second melting temperature and detect the third detection oligonucleotide probe. This third detection oligonucleotide
5 probe could have the same fluorophore as one of the other two detection oligonucleotide probes as it is detected at a separate time. The user may, therefore, use combinations of these should fluoresce at different wavelengths so that the user can establish the amount of each fluorophore that is present. Since the amount of ddNTP-labelling oligonucleotide probe that is present is proportional to the amount of at least one target
10 DNA sequence, this can be used to detect the presence, absence or relative amount of the at least one target DNA sequence. Optionally the fluorescence may be compared to reference levels. A suitable reference is a sample containing a known amount of a fluorophore.

Thus, steps of measuring the amount of the at least one hybridised ddNTP-labelling oligonucleotide probe can be carried out by:

- (i) measuring the fluorescence signal from the fluorophore;
- (ii) altering the conditions of the reaction mixture comprising the at least one hybridised ddNTP-labelling oligonucleotide probe to favour melting of the at least one hybridised ddNTP-labelling oligonucleotide probe;
- 20 (iii) measuring the fluorescence signal from the fluorophore; and
- (iv) determining the amount of the least one hybridised ddNTP-labelling oligonucleotide probe based on the difference between the fluorescence signal measured in (i) and the fluorescence signal measured in (iii).

Steps (i) and (iii) may be performed in a standard qPCT machine. Alternatively,
25 the fluorescence signal is detected by flow cytometry or spectrofluorometry.

By “altering the conditions of the reaction mixture” is meant that the user changes any condition which leads to melting of the hybridised ddNTP-labelling oligonucleotide probes. Suitable conditions include increasing the temperature of the reaction mixture or increase the salt content. Preferably the step of “altering the
30 conditions of the reaction mixture” comprises increasing the temperature of the reaction mixture, for example the temperature may be increased by between 2°C and 120°C, between 20°C and 100°C, between 50°C and 100°C or around 60°C.

Sequencing of DNA

Current technologies for analysing the bacteria in a bacterial culture are Single
5 Nucleotide Extension, Microarrays and Next Generation DNA Sequencing (NGS).
Single Nucleotide Extension has been used to identify larger groups of bacteria, at the
family, class and phylum level (Casen *et al.* (2015) *Aliment Pharmacol Ther.*
Jul;42(1):71-83). This method can detect imbalances in the gut microbiota (dysbiosis).
However, the method fails to give detailed information about the composition of the
10 bacterial culture at the genus and species level.

The use of DNA and RNA microarrays with overlapping sequences have also
been used to detect various bacteria in culture (Rajilić-Stojanović *et al.* (2009), *Environ*
Microbiol. Jul; 11(7): 1736–1751). The method uses Agilent Technologies microarrays,
and involves both labour-some and error-prone RNA technology, as well as high cost
15 microarrays.

Next generation sequencing (NGS) is used to describe a number of different
modern DNA sequencing technologies, made available on different hardware and
through different companies like Illumina (for example MiSeq, HiSeq and NextSeq)
sequencing, Roche 454 sequencing, Ion Torrent PGM sequencing, SOLiD sequencing,
20 PacMan and Nanopore to name a few.

NGS can be used to sequence DNA actually present in the sample, or DNA
amplified from DNA actually present in the sample so as to determine whether DNA
from a given target microorganism of the set is actually present in the sample.

In general, NGS is the preferred way of analysing the gut microbiome since it
25 has the potential to provide the most complete information about the microbiome.
Currently, there are generally two different approaches to analysing the gut microbiome
using NGS technology; Amplicon sequencing, typically with basis in the 16S rRNA
gene (16S Amplicon sequencing) or Whole Genome or Shotgun sequencing (WGS). In
16S Amplicon sequencing, a portion of the 16S rRNA gene is sequenced, and all the
30 different sequences that emerge are matched against a database of sequences of known
bacterial sequences. However, the resolution that can be obtained from 16S Amplicon
sequencing only goes to family and genus level, and it is very seldom possible to

accurately assign sequences to specific species and strains of bacteria. In addition, several publications have reported that 16S Amplicon sequencing result in a high number of false positives (*i.e.* identification of bacteria in a sample that does not contain those bacteria) (Fouhey *et al.* (2016), BMC Microbiol 16:123 and Jovel *et al.* (2016),
5 Front. Microbiol 7:459).

In spite of this, 16S Amplicon sequencing is the most commonly used method, since it is faster and cheaper than WGS. However, for a better understanding of the contribution of the individual gut bacteria on health and disease, it is imperative to be able to accurately detect and quantify bacteria at the species and strain level.

10 Using massively parallel sequencing technologies results in huge amount of data that is generated for each run. In order to detect rare bacteria at species and strain level, a high number of reads is required.

Details concerning processes, programs, data handling and processing can be found in Jovel *et al.* (2016, Front. Microbiol 7:459). In addition, targeted NGS
15 solutions have been described to target specific genes in the human genome, in order to use NGS to detect specific mutations leading to disease (Mertes *et al.* (2011) Breif Func Genom Vol 10, No 6, 374-386). This result in faster and more accurate detection of the mutations, and is a useful diagnostic tool for various cancer types. These techniques can be adapted for the present purposes.

20 For example, the most relevant equipment are: Illumina MiSeq, HiSeq, MiniSeq and NextSeq (see <https://www.illumina.com/>).

Amplicon sequencing can for example be performed according to the Illumina MiSeq protocol “*Overview of tailed amplicon sequencing approach with MiSeq*” (<http://www.Illumina.com>). This protocol provides a two-step PCR method utilising
25 sequence-specific primers and a specific DNA index kit. Primers are designed according to low diversity amplicon specifications. Adapter overhang sequences are added to the 5' end of both the forward and reverse primers. These 5'-primer regions are complementary to index sequences and thus permit the addition to the template of a unique sample index (barcode). The 5'-primer regions also allow the addition of
30 adapters to make the template compatible for hybridisation to the MiSeq flow cell.

Other sequencing methods are available. For example, base-specific release of hydrogen ions, which occurs during the incorporation process, can be detected in the

context of microwell systems (*e.g.* see the Ion Torrent system available from Thermo Fisher/Life Technologies using machines such as Ion S5, PGM or Ion Chef; <http://www.lifetechnologies.com/>). Similarly, in pyrosequencing the base-specific release of pyrophosphate (PPi) is detected and analysed. In nanopore technologies, DNA molecules are passed through or positioned next to nanopores, and the identities of individual bases are determined following movement of the DNA molecule relative to the nanopore. Systems of this type are available commercially *e.g.* from Oxford Nanopore (<https://www.nanoporetech.com/>). In an alternative method, a DNA polymerase enzyme is confined in a “zero-mode waveguide” and the identity of incorporated bases are determined with fluorescence detection of gamma-labeled phosphonucleotides (see *e.g.* Pacific Biosciences; <http://www.pacificbiosciences.com/>).

NGS techniques which can be used to implement methods of the present invention may be facilitated by various techniques known in the art. For example, a variety of techniques have been developed for the targeted enrichment of genomic regions of interest for the purposes of subsequently performing next generation sequencing (see for example Mertes *et al.* (2011, Briefings in Functional Genomics 10(6), 374-386) for a review of some specific techniques which may be used to implement the methods of the invention).

The current invention thus uses a targeted approach to microbiome sequencing. The invention for the first time makes it possible to obtain detailed information about the microbiome down to species and strain level, while reducing costs, time and complexity associated with sequencing and data analysis. This makes it possible to conduct larger studies, involving more subjects and more time-points compared to what is practically possible with current technologies. This will lead to increased understanding of the function of a given microbiome, and help towards the provision of better therapeutics and treatment options.

Since each amplicon is defined by a group of related pre-defined target microorganisms, the analysis of amplicons is greatly simplified, as only the known target DNA sequences of the pre-defined target microorganisms within that amplicon need to be explored. This eliminates the need for the extensive post-processing of data typically associated with NGS, and leads to faster and more accurate results.

Quantification of Target Microorganisms in a Sample Via Sequence Reads

As well as providing information as to the presence or absence of a target microorganism in a sample, the present methods also allow quantification of a target
5 microorganism in a sample.

When using DNA sequence conserved regions as a means to amplify all members of a group of microorganisms in a sample the relative quantity of each amplicon will provide a measure of the total amount of microorganisms in that particular group of microorganisms.

10 The number of sequence reads within each amplicon with sequence identity to the target microorganism will provide a measure of the abundance of each target microorganism relative to the other target microorganisms in that group, and it will provide a measure the abundance of each target microorganism relative to the other target microorganisms in the set of target microorganisms, *i.e.* in the sample as a whole.

15

A method for Analysis of a Plurality of Microorganisms in a Sample

The present methods allow a user to rapidly and accurately detect microorganisms in a sample. The methods involve detecting whether target DNA
20 sequences are present, absent and at what relative amount in a sample. As discussed above, detecting whether target DNA sequences are present, absent and at what relative amount in a sample can provide the user with information on whether that certain microorganism is present in the sample, and therefore allows the user to analyse the microorganisms present in the sample. Accordingly, the invention provides methods for
25 analysis of a plurality of microorganisms in a sample.

The microorganisms that are analysed may be any suitable microorganism. For example, the microorganisms may comprise bacteria, viruses, fungi or a combination of all three. Optionally the plurality of microorganisms is a collection of bacteria or yeast. Preferably the plurality of microorganisms is a collection of bacteria. Optionally the
30 method is a method for analysing a microbiome. In the content of the invention, the term “microbiome” is intended to refer to the genetic material of microorganisms present in a particular environment. For example, a microbiome of the invention may be

genetic material from microorganisms present in the gut of an animal, the microorganisms present in a source of water or the microorganisms present in the soil of a particular area.

The sample may be sourced from an animal, water, air, food, forensic sites, buildings or biofilms. For example, a sample may be a sample from an animal, for example a mammal. Preferably the sample is from a human, cat, dog, horse, cow, mouse, rat, guinea pig, zebrafish or bee. Optionally the sample is from a human.

If the sample is a sample from an animal, the sample may be a faecal, gut mucosal, skin, vaginal excretion, mouth, spit or toe sample from the animal. Preferably the sample is from the gut of an animal.

If the sample that is analysed is a sample from the gut of a human, the method can be used to analyse the gut microflora and to detect imbalances in the gut microflora. If the method is used to detect imbalances in the gut microflora it may comprise an additional step of determining a treatment that may be used to correct the gut microflora imbalance, for example the administration of a probiotic, prebiotic, specific diet or drug.

Preferably the method further comprises a step of purifying the sample, *i.e.*, if the sample is derived from the gut of a human animal it may comprise faecal matter that has been purified to enrich for DNA. Preferably the step of purifying the sample is before the step of amplifying target DNA sequences. Any method known in the art for purifying total genomic DNA can be used. Suitable purification methods may comprise addition of a detergent or surfactant to lyse any cells in the sample, addition of a protease and/or an RNase to break down proteins and/or RNA, centrifugation of the sample to remove cell debris or isolation of DNA using minicolumn purification. For example, the sample could be exposed to a detergent such as polysorbate 80 or sodium dodecylsulphate, the sample could be centrifuged and the supernatant applied to a column comprising glass beads in the presence of a chaotropic agent such as sodium iodide or sodium perchlorate.

Optionally, the method further comprises a step of measuring the amount of total genomic DNA, for example bacterial DNA, in the sample. This step can take place before the step of amplifying target DNA sequences. Optionally the step of measuring the total amount of genomic bacterial DNA in the sample is performed after a step of purifying the sample. The amount of total genomic bacterial DNA may be measured

using any conventional means. For example, the amount of total genomic bacterial DNA may be measured using a spectrophotometer. For example, the absorbance at 260 nm can provide a measure of total genomic bacterial DNA. Alternatively, other suitable methods includes quantitative PCR methods such as using the Fento™ bacterial DNA quantification kit (Zymo Research). The amount of total genomic DNA may be compared to a reference, for example a sample having a known amount of DNA present.

One or More Aliquots From the Sample

10

The sample may be divided into one or more aliquots. In such embodiments, the method may comprise a step of preparing one or more aliquots from the sample. In the context of the present invention, an “aliquot” is a portion of the sample. It is possible to use any number of aliquots in the method of the invention. Preferably around 4 aliquots are used. Suitably, the total number of aliquots is between 2 and 50, between 2 and 25, between 2 and 10 or around 4.

Separating the sample into multiple aliquots allows the user to detect a greater variety of target sequences in the sample. The method of the invention involves detecting the presence, absence, or relative amount of target DNA sequences. This step may involve hybridising the target DNA sequence to a labelling oligonucleotide probe. Several oligonucleotide probes may be used at the same time and, for example, their binding to a target DNA sequence could be measured using a multiplex fluorescence detection system. However, there is an upper limit on the number of oligonucleotide probes that can be used in the same aliquot. For example, there is a limit of the number of different flourophores which can be used in a multiplex fluorescence detection system. In addition, when different probes are present in the same aliquot, they may interact with each other, for example by binding to one another or to the same target sequence (albeit with different affinity), and this reduces the accuracy of the assay. By separating the sample into more than one aliquot, a smaller number of probes may be used in each aliquot, and this reduces unwanted interactions.

Optionally, a first ddNTP selected from the group consisting of ddC, ddT, ddA, ddG, ddI and ddU is used in some of the one or more aliquots,

a second ddNTP selected from the group consisting of ddC, ddT, ddA, ddG, ddI and ddU is used in some of the one or more aliquots, and

the first ddNTP and the second ddNTP are not the same ddNTP.

Optionally, a first ddNTP selected from the group consisting of ddC, ddT, ddA, 5 ddG, ddI and ddU is used in some of the one or more aliquots,

a second ddNTP selected from the group consisting of ddC, ddT, ddA, ddG, ddI and ddU is used in some of the one or more aliquots,

a third ddNTP selected from the group consisting of ddC, ddT, ddA, ddG, ddI and ddU is used in some of the one or more aliquots, and

10 the first ddNTP is not the same as the second ddNTP or the third ddNTP and the second ddNTP is not the same as the third ddNTP.

Optionally, a first ddNTP selected from the group consisting of ddC, ddT, ddA, ddG, ddI and ddU is used in some of the one or more aliquots,

a second ddNTP selected from the group consisting of ddC, ddT, ddA, ddG, ddI 15 and ddU is used in some of the one or more aliquots,

a third ddNTP selected from the group consisting of ddC, ddT, ddA, ddG, ddI and ddU is used in some of the one or more aliquots,

a fourth ddNTP selected from the group consisting of ddC, ddT, ddA, ddG, ddI and ddU is used in some of the one or more aliquots and

20 the first ddNTP is not the same as the second ddNTP, the third ddNTP or the fourth ddNTP, the second ddNTP is not the same as the third ddNTP or the fourth ddNTP and the third ddNTP is not the same as the fourth ddNTP.

By generating the same amplicon in different aliquots, it is possible to use labelling oligonucleotide probes in these different aliquots that otherwise would 25 interfere with each other in unwanted ways in the same aliquot. In addition, by using the same labelling oligonucleotide probe with different amplicons in different aliquots, the same labelling oligonucleotide probe could be used to detect different target bacteria, without detecting false positives.

Alternative Detection Platforms

In principle any suitable detection platform may be used to implement the core amplicon-based approaches described herein, and as such the methods are not intended
5 to be limited to probe-based approaches involving single nucleotide extension and DNA sequencing as described herein.

Alternative detection platforms envisaged include, for example, bead-based technologies and variants thereof.

One particular platform may utilise specific bead comprising *e.g.* polystyrene
10 microspheres/beads or paramagnetic microspheres/beads. Such beads possess internal dyes *e.g.* comprising red and infrared fluorophores of differing intensities, or beads may possess different distinguishable shapes. Each bead may be provided with a unique number/barcode, colour or other distinguishing feature, allowing one type of bead to be differentiated from another. Detection oligonucleotide probes may be applied to the
15 surfaces of such beads, wherein the detection oligonucleotide probes are complementary to each labelling oligonucleotide probe which has incorporated a labelling entity, such as a fluorophore, during the single nucleotide extension reaction. Detection instruments may be provided that are capable of detecting *e.g.* a specific coloured bead plus the hybridised labelling oligonucleotide probe-specific fluorophore, or *e.g.* the
20 specific shape of the bead plus the labelling oligonucleotide probe-specific fluorophore.

Techniques offered by *e.g.* Luminex and Applied Biocode may readily be adapted to the current techniques
(<https://www.thermofisher.com/uk/en/home/references/protein-analysis-guide/multiplex-assays-luminex-assays/how-luminex-technology-works.html>;
25 <http://www.apbiocode.com/>; see also Figure 2).

Kits

The present invention is directed towards kits suitable for use in any of the
30 methods described above.

For example, the present invention provides a kit that is adapted for use in any of the methods described above.

The present invention also provides a kit comprising:

- (i) at least one labelling oligonucleotide probe;
- (ii) at least one detection oligonucleotide probe;
- (iii) a ddNTP conjugated to at least one labelling moiety; and
- 5 (iv) primers;

wherein the at least one labelling oligonucleotide probe is complementary to a target DNA sequence and the at least one labelling oligonucleotide probe is complementary to the at least one detection oligonucleotide probe.

The kits comprise primers. The kit may be used to detect at least 3 target DNA
10 sequences from a genomic region. In this case, the primers are complementary to conserved regions of the genomic region of interest. Optionally primers complementary to conserved regions of more than one genomic region of interest are included.

Optionally the kit further comprises a manual containing instructions for performing a method of the invention. Optionally the kit further comprises components
15 that can be used to purify a reaction mixture, such as an affinity chromatography column.

Suitably the kit further comprises at least one buffer. For example, the kit may comprise buffer components that can be used at different stages of the methods of the invention. Suitable buffers include TAPS
20 (Tris(hydroxymethyl)methylaminopropanesulphonic acid) buffer, bicine buffer, tris buffer, tricine buffer, TAPSO (3-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino-2-hydroxypropane-1-sulphonic acid) buffer, HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulphonic acid) buffer, TES (2-[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulphonic acid) buffer, PIPES (1,4-
25 piperazinediethanesulphonic acid) buffer, cacodylate buffer and MES (2-morpholin-4-ylkethanesulphonic acid) buffer. Preferably the buffer is a Tris-EDTA or Tris buffer.

Optionally the kit comprises enzymes. As will be described in more detail, the methods of the invention comprise steps which may use enzymes and any of these enzymes may be included in a kit of the invention. For example the kit of the invention
30 may comprise a DNA polymerase such as Taq polymerase, two polymerases, Exonuclease I, proteinase K and/or Shrimp Alkaline Phosphatase.

EXAMPLES

The following Examples are provided to illustrate the invention but not to limit the invention.

5

Example 1: Defining Genomic Sequence Regions For Use in Microorganism Identification Assays.

10 Example outline methodology for defining genomic sequence regions for use in microorganism identification assays is described below.

Current estimates of the numbers of bacteria in the gut are between approximately 200 to approximately 400 different bacteria (Faith *et al.*, Science 341, 1237439 (2013), but there may be more or less. Furthermore, the number of bacteria with known functions and known variations between people with different health conditions could vary between approximately 50 to approximately 500 or more. Typically it is likely to vary between approximately 100 and approximately 150. Nevertheless, in any approach for studying a given environment, it will be beneficial to pre-define the exact microorganisms which are candidate target microorganisms whose presence or absence is to be established in a given assay. Establishing pre-defined candidate microorganisms for a given environment can be undertaken by the following non-limiting example methodologies.

Microorganisms, typically bacteria, can be divided into groups dependent on their sequence similarities. For example, it is difficult to distinguish the different Bifidobacteria that are present in the human gut just based on their 16S rRNA sequence. Bifidobacteria are important for good health. Ingestion of Bifidobacteria compositions, and other mechanisms of promoting Bifidobacteria in gut flora, have been suggested as a probiotic treatment for many different diseases, such as Irritable Bowel Syndrome (IBS), Ulcerative Colitis (UC) and Necrotizing Enterocolitis (NEC) (Tojo *et al.* (2014) Nov 7, 20(41): 15163-15176). Correct detection of the different bacteria can be helpful in optimizing diets and treatments options, and for monitoring the effect of treatments.

Likewise other bacteria, such as Lactobacillus, Clostridia and Enterobacteria may also be difficult to detect down to species and strain level just based on 16S rRNA

sequence. However it has been established herein that these and other groups of bacteria have distinct features, and therefore also unique gene sequences, which can be utilized for the purposes of detection in microbiome analysis.

As discussed above, the present methods involve determining the presence,
5 absence or quantity of target DNA sequences from a sample which can uniquely identify a target microorganism and distinguish that target microorganism from all other target microorganisms in a pre-defined set of target microorganisms for the sample.

The group of microorganisms may be, for example, a microorganism genus and the target microorganism may be, for example, a microorganism species or strain within
10 the genus.

The methods involve pre-defining a set of target microorganisms to be detected, wherein the set of target microorganisms comprises groups of related target microorganisms. The methods further involve pre-defining first and second conserved regions of DNA sequence which are conserved across members of a specific group of
15 related microorganisms, which are unique to the group of related microorganisms, and which can give rise to an amplicon specific for that group. In pre-defining the first and second conserved regions of DNA sequence target DNA sequence regions are pre-defined, wherein the target DNA sequence regions are located between the first and second conserved regions and wherein each target DNA sequence region comprises
20 sequences which are unique for any given species or strain of a group, within the group-specific amplicon.

In an example method for pre-defining candidate microorganisms for a given environment, sequence regions within a group of bacteria (such as Bifidobacteria) can be identified that are characteristic of that group. These regions can be used as a basis
25 for applying methods, such as those described and defined herein, to detect and quantify all bacterial species and strains within that group in any test assay.

An example method for pre-defining a set of candidate microorganisms in an environment, *e.g.* bacteria from the gut, may consist of the steps outlined below. Pre-defining a set of candidate microorganisms from other environments of interest can be
30 achieved using the same principles.

1. Obtain whole genome bacterial sequences for all bacteria expected to be present in the human gut. This can be obtained for example from <https://www.ncbi.nlm.nih.gov/genome/browse/>.
2. Clean up the sequences. This may be performed as a two step approach as follows.
 - i. Create a consensus genome sequence, if there are several genome sequences for the same bacteria. Identify and mark areas of differing sequences between the different genome sequences of the same bacteria.
 - ii. Mark or delete any non-identifiable sequences (which can be designated as “N” in a sequence listing).
3. Search through the entire library of cleaned up bacterial genome sequences for regions that can be used to identify a group of bacteria, for example Bifidobacteria. Two different approaches can be used to achieve this:
 - i. Either pre-define the group of bacteria to identify, for example Bifidobacteria, Clostridia and Enterobacteria.
 - ii. Alternatively, a search algorithm can be set up that searches for groups of bacteria without any pre-existing input.

The search criteria may include two sequence regions with conserved sequences within the group, interspersed with a sequence region with variations between the target bacteria within that group. By a sequence region with conserved sequences within the group it is meant either a sequence region of defined length (*i.e.* 10 – 30 bases), or that is able to hybridise a homologous oligonucleotide at a defined melting temperature (T_m). Several different types of oligonucleotides (DNA, RNA, LNA etc.) can be used to define this. By close to homologous sequence it is meant a sequence with a defined length containing a limited number of mismatch sequences (typically 1 to 3 mismatches), or is defined by an oligonucleotide that can hybridise at a defined T_m within a given range (for example ± 5 degrees Celsius). The distance between the two homologous sequence regions containing the variable region is typically from 50 – 1500 bases (optionally 75 – 500 bases).

Using this approach the Inventor has shown that it is possible to identify at least more than 20 different target DNA sequence regions that could be used to distinguish species of the Bifidobacteria genus.

An example sequence analysis is shown in Figure 3. Relevant genomic
5 sequences of several Bifidobacteria species are shown in an alignment. Species and strain specific sequence areas (target DNA sequence regions) are shown in the boxed area together with example putative forward and reverse primer areas (conserved regions) for generating a Bifidobacteria-specific genus amplicon containing amplification products comprising species- and strain-specific target DNA sequences.
10

Example 2: Preparing a Sample & Amplifying Sample DNA.

The following example sets out general exemplary methodologies which may be employed for the purposes of obtaining and preparing samples for analysis.

15 Biological samples are collected. Samples can be processed immediately following collection or stored for future use.

DNA is extracted and purified from samples according to standard procedures known in the technical field. Since the methods of the invention are applicable to any suitable sample, the precise protocol for DNA extraction will vary. For example, the
20 precise protocol for DNA extraction from soil will differ from the precise protocol for DNA extraction from a biological sample such as saliva. DNA extraction protocols will therefore be optimized using routine procedures known in the art depending on the particular sample to be studied.

For biological materials, the nature of the sample will be dependent upon the
25 environment to be analysed *e.g.* the body region of interest. Typically, a biological sample will comprise nasal mucous, saliva, sputum, oesophageal mucus, vomit, faeces, urine, vaginal mucous or skin.

Forward and reverse PCR primers containing sequences specific to amplify the DNA sequence regions identified, *e.g.* as described in Example 1, are added to a
30 solution of the purified DNA and other reagents in order to carry out an amplification reaction, preferably a PCR amplification reaction. Primers may be designed to include degenerative sequences if necessary.

The number of cycles, the type of amplification (for example: linear PCR followed by cyclic PCR, ligase chain reaction, loop mediated isothermal amplification, multiple displacement amplification) and other conditions may vary or may be adapted. As noted above, in preferred methods DNA sequence regions are amplified by PCR.

5 Since the methods will typically involve determining the presence or absence of many microorganism species within an environment, a number of DNA sequence regions may be amplified in parallel, and this will require multiplex amplification techniques.

10 The number of primer pairs that can be used in the same reaction vessel in a singleplex or multiplex manner, and therefore the number of amplification reactions that can be performed in the same reaction vessel can be determined empirically using routine optimization tests.

15 If necessary, the methods of the present invention allow the sample to be divided into several aliquots and singleplex or multiplex PCR performed on the same sample material in different reaction vessels, with optionally multiple sets of primer pairs added to each reaction vessel. If required, samples from completed reactions in separate reaction vessels can then be combined back into one or more combined aliquot before being further processed. The methods of the present invention allow different conditions to be used in each aliquot (*e.g.* temperature, time, salt concentrations, etc.).
20 This may provide greater flexibility in designing amplification primers. Alternatively, the same conditions may be applied for all aliquots. After a clean-up of amplified DNA to remove primers, template, etc., a further amplification may be performed with instrument-specific sequence tags and indexes as required.

25 **Example 3: Identification of Bifidobacteria amplicons.**

Whole genome bacterial sequences were obtained from NCBI/Genome of the bacteria listed in Table 1 (Figure 8).

30 Full genome sequences of the five Bifidobacterium strains were aligned, using Mugsy with default settings. This resulted in 286 aligned regions where all five genomes were represented. Inside each of these regions search was performed for fully conserved sub-regions of minimum 25 bases, as possible targets for PCR primers

(primer sites). Every region of up to 1000 bases enclosed by two such primer sites was listed as a potential amplicon.

In total 35 such potential amplicon regions were found.

Next, all the primer sites of the potential amplicons were BLASTed against all
5 genomes (both *Bifidobacterium* and the other), and the melting temperature for the best BLAST hits were computed. This was done to discard amplicons with primer sites giving false positive hits in other genomic regions of interest. No potential amplicons were discarded by this, and 35 potential amplicons were left prior to the probe search.

10 **Example 4: Feasibility of FRET technology for detection.**

Specific PCR primers and labelling oligonucleotide probes (LP) and detection
oligonucleotide probes (DP) used are listed in Table 2 (16S rRNA gene; Figure 8) and
Table 3 (Bifidobacteria specific amplicon 4; Figure 8). The sequence identical to the
15 genomic sequence is underlined, while the sequence complementary to LP and DP is shown in grey shade.

LP containing an additional C and ddCTP-Black-Hole-Quencher2 (Jena
Sciences) and DP containing a fluorophore and specific melting temperature ('T_m') as
listed in Table 3 and 4 was used to assess the feasibility of using FRET technology to
20 detect labelled probes.

10µM each of LP and DP and 0.5mM MgCl₂ in a total volume of 20µl was
mixed as indicated in Table 4.

The samples were mixed in a LightCycler® 480 Multiwell Plate 96, white
(Roche) and run on a LightCycler 480 II using a melting point programme with the
25 following parameters:

60 sec at 95°C

60 sec at 25°C

10 min 25°C - 85°C

30

The analysis was performed as a melting point analysis, and approximate
numbers for T_m, increase in fluorescence signal and signal peak was estimated from the

read-out as shown in Table 5 (since the numbers were negative, the software was not able to calculate these numbers).

Example 5: Mock library of genomic bacterial DNA.

5

Mock libraries of different compositions of the 12 genomic bacterial DNA listed in Table 1 was prepared by 10-fold dilutions from 100 ng to 1 ng, as shown in Table 6 (Figure 8).

Example 6: PCR of 16S rRNA and Bifidobacteria amplicon.

10

Total genomic DNA listed in Table 1 (Figure 8) was amplified using either primer pair 16SV3V9F and 16SV3V9R for 16S rRNA amplicon amplification, and Bifi4F and BIFI4R for Bifidobacteria amplicon 4 amplification. 1.5 U HotFirePol (Solis Biodyne, Tartu, Estonia), 1x B2 buffer (Solis Biodyne), 2.5 mM MgCl₂ (Solis Biodyne), 200 µM deoxynucleo-triphosphate (dNTP) (Thermo Fisher Scientific, Waltham, MA), 0.2 µM each forward and reverse primer, and approximately 10 to 50 ng template in a total volume of 25 µl was used for the amplification. The amplification included a 15-min activation stage at 95°C, followed by 30 cycles with 30 sec denaturation at 95°C, 30 sec annealing at 55°C for 16S rRNA amplification and 65°C for Bifidobacteria amplification, and 90 sec extension at 72°C. A final elongation for 7 min at 72°C was included for completion of all the PCR products. 3 µl of each PCR reaction was analysed by gel electrophoresis, and visualized by UV light.

Example 7: Test of labelling and detection on LightCycler.

25

PCR products made according to Example 4 were treated with 3 U exonuclease I (New England BioLabs, Ipswich, MA) and 8 U shrimp alkaline phosphatase (New England BioLabs, Ipswich, MA) at 37°C for 2 h and inactivated at 80°C for 15 min, and quantified by gel electrophoresis. Alternatively, the PCR products were purified using MinElute (Qiagen) according to manufacturers recommendation, and the amount of purified PCR product was measured using NanoDrop.

From the purified PCR product, 100 ng was used in the following labelling reaction mixture: in a total reaction volume of 20 µl, 2.5 U Hot TermiPol (Solis Biodyne), 2 µl buffer C (Solis Biodyne), 4 mM MgCl₂ (Solis Biodyne), 0.4 µM ddCTP-Black-Hole-Quencher2 (Jena Bioscience, Jena, Germany) and 0.2 µM of each labelling probe (LP). The labelling protocol included a 12-min activation stage at 95°C, followed by 10 cycles with 20 sec denaturation at 96°C and 35 sec combined annealing and extension at 60°C.

The 20µl sample was transferred to LightCycler® 480 Multiwell Plate 96, white (Roche), and 2 µl of a 0.1 µM solution of DP for each LP was added to each well. The samples were run on a LightCycler 480 II using a melting point programme with the following parameters:

60 sec at 95°C
 60 sec at 25°C
 15 10 min 25°C - 85°C

The analysis was done as a melting point analysis, and the results of the run is shown in Figures 6 and 7.

20 **Example 8: PCR of specific Bifidobacteria amplicon.**

Total genomic DNA listed in Example 8 Table A below was amplified using either primer pair B70_2_7F and B70_2_7R, or B194_15_1F and B194_15_1R. 1.5 U HotFirePol (Solis Biodyne, Tartu, Estonia), 1x B2 buffer (Solis Biodyne), 2.5 mM MgCl₂ (Solis Biodyne), 200 µM deoxynucleo-triphosphate (dNTP) (Thermo Fisher Scientific, Waltham, MA), 0.2 µM each forward and reverse primer, and approximately 10 to 50 ng template in a total volume of 25µl was used for the amplification. The amplification included a 15-min activation stage at 95°C, followed by 30 cycles with 30 sec denaturation at 95°C, 30 sec annealing at 55°C for 16S rRNA amplification and 65°C for Bifidobacteria amplification, and 90 sec extension at 72°C. A final elongation for 7 min at 72°C was included for completion of all the PCR products. 3µl of each PCR reaction was analysed by gel electrophoresis, and visualized by UV light.

Table A

Labelling probes:		Detection probes	
B70_Blong1	CCGACGCTGCACC	B70_Blong1RC-TAM	GGTGCAGCGTCGG
B70_Bbrev1	CCGCAAGCATCCA GT	B70_Bbrev1RC-TAM	ACTGGATGCTTGCGG
B194_Bbrev1	GAAGGCCGTCATC GT	B194_Bbrev1RC-TAM	ACGATGACGGCCTTCTTT
B70_Bkash1	CAACGGCCTTTGTG G	B70_Bkash1RC-TAM	CCACAAAGGCCGTTGTTTTTT
B194_Badol1	GAAGGCCAAGCTG GT	B194_Badol1RC-TAM	ACCAGCTTGGCCTTCTTTTTTT TT

5 Bifidobacteria PCR primers used:

B70_2_7F GGGGTGACGGCGTG

B70_2_7R CTCATGTCTGCATTCTCACTC

B194_15_1F AACATGCAGTGGTTCAAGAT

B194_15_1R CGGTGATCGGGTACTTSTA

10

Example 9: Labelling and detection of probes based on size.

PCR products made according to Example 8 were purified using MinElute (Qiagen) according to manufacturers recommendation, and the amount of purified PCR
15 product was measured using NanoDrop.

From the purified PCR product, 100 ng was used in the following labelling reaction mixture: in a total reaction volume of 20 µl, 2.5 U Hot TermiPol (Solis Biodyne), 2 µl buffer C (Solis Biodyne), 4 mM MgCl₂ (Solis Biodyne), 0.4 µM ddCTP-biotin (Jena Bioscience, Jena, Germany) and 0.2 µM of each labelling probe (LP). The
20 labelling protocol included a 12-min activation stage at 95°C, followed by 10 cycles with 20 sec denaturation at 96°C and 35 sec combined annealing and extension at 60°C.

Labeled probes were captured on Streptavidin beads (Thermo Fisher) and all unbound oligos were washed away. Complimentary detection probes (DP) listed in Example 8 Table A above were added, and the hybridisation took place at 55°C for 60
25 minutes, followed by two washing steps in Tris buffer (pH 8.0). 10ul dH₂O was then added, and elution of the bound DP was carried out at 80°C for 15 minutes. The eluate

was analysed on Urea-PAGE gel, and labelled probes were visualized on GelBox using TAMRA direct settings (see Figure 9).

It is known that biotin–streptavidin bonds can be broken in the presence of de-ionized dH₂O at elevated temperature. It is therefore possible to follow the above
5 example and use a LP that has a fluorophore attached to 5' end, and detect the labelled labelling probe directly. A combination of different lengths and different fluorophores can be used to detect a high number of different labelled labelling probes from each aliquot.

10 **Example 10: Sequencing DNA Amplified From a Sample.**

As noted previously, “next generation sequencing” (NGS) technologies may be performed to sequence amplified regions of DNA generated in amplification methods described above. Sequencing can be performed in any suitable massively parallel NGS
15 DNA sequencing machines, as described above.

Any number of read lengths and configurations can be used. A preferred method involves a single read length of around 100 bases (Illumina has 75 and 150 as standard length) in only one direction (*i.e.* unpaired reads).

As few reads as possible are used in order to obtain sufficient sequence
20 information to determine accurately each microorganism species or strain. This may be determined empirically for each machine and setup using routine optimization set-up tests. The fewer the number of reads and the shorter the read length will translate into a faster method with a higher throughput, thus lowering cost per sample.

25 **Example 11: Analysis of DNA Sequence Reads.**

After the sequencing runs, standard de-multiplexing will need to be performed. Then, instead of the normal OTU assignment, the result of the run will be compared to the known DNA sequences of the target microorganisms, *i.e.* sequences specific for
30 target microorganisms within the set of target microorganisms to be detected. This will be further simplified by only comparing DNA sequences within the particular DNA sequence region/amplicon that the target microorganism belongs to. For example, in

the exemplary case of Bifidobacteria, in the Bifidobacteria DNA sequence region, the reads from the sequence run will only be compared to the 13 Bifidobacteria known to be present in human gut microbiome. These Bifidobacteria are: Bifidobacterium adolescentis, Bifidobacterium angulatum, Bifidobacterium animalis, Bifidobacterium
 5 bifidum, Bifidobacterium breve, Bifidobacterium catenulatum, Bifidobacterium dentium, Bifidobacterium gallicum, Bifidobacterium kashiwanohense, Bifidobacterium longum subsp. longum, Bifidobacterium longum subsp. infantis, Bifidobacterium pseudocatenulatum and Bifidobacterium stercoris.

10 A sequence homology of 98% - 100% (or even down to 95%) will be used to identify the correct bacteria.

Similarly, other DNA sequence regions can be analysed in the same manner. The commonly used 16S sequence region will be used to detecting unique microbial species and strains that can be adequately distinguished within the 16S sequence region. Examples of such bacteria that can be adequately distinguished within the 16S sequence
 15 region are *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Methanobrevibacter smithii* and *Bacteroides thetaiotaomicron*.

However, one of the benefits of the methods of the present invention is that they can exclude all other sequences. This overcomes a main challenge with 16S amplicon sequencing (or any amplification-based sequencing approach), namely the presence of
 20 chimeras, that leads to incorrect identification of bacteria that may not be present in the sample at all. Since the methods of the present invention do not “see” these sequences, it is not necessary to consider them.

Example 12: Targeted Amplicon Sequencing.

25

Bacterial DNA used was obtained from DSMZ (Germany) and is listed in Table B below. PCR primers with adaptors and indexes used are listed in Table C below. Mock libraries were made as outlined in Table D below.

30 Table B

Bifidobacterium longum subsp. infantis	DSM 20088
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	Bifidobacterium breve	DSM20213
	Bifidobacterium animalis lactis	DSM10140
	Bifidobacterium adolescentis	DSM20083
	Bifidobacterium pseudocatenulatum	DSM20438
5	Bifidobacterium bifidum	DSM20456
	Bifidobacterium catenulatum	DSM16992
	Bifidobacterium gallicum	DSM20093
	Bifidobacterium kashiwanohense	DSM21854
	Bifidobacterium longum subsp. longum	DSM20219
10	Bacteroides thetaiotaomicron	DSM2079
	Akkermansia muciniphila	DSM22959
	Faecalibacterium prausnitzii	DSM17677
	Methanobrevibacter smithii	DSM861

15 Table C

B70_2_7 amplicon adaptor primers:

20 B70_2_7FA:
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGGGTGACGGCGTGC

B70_2_7RA:
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTCATGTCTGCATTCTCA
25 CTC

B194_15_1 amplicon adaptor primers:

B194_15_1FA:
30 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAACATGCAGTGGTTCAAG
AT

B194_15_1RA:
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCGGTGATCGGGTACTTST
35 A

Univ16S amplicon adaptor primers:

Univ16SFA:
40 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

Univ16SRA:

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTA
ATCC

5

Table D

Mock library theoretical and actual numbers measured by Qubit.

10

Mock library theoretical amount			M2	M3	M4	M5
Bifido amplicons B70 and B194 and 16S amplicon	8	B_longum_infantis	C (1000pg)	D (100pg)	E (10pg)	0
	9	B_breve	0	C (1000pg)	D (100pg)	E (10pg)
	10	B_animalis_lactis	E (10pg)	0	C (1000pg)	D (100pg)
	11	B_adolescentis	D (100pg)	E (10pg)	0	C (1000pg)
	12	B_pseudocatenulatum	C (1000pg)	D (100pg)	E (10pg)	0
	14	B_bifidum	E (10pg)	0	C (1000pg)	D (100pg)
	15	B_catenulatum	D (100pg)	E (10pg)	0	C (1000pg)
	16	B_gallicum	C (1000pg)	D (100pg)	E (10pg)	0
	17	B_kashiwanohense	0	C (1000pg)	D (100pg)	E (10pg)
16S amplicon	18	B_longum_longum	E (10pg)	0	C (1000pg)	D (100pg)
	5	B_thetaiotaomicron	C (1000pg)	D (100pg)	E (10pg)	0
	20	A_muciniphila	0	C (1000pg)	D (100pg)	E (10pg)
	21	F_prausnitzii	E (10pg)	0	C (1000pg)	D (100pg)
	22	M_smithii	D (100pg)	E (10pg)	0	C (1000pg)

Measured amount by Qubit (ng/ul)			M2	M3	M4	M5
Bifido amplicons B70 and B194 and 16S amplicon	8	B_longum_infantis	0,988	0,188	0,0188	0
	9	B_breve	0	1,38	0,129	0,0129
	10	B_animalis_lactis	0,0266	0	1,56	0,266
	11	B_adolescentis	0,106	0,0106	0	1,05
	12	B_pseudocatenulatum	0,76	0,08	0,008	0
	14	B_bifidum	0,0102	0	1,01	0,102
	15	B_catenulatum	0,162	0,0162	0	1,52
	16	B_gallicum	0,844	0,117	0,0117	0
	17	B_kashiwanohense	0	0,968	0,0544	0,00544
16S amplicon	18	B_longum_longum	0,00708	0	1,04	0,0708
	5	B_thetaiotaomicron	1,03	0,0864	0,00864	0
	20	A_muciniphila	0	1,00	0,0908	0,00908
	21	F_prausnitzii	0,0138	0	1,15	0,138
	22	M_smithii	0,105	0,0105	0	1,09

italics = calculated dilution

The method was essentially as outlined in Illumina manual for 16S amplicon sequencing, expect that MinElute was used to clean up the PCR reaction, and a final
15 Exo I treatment was used in the end to eliminate large primer-dimers that would interfere with the sequencing reaction.

A first PCR reaction were performed for each mock library and amplicon in separate tubes. Each tube contained 2.5 ng of bacterial DNA, 0.2uM each of forward and reverse primer med adaptor from Table C, 1,5U HotFirePol (Solis BioDyne), 2.5 mM MgCl₂, 1 x B2 buffer (Solis BioDyne) and 0.2 uM dNTP in a 20ul reaction. The
 5 amplification reaction consisted of 15 min at 95°C, followed by 25 cycles of: 30 sec each at 95°C, 55°C and 72°C; 5 min 72°C, then 4°C.

Each amplicon of the same mock library was then pooled, and MinElute (Qiagen) was used according to manufacturers recommendation to purify amplified PCR product containing sequence adaptors. Eluted volume was diluted to 50ul.

10 A second PCR reaction, attaching Illumina indexes was then performed, according to 16S Metagenomic Sequencing Library Preparation (https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf). The Nextera XT DNA Library Preparation Kit (24
 15 samples) (FC-131-1024) was used. 5ul of purified sample from the first PCR reaction was added to 5ul each of Nextera Indexes N7 and N5 as shown in Table D, as well as 1,5U HotFirePol (Solis BioDyne), 2.5 mM MgCl₂, 1 x B2 buffer (Solis BioDyne) and 0.2 uM dNTP in a final reaction volume of 50ul. The amplification reaction consisted of 15 min at 95°C, followed by 8 cycles of: 30 sec each at 95°C, 55°C and 72°C; 5 min
 20 72°C, then 4°C. Two ul from each reaction was analyzed on 1% agarose gel w/TBE buffer.

Table E.

25 Forward (N702 – N705) and reverse (S502) index primers used.

	N702	N703	N704	N705
S502	M2	M3	M4	M5

Agarose gel electrophoresis analysis showed higher MW bands in indexed samples, indicating successful addition of indexes. Samples were then pooled into one
 30 tube. An additional clean-up step was performed to eliminate primer-dimer formation

that may interfere with the sequencing reaction. This was done by adding 5,5ul Exo I buffer and 3ul Exo I (NEB) to a 50ul tube containing the pooled samples. This was incubated at 37C for 2 hours, followed by an activation step of 80C for 15 minutes. A MinElute purification step was then performed, and the final eluate was diluted to 50ul.

- 5 20ul of 18ng/ul solution was sent to sequencing at the Norwegian Sequencing Centre, Ullevål using standard setup for a 300bp Paired end read on MiSeq (Illumina) machine.

- Approximately 1 mill reads per sample in both directions were obtained. After standard de-multiplexing, each read was compared to the sequence of the target bacteria in each sample, using 99% sequence homology as a cutoff. The result from this, and the
10 expected abundance of the different bacteria in each sample is listed below.

Table F.

B194 99% R1	M2	M3	M4	M5
B_longum_ssp_infantis	74 101	15 736	1 139	9
B_breve	59	135 968	11 638	1 345
B_animalis_ssp_lactis	702	33	89 615	6 338
B_adolescentis	12 160	1 386	71	148 135
B_pseudocatenulatum	97 600	6 738	932	22
B_bifidum	359	14	28 031	2 477
B_catenulatum	14 200	1 636	76	163 052
B_gallicum	155 089	21 724	1 658	23
B_kashiwanohense	46	99 376	4 908	1 069
Bm_longum_ssp_longum	874	160	142 917	7 595

15

All prior-published references cited herein are incorporated by reference in their entirety.

CLAIMS

1. A method of determining the presence, absence or quantity of target microorganisms in a sample containing multiple microorganisms, wherein each target
5 microorganism is a member of a group of related microorganisms and each target microorganism is a member of a pre-defined set of target microorganisms for the sample, and wherein each target microorganism in the set of target microorganisms can be distinguished from the other target microorganisms in the set, the method comprising:
- 10 1. providing test DNA from the sample;
2. providing reaction conditions wherein amplification products from the DNA of each target microorganism in the pre-defined set can be generated;
3. generating a plurality of amplicons from the DNA, wherein each amplicon is
15 specific for a different group of related target microorganisms of the pre-defined set; wherein amplification products within each amplicon comprise first and second conserved regions of DNA sequence interspersed with a target DNA sequence region, wherein both first and second conserved regions are conserved across members of the group of related
20 target DNA sequence that is a sequence within an amplicon which is unique to each target microorganism within the pre-defined set of microorganisms; and
4. determining the presence, absence or quantity of target DNA sequences of amplification products within each amplicon and thus determining the
25 presence, absence or quantity of each target microorganism in the pre-defined set.
2. A method according to claim 1, wherein the step of generating a plurality of amplicons comprises performing amplification reactions using forward and reverse
30 primers designed to anneal to DNA sequence regions comprising respectively the sequences of first and second conserved regions.

3. A method according to claim 1 or claim 2, wherein: (a) amplification reactions are performed by multiplex amplification in the same reaction vessel, preferably wherein multiplex amplification is performed by PCR; or (b) DNA from the sample is divided into aliquots and added to different reaction vessels, and wherein amplification reactions are performed by multiplex amplification in each reaction vessel, preferably wherein multiplex amplification is performed by PCR.
4. A method according to any one of the preceding claims, wherein for each amplicon generated the step of determining the presence, absence or quantity of target DNA sequences of amplification products (step 4) comprises detecting the presence of target DNA sequences using probes, wherein each probe is specific for a target DNA sequence and wherein the sequence of each probe is designed to distinguish a given target DNA sequence from all other target DNA sequences of target microorganisms in the pre-defined set.
5. A method according to claim 4, wherein each probe comprises DNA and is designed to hybridize under stringent conditions to a target DNA sequence from the given target microorganism of the pre-defined set and not to hybridize under stringent conditions to target DNA sequences of all other target microorganisms in the pre-defined set.
6. A method according to claim 5, wherein each probe is designed to act as a primer in an enzymatic extension reaction and wherein the method further comprises performing a single nucleotide extension reaction (SNE) to add a single ddNTP to a terminal end of the probe, wherein each probe is designed to hybridise to the target DNA sequence in such a way that the next nucleotide to be added to the probe in the SNE is complementary to a nucleotide which is unique to the target DNA sequence, and wherein the SNE is performed in a reaction mixture which comprises only ddNTPs which are complementary to said nucleotide which is unique to the target DNA sequence.

7. A method according to claim 6, wherein the ddNTPs provided in the reaction mixture are conjugated to a quencher of a signal, a labelling entity or a binding entity.

8. A method according to claim 7, wherein for amplification products the step of determining the presence, absence or quantity of each target DNA sequence within each amplicon (step 4) comprises the steps of:

- (a) providing at least one labelling oligonucleotide probe to form at least one labelling oligonucleotide probe-amplification product hybrid if an amplification product having target DNA sequence complementary with that of the probe is present;
- (b) performing a single nucleotide extension reaction to add a ddNTP-conjugate to the at least one labelling oligonucleotide probe-amplification product hybrid to form at least one labelling oligonucleotide probe-ddNTP-conjugate;
- (c) measuring the amount of the at least one labelling oligonucleotide probe-ddNTP-conjugate; and
- (d) determining the presence, absence or quantity of the target microorganism in the set (step 4) based on the amount of the at least one labelling oligonucleotide probe-ddNTP-conjugate.

9. A method according to claim 8, wherein a labelling oligonucleotide probe-ddNTP-conjugate purification step is performed before step (c), preferably wherein step (b) comprises providing ddNTPs conjugated to a binding entity and wherein the binding entity can bind to a capture entity.

10. A method according to claim 8 or claim 9, wherein step (c) comprises hybridising the at least one labelling oligonucleotide probe-ddNTP-conjugate of step (b) to at least one detection oligonucleotide probe to form a reaction mixture comprising at least one labelling oligonucleotide probe-ddNTP-conjugate-detection oligonucleotide probe hybrid, and wherein in step (c) a signal can be generated when the nucleic acid strands of the at least one labelling oligonucleotide probe-ddNTP-conjugate-detection oligonucleotide probe hybrid are separated.

11. A method according to claim 10, wherein the detection oligonucleotide probe is coupled to at least one labelling entity and the step of measuring the amount of the at least one labelling oligonucleotide probe-ddNTP-conjugate comprises quantifying a
5 signal generated via the labelling entity.

12. A method according to any one of claims 7 to 11, wherein the labelling entity is a quencher and the signal is an increase in energy emitted by a fluorophore coupled to the detection oligonucleotide when the nucleic acid strands of a labelling
10 oligonucleotide probe-ddNTP-conjugate-detection oligonucleotide probe hybrid are separated.

13. A method according to any one of claims 7 to 11, wherein: (a) the labelling entity coupled to a probe for the detection of a given target microorganism differs from
15 the the labelling entities coupled to probes for the detection of every other target microorganism in the pre-defined set; or (b) wherein the labelling entity coupled to a probe for the detection of a given target microorganism is the same as the labelling entities coupled to probes for the detection of every other target microorganism in the pre-defined set.

20

14. A method according to claim 13, wherein each labelling oligonucleotide probe-ddNTP-conjugate-detection oligonucleotide probe with the same labelling entity has a different melting temperature.

25 15. A method according to claim 14, wherein different detection oligonucleotide probes have different labelling entities.

16. A method according to claim 12, wherein the step of measuring the amount of the at least one detection oligonucleotide coupled fluorophore comprises:

- 30 (i) measuring the fluorescence signal from the fluorophore;
(ii) altering the conditions of the reaction mixture comprising the at least one hybridised labelling oligonucleotide probe-ddNTP-conjugate to favour melting

of the at least one hybridised labelling oligonucleotide probe-ddNTP-conjugate from the detection oligonucleotide containing the fluorophore;
(iii) measuring the fluorescence signal from the fluorophore; and
(iv) determining the amount of the least one hybridised labelling oligonucleotide probe-ddNTP-conjugate based on the difference between the fluorescence signal measured in (i) and the fluorescence signal measured in (iii).

17. A method according to claim 10, wherein the labelling oligonucleotide probe has a labelling entity coupled to the 5' end.

18. A method according to claim 17, wherein the ddNTP is conjugated to a binding moiety and the detection oligonucleotide probe is coupled to at least one quencher, and the step of measuring the amount of the at least one labelling oligonucleotide probe-ddNTP-conjugate comprises quantifying a signal generated via the labelling entity.

19. A method according to claim 10, wherein each detection oligonucleotide probe is conjugated to a labelling entity, and wherein each detection oligonucleotide probe corresponding to a specific target DNA sequence can be distinguished from the other detection oligonucleotide probes corresponding to all other specific target DNA sequences in the reaction vessel by different lengths.

20. A method according to claim 19, wherein different labelling entities are conjugated to different detection oligonucleotide probes.

21. A method according to claim 20, where any combination of labelling entities and length of detection oligonucleotide probes are used to uniquely determining the presence, absence or quantity of the target microorganism in the set (step 4) based on the amount of the at least one detection oligonucleotide probes.

22. A method according to claim 17, wherein the ddNTP is conjugated to a labelling moiety and wherein each labelling oligonucleotide probe corresponding to a specific target DNA sequence can be distinguished from the other labelling oligonucleotide

probes corresponding to all other specific target DNA sequences in the reaction vessel by different lengths.

23. A method according to claim 22, wherein different labelling entities are
5 conjugated to different labelling oligonucleotide probes.

24. A method according to claim 23, wherein any combination of labelling entities
and length of labelling oligonucleotide probes are used to uniquely determine the
presence, absence or quantity of the target microorganism in the set (step 4) based on
10 the amount of the at least one labelling oligonucleotide probes.

25. A method according to any one of claims 7 to 24, wherein the labelling moiety
is a fluorophore.

15 26. A method according to any one of claims 7 to 24, wherein a binding moiety is
able to be captured by an antibody or other capture entity.

27. A method according to any one of claims 7 to 26, wherein a binding moiety is
biotin and a capture entity is streptavidin.
20

28. A method according to any one of claims 7 to 24, wherein a labelling entity is a
quencher and wherein the quencher is selected from the group consisting of BHQ0,
BHQ1, BHQ2, BHQ3, BHQ10, TAMRA, QXL520, EDQ, QXL570, EDQ1, QXL610,
DDQ-II, QXL670, QXL, DDQ-1, Dabcyl, Eclipse, Iowa Black FQ, QSY-7, QS-9,
25 QSY-21, QS-35, Iowa Black RQ, malachite green, blackberry quencher 650,
ElleQuencher and QSY-21.

29. A method according to any one of claims 7 to 24, wherein the ddNTP is selected
from the group consisting of ddC, ddT, ddA, ddG, ddI or ddU.
30

30. A method according to any one of claims 1 to 3, wherein the step of determining
the presence, absence or quantity of target DNA sequences of amplification products

within each amplicon and thus determining the presence, absence or quantity of each target microorganism in the pre-defined set (step 4) comprises:

- 5 A. for each target microorganism in the set, sequencing the target DNA sequence in the target DNA sequence region to identify the sequence and quantifying the amount thereof;
- B. comparing the sequence results of step A against known DNA sequences of all of the target microorganisms in the set; and
- C. determining the presence, absence or quantity of each target microorganism in the set.

10

31. A method according to claim 30, wherein amplicons are sequenced by next generation sequencing (NGS) methods.

15 32. A method according to claim 30 or claim 31, wherein adaptor sequences are attached to amplicons as part of the amplification steps to facilitate DNA sequencing.

33. A method according to any one of claims 30 to 32, wherein to facilitate DNA sequencing index sequences are attached to amplicons.

20 34. A method according to claim 33, wherein to facilitate DNA sequencing index sequences are attached to amplicons in the same amplification reaction performed to attach adaptor sequences, or wherein index sequences are attached to amplicons in an amplification reaction which is separate from the reaction performed to attach adaptor sequences.

25

35. A method according to any one of the preceding claims, wherein the presence, absence or quantity of at least 2, at least 4, at least 6, at least 8, at least 10 or at least 20 different target microorganisms in the sample is determined, optionally wherein at least 50-200 different target microorganisms in the sample is determined.

30

36. A method according to any one of the preceding claims, wherein the target DNA sequence of any or all target microorganisms has a length of between about 50 to about 1500 bases.

5 37. A method according to any one of the preceding claims, further comprising quantifying the target microorganisms in the sample.

38. A method according to claim 37, comprising determining the proportions of target microorganisms present within the sample.

10

39. A method according to any one of the preceding claims, wherein target microorganisms include bacteria, including gram-negative and gram-positive bacteria; and/or wherein target microorganisms include fungi; and/or wherein target microorganisms include algae; and/or wherein target microorganisms include viruses,
15 including eukaryotic viruses and prokaryotic viruses.

40. A method according to any one of the preceding claims, wherein the set of target microorganisms is a set of microorganisms which inhabit a body region of an individual.

20

41. A method according to claim 40, wherein the body region is a region of the gastrointestinal tract.

42. A method according to claim 41, wherein the sample is from a mucosal layer of
25 a region of the gastrointestinal tract or wherein the sample is from the lumen of a region of the gastrointestinal tract.

43. A method according to claim 41 or claim 42, wherein the region of the gastrointestinal tract is the mouth, tongue, throat, oesophagus, stomach, small
30 intestine, large intestine, colon or rectum.

44. A method according to claim 43, wherein the body region is the eye, ear, nasal cavity, skin, vagina or urethra.
45. A method according to claim 42, wherein the body region is a region of biofilm
5 on a surface of the individual.
46. A method according to claim 42, wherein the sample is nasal mucous, saliva, sputum, oesophageal mucus, vomit, faeces, urine, vaginal mucous or skin.
- 10 47. A method according to any one of the preceding claims, wherein the sample is a sample from an individual, and wherein the individual is an animal, preferably a mammal such as an equine animal, a bovine animal, a porcine animal, a canine animal, a feline animal, an ovine animal, a rodent animal such as a murine animal including species of the genus mus and species of the genus rattus, preferably the individual is
15 human.
48. A method according to any one of claims 1 to 39, wherein the set of target microorganisms is a set of microorganisms which inhabit an environment or medium, and wherein the sample is a sample from the environment or medium.
20
49. A method according to claim 48, wherein the medium is soil.
50. A method according to any one of the preceding claims, wherein the group of microorganisms is a microorganism genus and the target microorganism is a
25 microorganism species or strain.
51. A method according to any one of the preceding claims, wherein target microorganisms are bacteria, optionally wherein the first and second DNA sequence conserved regions are outside of a ribosomal 16S sequence region.
30
52. A method according to claim 51, wherein target microorganisms are bifidobacteria, enterobacteria, archebacteria, lactobacilli or chlostridia.

53. A method according to claim 52, wherein target microorganisms are *bifidobacterium adolescentis*, *bifidobacterium angulatum*, *bifidobacterium animalis*, *bifidobacterium bifidum*, *bifidobacterium breve*, *bifidobacterium catenulatum*,
5 *bifidobacterium dentium*, *bifidobacterium gallicum*, *bifidobacterium kashiwanohense*, *bifidobacterium longum subsp. longum*, *bifidobacterium longum subsp. infantis*, *bifidobacterium pseudocatenulatum* and/or *bifidobacterium stercoris*.
54. A kit adapted for use in the method of any one of claims 1-53.
- 10 55. A kit comprising:
(i) at least one labelling oligonucleotide probe;
(ii) at least one detection oligonucleotide probe;
(iii) a ddNTP conjugated to at least one labelling moiety; and
15 (iv) primers;
wherein the at least one labelling oligonucleotide probe is complementary to a target DNA sequence in at least one genomic region and the at least one labelling oligonucleotide probe is complementary to the at least one detection oligonucleotide probe.
- 20 56. The kit of claim 55, wherein the kit comprises at least two, at least three, at least four, at least five, at least ten or at least twenty labelling oligonucleotide probes.
57. The kit of claim 55 or claim 56, wherein the at least one labelling entity is a
25 quencher or a molecule to which a quencher may be attached.
58. The kit of any one of claims 55 to 57, wherein the at least one labelling moiety is a molecule to which a quencher may be attached which is a first binding moiety, and wherein the method comprises attaching the ddNTP conjugated to the first binding
30 moiety to a quencher by exposing the ddNTP conjugated to the first binding moiety to a quencher-second binding moiety conjugate, wherein the first binding moiety and the second binding moiety have affinity for one another.

59. The kit of any one of claims 55 to 58, wherein the quencher is selected from the group consisting of BHQ0, BHQ1, BHQ2, BHQ3, BHQ10, TAMRA, QXL520, EDQ, QXL570, EDQ1, QXL610, DDQ-II, QXL670, QXL, DDQ-1, Dabcyl, Eclipse, Iowa
5 Black FQ, QSY-7, QS-9, Iowa Black RQ, malachite green, blackberry quencher 650, ElleQuencher and QSY-21.
60. The kit of any one of claims 55 to 59, wherein the kit comprises at least 2, at
10 least 4, at least 6, at least 8, at least 10 or at least 20 different labelling oligonucleotide probes.
61. The kit of any one of claims 55 to 60, wherein the at least one detection oligonucleotide probe is conjugated to a fluorophore.
- 15 62. The kit of any one of claims 55 to 61, wherein the kit comprises at least 2 at least 4, at least 6, at least 8, at least 10 or at least 20 different detection oligonucleotide probes.
63. The kit of claim 60, wherein each detection oligonucleotide probe is unique in
20 that it has either a different fluorophore or a different melting temperature compared to other detection oligonucleotide probes present.
64. The kit of any one of claims 55 to 63, further comprising a manual containing instructions for performing the method of any one of claims 1-53.
25
65. The kit of any one of claims 55 to 64, further comprising at least one affinity chromatography column.
66. The kit of any one of claims 55 to 65, further comprising at least one buffer.
30
67. The kit of any one of claims 55 to 66, further comprising enzymes.

68. The kit of claim 67, wherein the enzymes comprise Taq polymerase.

69. The kit of claim 68, wherein the enzymes comprise two different Taq polymerases.

5

70. The kit of any one of claims 67 to 69, wherein the enzymes comprise Exonuclease I.

71. The kit of any one of claims 67 to 70, wherein the enzymes comprise Shrimp
10 Alkaline Phosphatase.

Figure 1

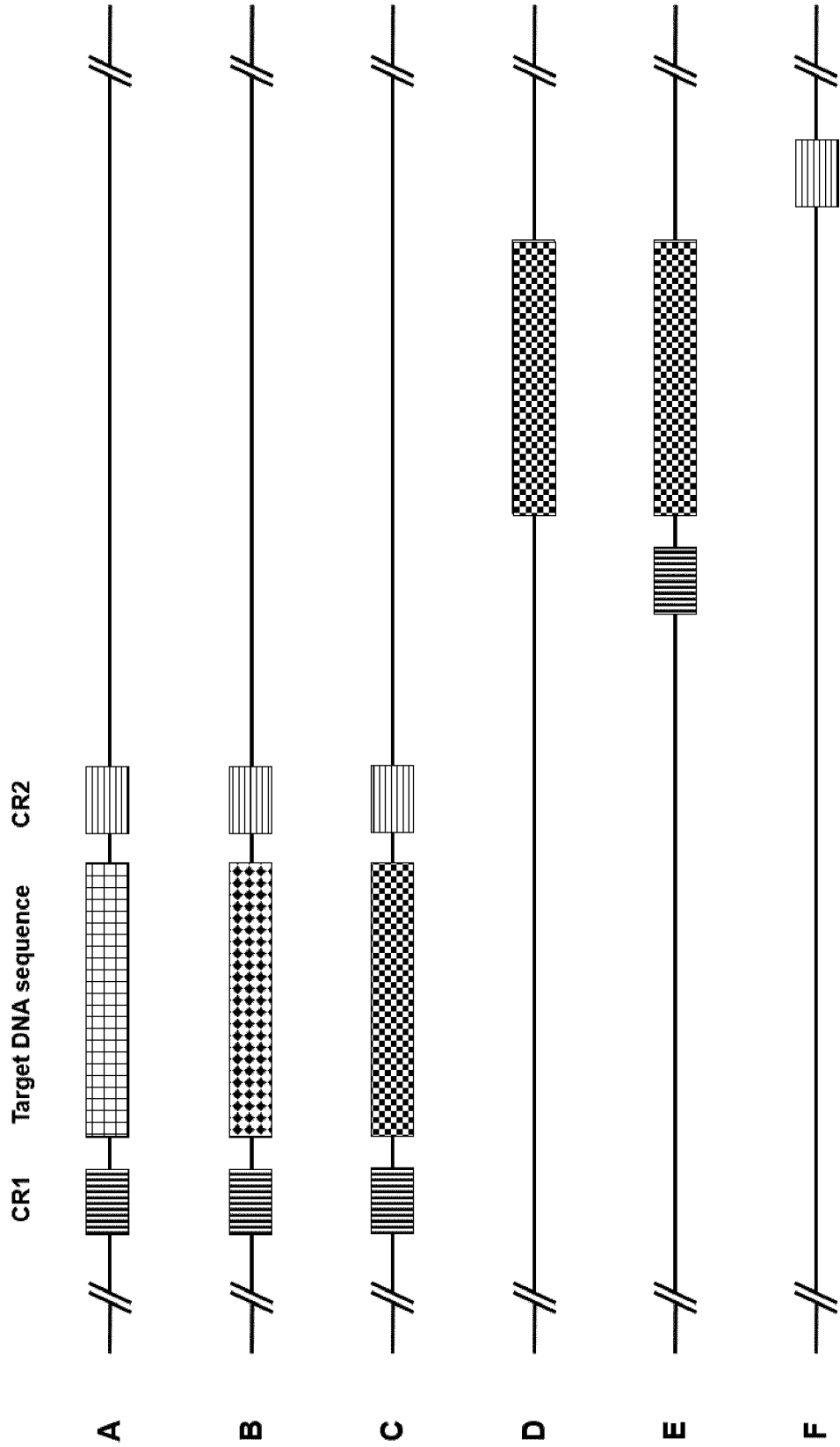


Figure 2

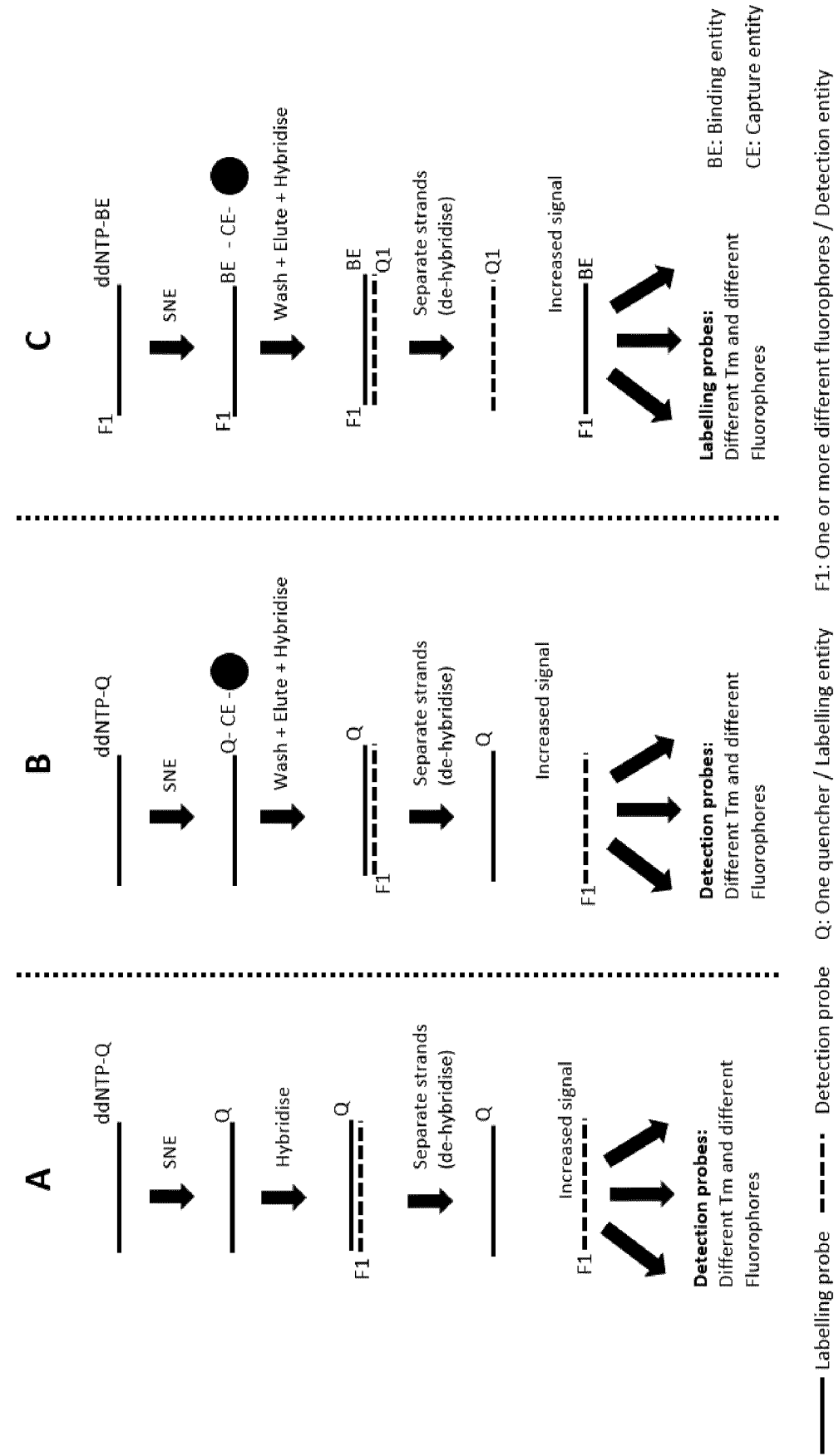


Figure 2 (continued)

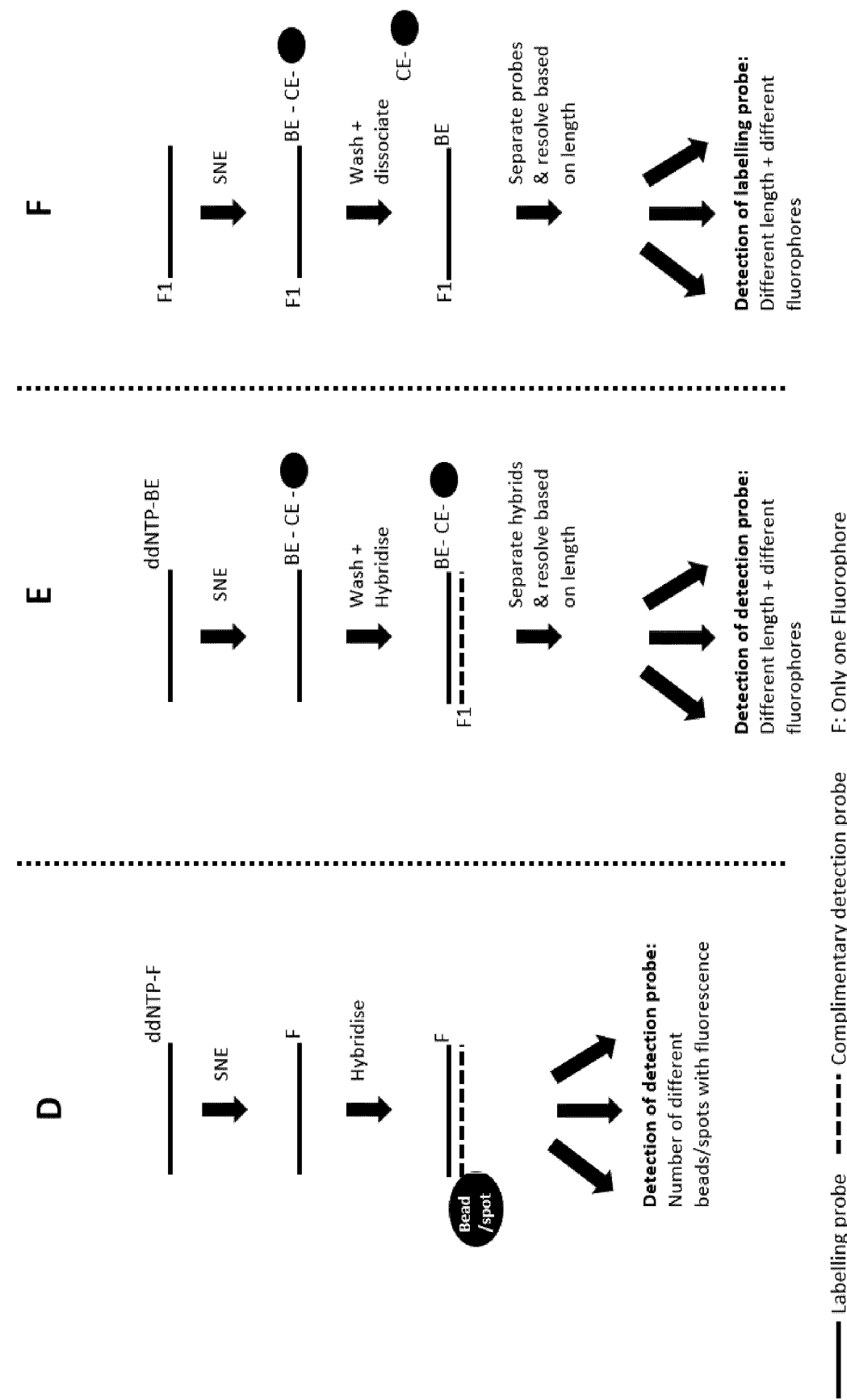


Figure 3

B. longum	CACGTCGACTGCCCGGGCCACGCCGACTTCGTGAAGAACAATGATCACCGGTGCCGCCAG
B. breve	CACGTCGACTGCCCGGGCCACGCCGACTTCGTGAAGAACAATGATCACCGGTGCCGCCAG
B. animalis	CACGTCGACTGCCCGGGCCACGCCGACTTCGTGAAGAACAATGATCACCGGTGCCGCCAG
B. adolescentis	CACGTCGACTGCCCGGGCCACGCCGACTTCGTGAAGAACAATGATCACCGGTGCCGCCAG
B. pseudocatenulatum	CACGTCGACTGCCCGGGCCACGCCGACTTCGTGAAGAACAATGATCACCGGTGCCGCCAG
	***** ** ** *
B. longum	ATGATGGCGCCATCCTCGTTGTGGCCGCCACCGACGGCCCGATGGCCAGACCCGCGAG
B. breve	ATGATGGCGCCATCCTCGTTGTGGCCGCCACCGACGGCCCGATGGCTCAGACCCGCGAG
B. animalis	ATGATGGCGCCATCCTCGTTGTGGCCGCCACCGACGGCCCGATGGCCAGACCCGCGAG
B. adolescentis	ATGATGGCGCCATCCTCGTTGTGGCCGCCACCGACGGCCCGATGGCTCAGACCCGCGAG
B. pseudocatenulatum	ATGATGGCGCCATCCTCGTTGTGGCCGCCACCGACGGCCCGATGGCTCAGACCCGCGAG
	***** ***** ***** ***** *****
B. longum	CACGTGCTGCTCGCCCGTCAGGTCGGCGTTCCGAAGATCCTCGTCGCCCTCAACAAGTGC
B. breve	CACGTGCTGCTCGCTCGTCAGGTGGCGTCCCGAAGATCCTTGTCGCCCTGAACAAGTGC
B. animalis	CACGTGCTGCTCGCCCGTCAGGTCGGCGTCCCGAAGATCCTCGTCGCTCTGAACAAGTGC
B. adolescentis	CACGTGCTGCTCGCTCGTCAGGTGGCGTCCCGAAGATCCTCGTCGCTCTGAACAAGTGC
B. pseudocatenulatum	CACGTGCTGCTCGCTCGTCAGGTGGCGTCCCGCGTATCCTCGTCGCTCTGAACAAGTGC
	***** ***** ***** ***** *****
B. longum	GACATGGTCGACGATGAAGAGCTCATCGAGCTCGTCGAAGAAGAGGTCCGTGACCTCCTC
B. breve	GACATGGTTGAGGATGAGGAGCTCATCGAGCTCGTCGAAGAAGAGGTCCGCGACCTCCTC
B. animalis	GATATGGTCGATGACGAAGAGCTCATCGAGCTCGTCGAAGAAGAGGTCCGCGACCTCCTC
B. adolescentis	GATATGGTCGACGACGAGCTCATCGAGCTCGTTGAGGAAGAGGTCCGTGACCTCCTC
B. pseudocatenulatum	GATATGGTCGACGACGAGCTCATCGAGCTTGTGAGGAAGAGGTCCGTGACCTCCTC
	** ***** ** ** ***** ** ** ***** ***** *****

Figure 3 (continued)

B. longum	GACGAGACGGCTTCGATCGCGACTGCCCGGTCAATCCACACCTCCGCCTACGGGCGCCCTG
B. breve	GACGAGACGGCTTCGACCGCGACTGCCCGGTCAATCCACACCTCCGCTTACGGTGTCTCTG
B. animalis	GACGAGACGGCTTCGACCGCGACTGCCCGGTCAATCCACACCTCCGCTTACGGGCGCTCTG
B. adolescentis	GACGAAATGGCTTCGATCGCGATTGCCCGGTCAATCCACAGTGTCCGCTTACGGGCGCACTG
B. pseudocatenulatum	GACGAAACGGCTTCGATCGCGATTGCCCGGTCAATCCACACCTCCGCTTACGGGCGCGCTG
	**** ** ***** **** * **** * **** * **** * **** * **** *
	Forward PCR primer area
B. longum	CACGACGACGCTCCGGACCAACGAGAGTGGGTTCCAGTCCGTCAAGGACCTCATGCGCGCC
B. breve	CACGACGACGCTCCGGACCAACGAGAGTGGGTTCCAGTCCGTCAAGGACCTCATGAGCGCC
B. animalis	CATGACGACGCTCCGGATCACGACAAGTGGGTTGCCACCATCAAGGAGCTCATGGACGAC
B. adolescentis	CACGATGACGCTCCGGACCAACGAGAGTGGGTTGAGCAGATCAAGAGCTCATGGACGCC
B. pseudocatenulatum	CACGACGACGCTCCGGATCACGAGAGTGGGTTGAGTCCGTCAAGGAACCTCATGAAGGCT
	** ** ***** **** * **** * **** * **** * **** * **** *
	Species and strain specific sequence area
B. longum	GTCTGATGACTACATCCCAACCCCGGTTACGACCTGGACAAGCCGTTCTCTGATGCCGATC
B. breve	GTCTGACGACTACATCCCGACCCCGGTTCCAGACCTCGACAAGCCGTTCTCTGATGCCGATC
B. animalis	GTCTGACGAGTACATCCCGACCCCGGTTCCAGACCTCGACAAGCCGTTCTCTGATGCCGATC
B. adolescentis	GTCTGATGACTACATCCCGACCCCGGTTCCAGATCTGGACAAGCCGTTCTCTGATGCCGATC
B. pseudocatenulatum	GTCTGACGAGTACATCCCGACCCCGACTCACGATCTGGACAAGCCGTTCTCTGATGCCGATC
	***** ** ***** **** * **** * **** * **** * **** * **** *
	Reverse PCR primer area
B. longum	GAGGACGTCTTACCATCTCCGGCCGTTGGTACCGTCGTACCCGGTCGTGTCGAGCGTGGC
B. breve	GAGGACGTCTTACCATCTCCGGCCGTTGGTACCGTTGTACCCGGTCGTGTTGAGCGTGGC
B. animalis	GAGGACGTCTTACCATCTCCGGCCGTTGGTACCGTCGTACCCGGTCGTGTCGAGCGCGGC
B. adolescentis	GAAGATGTCTTACCATCTCCGGCCGTTGGTACCGTGGTACCCGGCCGTTGTCGAGCGTGGT
B. pseudocatenulatum	GAAGATGTCTTACCATCTCCGGCCGTTGGTACCGTGGTACCCGGCCGTTGTCGAGCGTGGT
	** ** ***** **** * **** * **** * **** * **** * **** *
B. longum	CAGCTGGCCGTCAACACCCCGGTGAGATCGTGGCATCCGTCCGACCCAGACCAACACC
B. breve	CAGCTGGCCGTCAACACTCCGGTCGAGATCGTTGGTATCCGTCCGACCCAGACCAACACC
B. animalis	AAGCTGCCGATCAACACGAACGTGAGATCGTGGCATCCGCCGACCCAGACCAACACC
B. adolescentis	AAGTCCCGGTCAACTCCAACGTGAGATCGTGGCATCCGTCCGACCCAGACCAACACC
B. pseudocatenulatum	AAGTCCCGGTCAACTCCAACGTGAGATCGTTGGTATCCGTCCGACCCAGACCAACACC
	**** * ***** * ***** **** * **** * **** * **** * **** *

Figure 3 (continued)

B. longum	GTCACCTCCATCGAGACCTTCCACAAGACCATTGGACGCTGCGAGGCTGGCGACAACACC
B. breve	GTCACCTCCATCGAGACCTTCCACAAGACCATTGGACGCTGCGAGGCTGGCGACAACACC
B. animalis	GTCACCTCCATCGAGACCTTCCACAAGACAGATGGATGAGTGGAGGCCGGCGACAACACC
B. adolescentis	GTCACCTCCATCGAGACCTTCCACAAGACAGATGGACGAGTGGAGGCTGGCGACAACACC
B. pseudocatenulatum	GTCACCTCCATCGAGACCTTCCACAAGACAGATGGATGAGTGGAGGCTGGCGACAACACC
	***** * *****
B. longum	GGTCTGCTGCTGGCCCTGGCCGCTGAGGATGTCGAGCGCGGTGAGTCTGGCCAAAG
B. breve	GGTCTGCTGCTGGTGTCTTGGTCGTGAAGATGTCGAGCGTGGCCAGGTTGTGGCCAAG
B. animalis	GGTCTGCTGCTCCGGGCATCAACCGCACCGACGTCGAGCGTGGCCAGGTCGTGGCTGCT
B. adolescentis	GGTCTGCTGCTCCGGGCATCAACCGTACCCAGTTCGAGCGTGGCCAGGTTCTGGCTGCT
B. pseudocatenulatum	GGTCTGCTGCTCCGGGCATCAACCGTACCCAGTTCGAGCGTGGCCAGGTTCTGGCTGCT
	***** ** * * ***** ** *****
B. longum	CCGGGCTCCGTCAACCCCGCACACCAAGTTCGAGGGCGAAGTCTACGTGCTGACCAAGGAC
B. breve	CCGGGCTCCGTCAACCCCGCACACCAAGTTCGAGGGCGAAGTCTACGTGCTGACCAAGGAC
B. animalis	CCGGGTTCCGTCAACCCCGCACACCAAGTTCGAAGGCGAAGTCTACGTCTTACCAAGGAT
B. adolescentis	CCGGGCTCCGTGACCCCGCACACCAAGTTCGAGGGCGAAGTCTACGTGCTGACCAAGGAC
B. pseudocatenulatum	CCGGGCTCCGTGACCCCGCACACCAAGTTCGAGGGCGAAGTCTACGTGCTGACCAAGGAC
	***** ** * ***** ***** ***** ** *****
B. longum	GAAGGCGCGGTCACTCGCCGTTCTTCTCCAACCTACCGTCCGCGAGTCTACTTCCG
B. breve	GAGGCGGCGGTCACTCGCCGTTCTTCTCCAACCTACCGTCCGCGAGTCTACTTCCG
B. animalis	GAGGCGGCGGTCACTCGCCGTTCTTCTCGAACTACCGTCCGCGAGTCTACTTCCG
B. adolescentis	GAAGGCGCGGTCACTCGCCGTTCTTCTCCAACCTACCGTCCGCGAGTCTACTTCCG
B. pseudocatenulatum	GAAGGCGCGGTCACTCGCCGTTCTTCTCCAACCTACCGTCCGCGAGTCTACTTCCG
	** ***** ***** ***** ***** *****

Figure 4

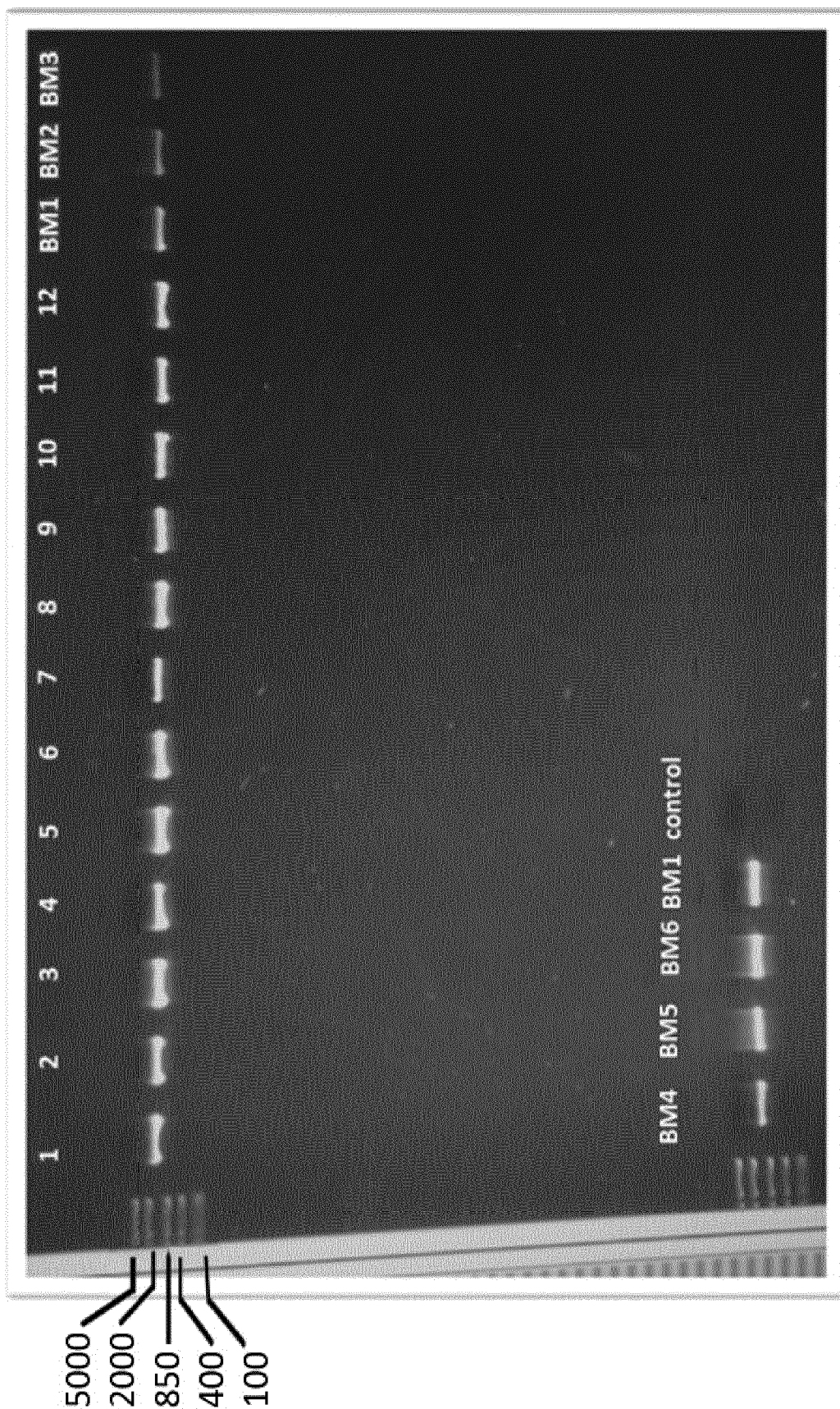


Figure 5



Figure 6

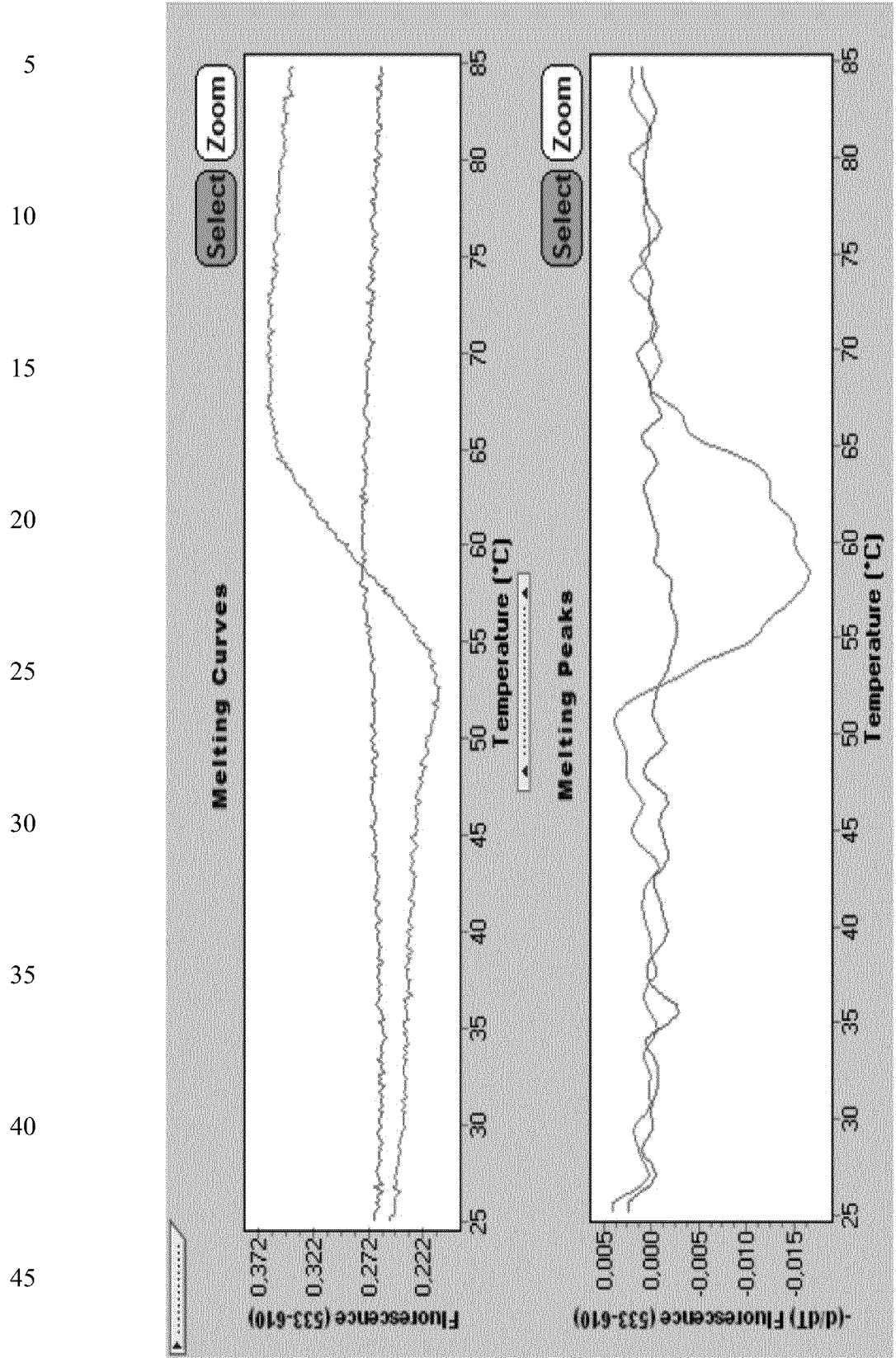


Figure 7

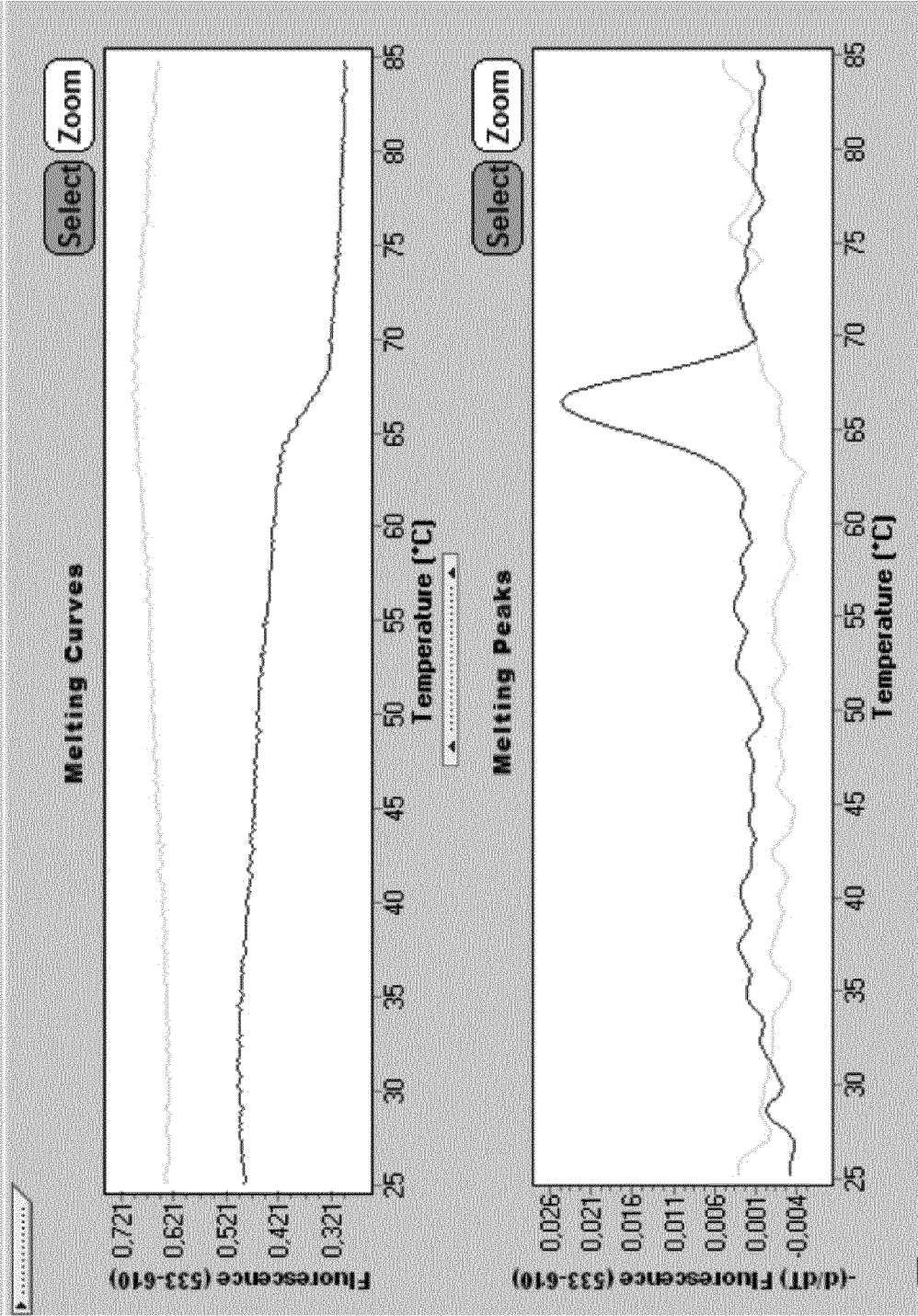


Figure 8A - TABLE 1

No.	Bacteria name	Strain no
1	<i>Streptococcus sanguinis</i>	DSM20567
2	<i>Clostridium ramosum</i>	DSM1402
3	<i>Streptococcus pyogenes</i>	DSM20565
4	<i>Listeria monocytogenes</i>	DSM20600
5	<i>Bacteroides thetaiotaomicron</i>	DSM2079
6	<i>Enterobacter cloacae</i> subsp. <i>cloacae</i>	DSM30054
7	<i>Lactobacillus acidophilus</i>	DSM20079
8	<i>Bifidobacterium longum</i> subsp. <i>infantis</i>	DSM 20088
9	<i>Bifidobacterium breve</i>	DSM20213
10	<i>Bifidobacterium animalis lactis</i>	DSM10140
11	<i>Bifidobacterium adolescentis</i>	DSM20083
12	<i>Bifidobacterium pseudocatenulatum</i>	DSM20438

Figure 8B - TABLE 2

Bacteria	Strain no	BM ID	Sequence 5' - 3'	Modification	T _m lab/det)
Streptococcus sanguinis DSM20567	1SsangL		CACTCTCACACCCGTT		<u>60,2</u>
	1SsangL-Q		CACTCTCACACCCGTT-C-BHQ2	ddCTP-BHQ2	
	R 3' - 5'		GAGTGTGGCA-		
Clostridium ramosum DSM1402	1SsangD-610-50		CAL-610-ACGGGTGTGAG	CAL Fluor Red 610	<u>49,5</u>
	2CramsL		GTCACCTCGGCTACCATTTTC		<u>61,8</u>
	2CramsL-Q		GTCACCTCGGCTACCATTTTC-C-BHQ2	ddCTP-BHQ2	
Streptococcus pyogenes DSM20565	R 3' - 5'		AGCCGATGGTAA-*		
	2CramsD-560-50		CAL-560-AATGTTAGCCGA	CAL Fluor Orange 560	<u>49,7</u>
	3SpyogL		GATTTTCCACTCCCACCAT		<u>61,7</u>
Listeria monocytogenes DSM20600	3SpyogL-Q		GATTTTCCACTCCCACCAT-C-BHQ2	ddCTP-BHQ2	
	R 3' - 5'		GTGAGGGTGGTA-*		<u>50,4</u>
	3SpyogD-FAM-50		FAM-ATGGTGGGAGTG	FAM	
Bacteroides thetaiota... DSM2079	4lmonoL		CCGTCAAGGGACAAG		<u>57,7</u>
	4lmonoL-Q		CCGTCAAGGGACAAG-C-BHQ2	ddCTP-BHQ2	
	R 3' - 5'		GCAGTTCCGT-*		<u>43,3</u>
Enterobacter cloacae DSM30054	4lmonoD-560-40		CAL-560-TCCCTTGACG	CAL Fluor Orange 560	<u>43,3</u>
	5BthetL		AAGACAGCCAGTATCAACTG		<u>61,6</u>
	5BthetL-Q		AAGACAGCCAGTATCAACTG-C-BHQ2	ddCTP-BHQ2	
Lactobacillus acidophilus DSM20079	R 3' - 5'		TCTGTGCGTCAATAGTGAC-*		<u>60,9</u>
	5BthetD-560-60		CAL-560-CAGTTGATAGTGGCTGTCT	CAL Fluor Orange 560	
	6EcloaL		GCGCCCCCGGGGATTTTACATC		<u>61,3</u>
Lactobacillus acidophilus DSM20079	6EcloaL-Q		GCGCCCCCGGGGATTTTACATC-C-BHQ2	ddCTP-BHQ2	
	R 3' - 5'		GCGGGGGCCCCCTAAAGTGA-*		<u>70,3</u>
	6EcloaD-560-70		CAL-560-ATGTGAATCCCCGGGGGCG	CAL Fluor Orange 560	
16S rRNA PCR primers	7LacidL		GGCGGGCCCCCGGCCACATGCTGGGTTCCTC		<u>61,5</u>
	7LacidL-Q		GGCGGGCCCCCGGCCACATGCTGGGTTCCTC-C-BHQ2	ddCTP-BHQ2	
	R 3' - 5'		CGCGCGGGGGCGGTGTACGACCCAA-*		<u>79,4</u>
16S rRNA PCR primers	7LacidD-560-80		CAL-560-AACCCAGCATGTGGCCGGGGCGGCG	CAL Fluor Orange 560	
	16SV3V9F		TCCTACGGGAGGCAGCAG		
	16SV3V9R		CGGTTACCTTGTACGACTT		

Figure 8C - TABLE 3

Bacteria	Strain no	BM ID	Sequence 5' - 3'	Modification	T _m (Lab/det)
Bifidobacterium longum	DSM20219	8BlongL	CGCGCTCGACATC	ddCTP-BHQ2	57
		8BlongL-Q	CGCGCTCGACATC-C-BHQ2		
		R 3' - 5'	GCGAGCTG-*		
		8BlongD-FAM-40	FAM-GTCGAGCG		
Bifidobacterium breve	DSM20213	9Bbrev1L	GGAATCGATGAGCTCCTCATC	ddCTP-BHQ2	59
		9Bbrev1L-Q	GGAATCGATGAGCTCCTCATC-C-BHQ2		
		R 3' - 5'	CCTTAGCTACTCGAGGAGTA-*		
		9Bbrev1D-FAM-60	FAM-ATGAGGAGCTCATCGATTCC		
Bifidobacterium breve	DSM20213	9Bbrev2L	GCTTGACATGGTTGAGGATGA	ddCTP-BHQ2	57
		9Bbrev2L-Q	GCTTGACATGGTTGAGGATGA-C-BHQ2		
		R 3' - 5'	CGAACTGTACCAACTCCTAC-*		
		9Bbrev2D-FAM-60	FAM-CATCCTCAACCATGTCAAGC		
Bifido animalis	DSM10140	10BanimL	GACAAGTGGGTTGC	ddCTP-BHQ2	56
		10BanimL-Q	GACAAGTGGGTTGC-C-BHQ2		
		R 3' - 5'	GTTACCCAA-*		
		10BanimD-610-40	CAL-610-AACCCACATTG		
Bifido adolescentis	DSM20083	11Badoll	GGAACCGTAAGCGGACA	ddCTP-BHQ2	56
		11Badoll-Q	CCTTCGGTAAGCGGACA-C-BHQ2		
		R 3' - 5'	GGAAGGCATTGGCCTG-*		
		11BadollD-610-60	CAL-610-GTCCGCTTACGGAAGG		
Bifido pseudocatenulatus	DSM20438	12BpseuL	CGCGCCCGACCCCGA	ddCTP-BHQ2	56
		12BpseuL-Q	CGCGCCCGACCCCGA-C-BHQ2		
		R 3' - 5'	GCGCGGGCTGGGGCT-*		
		12BpseuD-610-70	CAL-610-TCGGGGTCGGGGCGCG		
Amplicon 4	PCR primers 1/3-17				T _m
	Bifi4F				63, 5
	BIFI4R				59, 1
	TAGAACTGCGGACGG				

Figure 8D - TABLE 4

Plate1		LP-Q3probes											
		1SsangL-Q ↓ 1	2Cramsl-Q ↓ 2	3SpyogL-Q ↓ 3	4Lmonol-Q ↓ 4	5BthetL-Q ↓ 5	6Ecloal-Q ↓ 6	7LacidL-Q ↓ 7	8BlongL-Q ↓ 8	9Bbrev1L-Q ↓ 9	10BanimL-Q ↓ 10	11Badoll-Q ↓ 11	12BpseuL-Q ↓ 12
1SsangD-610-50	A	10	10	10	10	10	10	10	10	10	10	10	10
	→	10	10	10	10	10	10	10	10	10	10	10	10
2CramsD-560-50	B	10	10	10	10	10	10	10	10	10	10	10	10
	→	10	10	10	10	10	10	10	10	10	10	10	10
3SpyogD-FAM-50	C	10	10	10	10	10	10	10	10	10	10	10	10
	→	10	10	10	10	10	10	10	10	10	10	10	10
4Lmonod-560-40	D	10	10	10	10	10	10	10	10	10	10	10	10
	→	10	10	10	10	10	10	10	10	10	10	10	10
5BthetD-560-60	E	10	10	10	10	10	10	10	10	10	10	10	10
	→	10	10	10	10	10	10	10	10	10	10	10	10
6EcloaD-560-70	F	10	10	10	10	10	10	10	10	10	10	10	10
	→	10	10	10	10	10	10	10	10	10	10	10	10
7LacidD-560-80	G	10	10	10	10	10	10	10	10	10	10	10	10
	→	10	10	10	10	10	10	10	10	10	10	10	10
dH2O	H	10	10	10	10	10	10	10	10	10	10	10	10
	→	10	10	10	10	10	10	10	10	10	10	10	10

Plate2		LP-Q3probes											
		1SsangL-Q ↓ 1	2Cramsl-Q ↓ 2	3SpyogL-Q ↓ 3	4Lmonol-Q ↓ 4	5BthetL-Q ↓ 5	6Ecloal-Q ↓ 6	7LacidL-Q ↓ 7	8BlongL-Q ↓ 8	9Bbrev1L-Q ↓ 9	10BanimL-Q ↓ 10	11Badoll-Q ↓ 11	12BpseuL-Q ↓ 12
8BlongD-FAM-40	A	10	10	10	10	10	10	10	10	10	10	10	10
	→	10	10	10	10	10	10	10	10	10	10	10	10
9BbrevD-FAM-60	B	10	10	10	10	10	10	10	10	10	10	10	10
	→	10	10	10	10	10	10	10	10	10	10	10	10
10BanimD-610-40	C	10	10	10	10	10	10	10	10	10	10	10	10
	→	10	10	10	10	10	10	10	10	10	10	10	10
11BadollD-610-60	D	10	10	10	10	10	10	10	10	10	10	10	10
	→	10	10	10	10	10	10	10	10	10	10	10	10
12BpseuD-610-70	E	10	10	10	10	10	10	10	10	10	10	10	10
	→	10	10	10	10	10	10	10	10	10	10	10	10
dH2O	F	10	10	10	10	10	10	10	10	10	10	10	10
	→	10	10	10	10	10	10	10	10	10	10	10	10

Figure 8E - TABLE 5

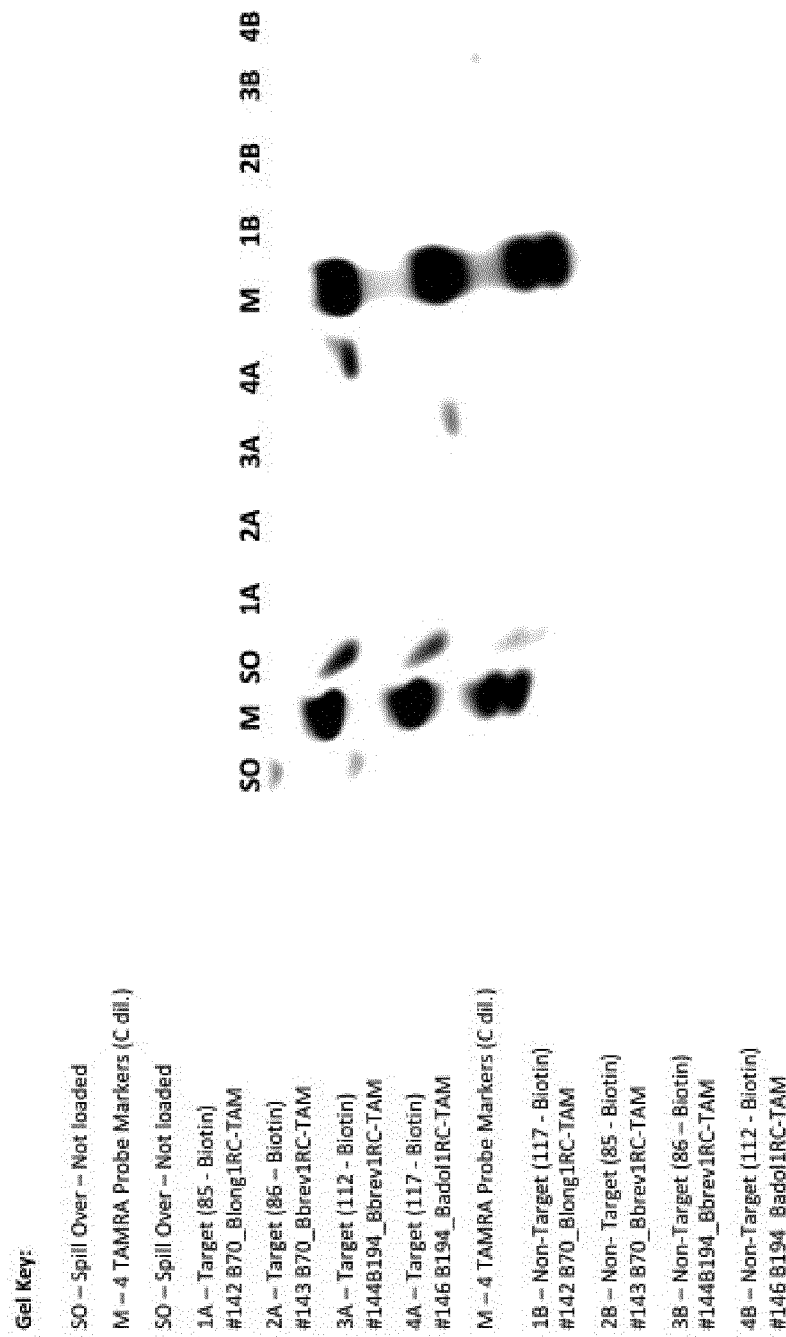
[illegible]

PlateID		LP-Q10probes											
		1SsangL-Q ↓ 1	2CramsL-Q ↓ 2	3SpvogL-Q ↓ 3	4Lmonol-Q ↓ 4	5BthetL-Q ↓ 5	6Ecloal-Q ↓ 6	7LacidL-Q ↓ 7	8BlongL-Q ↓ 8	9Bbrev1L-Q ↓ 9	10BanimL-Q ↓ 10	11BadolL-Q ↓ 11	12BpseuL-Q ↓ 12
A	8BlongD-FAM-40	0	0	0	0	0	0	0	40	0	0	0	0
		0	0	0	0	0	0	0	2,3/40	0	0	0	0
	9BbrevD-FAM-60	0	0	0	0	0	0	0	0	64	0	0	0
		0	0	0	0	0	0	0	0	10/80	0	0	0
C	10BanimD-610-40	0	0	0	0	0	0	0	0	0	42	0	0
		0	0	0	0	0	0	0	0	0	7,5/160	0	0
	11BadolD-610-60	0	0	0	0	0	0	0	0	0	0	69	0
		0	0	0	0	0	0	0	0	0	0	23/270	0
E	12BpseuD-610-70	0	0	0	0	0	0	0	41	0	0	0	62
		0	0	0	0	0	0	0	7/150	0	0	0	12/150
F		1SsangD ↓ ↓	2CramsD ↓ ↓	35pyogD ↓ ↓	4LmonoD ↓ ↓	5BthetD ↓ ↓	6EcloaD ↓ ↓	7LacidD ↓ ↓	8BlongD ↓ ↓	9Bbrev1D ↓ ↓	10BanimD ↓ ↓	11BadolD ↓ ↓	12BpseuD ↓ ↓
		0	0	0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0	0	0

Figure 8F - TABLE 6

Relative amount of genomic DNA	BM1	BM2	BM3	BM5	BM6	BM7
<i>Streptococcus sanguinis</i>	100	10	10	1	100	100
<i>Clostridium botulinum</i>	10	10	100	10	1	10
<i>Streptococcus pyogenes</i>	1	10	1	100	10	1
<i>Listeria monocytogenes</i>	1	10	0	100	10	1
<i>Bacteroides thetaiotaomicron</i>	100	10	10	0	1	100
<i>Enterobacteriaceae</i> sp. 1	10	10	100	1	0	10
<i>Lactobacillus acidophilus</i>	1	10	0	100	10	1
<i>Bifidobacterium adolescentis</i>	0	10	10	1	100	0
<i>Bifidobacterium animalis</i> lactis	100	10	0	10	1	100
<i>Bifidobacterium pseudocatenulatum</i>	10	10	100	0	1	10
<i>Bifidobacterium longum</i> subsp. infantis	1	10	1	100	0	1
<i>Bifidobacterium breve</i>	100	10	1	10	100	100

Figure 9



INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2018/058494

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/689
ADD.

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)
C12Q

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

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

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2016/289746 A1 (PARVEEN NIKHAT [US] ET AL) 6 October 2016 (2016-10-06)	1-5, 35-40, 47-51,54
Y	paragraphs [0008], [0009], [0012]; claims 1,10-20; examples 5-8	6-34, 41-46, 52,53
X	----- WO 2016/120494 A1 (NORWEGIAN UNIVERSITY OF LIFE SCIENCES [NO]; HEDMARK UNIVERSITY OF APPL) 4 August 2016 (2016-08-04)	54
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Y	paragraphs [0007], [0008], [0063] - [0069]; claims 1-22	1-53
	----- -/-	



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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"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

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Date of the actual completion of the international search

12 June 2018

Date of mailing of the international search report

20/08/2018

Name and mailing address of the ISA/

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Authorized officer

Schmitt, Anja

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2018/058494

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	claims 1-15, 20-27; figures 1, 2	1-53
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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2018/058494

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Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012/080754 A2 (GENETIC ANALYSIS AS [NO]; VEBOE HEIDI [NO]; RUDI KNUT [NO]; ROWE DANIE) 21 June 2012 (2012-06-21) cited in the application	54
Y	page 11; claims 1-27; example 1; tables 2-4 -----	1-53

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2018/058494

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.:
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:

see additional sheet

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 an 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.:

1-54

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.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-54

Method of determining the presence, absence or quantity of target microorganisms in a sample containing multiple microorganisms, wherein each target microorganism is a member of a group of related microorganisms and each target microorganism is a member of a pre-defined set of target microorganisms for the sample, and wherein each target microorganism in the set of target microorganisms can be distinguished from the other target microorganisms in the set, the method comprising generating a plurality of amplicons from the DNA, wherein each amplicon is specific for a different group of related target microorganisms of the pre-defined set; wherein amplification products within each amplicon comprise first and second conserved regions of DNA sequence interspersed with a target DNA sequence region, wherein both first and second conserved regions are conserved across members of the group of related microorganisms, and wherein a target DNA sequence region comprises a target DNA sequence that is a sequence within an amplicon which is unique to each target microorganism within the pre-defined set of microorganisms; and determining the presence, absence or quantity of target DNA sequences of amplification products within each amplicon and thus determining the presence, absence or quantity of each target microorganism in the pre-defined set.

A kit adapted for use in the method of any one of claims 1-53.

2. claims: 55-71

A kit comprising: (i) at least one labelling oligonucleotide probe; (ii) at least one detection oligonucleotide probe; (iii) a ddNTP conjugated to at least one labelling moiety; and (iv) primers; wherein the at least one labelling oligonucleotide probe is complementary to a target DNA sequence in at least one genomic region and the at least one labelling oligonucleotide probe is complementary to the at least one detection oligonucleotide probe.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2018/058494

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