METHODS AND KITS FOR QUANTITATIVE DETECTION OF SPECIFIC MICROORGANISMS AND NUCLEIC ACID SEQUENCES

FIELD OF THE INVENTION

This invention relates to a method for the qualitative and quantitative evaluation and detection of microorganisms or nucleic acid sequences in a sample. In particular, this application relates to the quantitative evaluation and detection of microorganisms and nucleic acid sequences in an environmental, medical, veterinary, agricultural and biotechnological sample.

BACKGROUND OF THE INVENTION

There are increasing public health concerns with respect to the contamination of food, water and other consumables with microscopic pathogens such as bacteria, viruses and parasites. Accordingly, most medical or veterinary products, as well as consumables like food and water are tested for microbiological contamination. This necessitates the sampling of these products as well as testing the surrounding environment.

There are many different chemical, biological and physical methods for the identification of microorganisms. For example, Table 1 describes some of the present methods of detection for microbiological contamination; however, it can be readily seen that most of the described methods are laborious and slow and require highly trained personnel. Moreover, as these methods have mainly been developed for epidemiological purposes, their usefulness as a quantitative detection system for microorganisms is poor.

Table 1.

Method	Brief Description, advantages/disadvantages
Phospholipid fatty acid profile	Only broad groups of microorganisms may be determined from the structure of specific fatty acids. Requires highly trained personnel. Time of analysis is 5-10 hours.
Protein and enzyme profiles	Solubilization, electrophoresis and comparison of microbial proteins or enzymes enhanced by specific colour-producing substrates (chromogens). Only broad groups of microorganisms may be determined from the structure of specific fatty acids. Requires highly trained personnel. Time of analysis is 5-10 hours.
Identification of specific biopolymers by mass-spectrometry	Rapid (0.5-1 hour) detection of specific biopolymers by modern kinds of mass-spectrometers. Requires expensive equipment. Requires preliminary concentration of microbial cells. Requires highly trained consumption of specific substrates in the wells of microplate. Requires highly trained personnel. Time of analysis is 2-3 days.
Serotyping	Rapid detection of specific antigenic structures; applicable for the detection of serovariants only. Requires preliminary concentration of microbial cells. Requires highly trained personnel. Applicable for limited epidermiological analysis only.

Analysis of chromosomal DNA	Sequencing of parts of DNA, specific splitting (restriction) of DNA, amplification of specific sequence by polymerase- chain-reaction (PCR), electrophoresis of DNA and its parts. Requires highly trained personnel.
Hybridisation DNA or RNA sequences	Hybridisation (specific binding) between single strand of DNA/RNA and sequence of synthetic oligonucleotide (probe).

In recent times, the use of modern molecular techniques have been employed. For example, the use of signal intensity in a nucleic acid hybridisation assay to estimate total nucleic acid present in a sample is known. In these methods, a sample mixture containing RNA or single-stranded DNA is hybridised with an oligonucleotide or polynucleotide probe which is specifically complementary to the nucleotide sequence to be identified and carries a detectable chemical group.

The most suitable approach for rapid microbiological analysis relies on the hybridisation of whole-cell rRNA with fluorescent-labelled oligonucleotide probes.

However, while these techniques have overcome some of the problems identified in Table 1, there are disadvantages associated with these methods which reduce their suitability. For example, these techniques require time consuming sample preparation steps to overcome the problems associated with the detection of a sequence or microorganism that may be at a low concentration. Moreover, the sequence or microorganism may be masked by associated protein in a complex biological sample such as faeces, microbial sludge and the like. This would also exacerbate problems associated with non-specific binding and high variability of data obtained by measurement of

the intensity of fluorescence in microscopic images, when fluorescent-labelled probes are used.

Accordingly, there is still a need for a simple, efficient and reliable method for the quantitative detection of microorganisms or nucleic acid sequences in a sample. As a consequence, the present invention attempts to overcome or at least alleviate the problems associated with the prior art methods.

The major advantages of the present invention over existing methods is that the quantitative detection of specific microorganisms and nucleic acid sequences is performed in the bulk of the sample and is measured as the difference in the binding of labelled probes to sequences of interest under low and high stringency conditions.

Quantitative and qualitative detection as provided in the present invention is statistically reliable and does not depend on the physical and chemical heterogeneity of the sample. Therefore, the invention may be used for the quantitative and qualitative detection of selected groups of microorganisms or specific nucleic acid sequences in a heterogeneous sample such as faeces, sludge and the like.

SUMMARY OF THE INVENTION

In a first aspect, the present invention provides a method for quantitative and/or qualitative detection of a microorganism or nucleic acid sequence in a sample, comprising the steps of:

(a) adding to the sample, a labelled oligonucleotide probe that specifically binds to a target sequence,

(b) determining the level of binding of the labelled probe with the target sequence under low stringency conditions,

(c) determining the level of binding of the labelled oligonucleotide probe with the target sequence under high stringency conditions,

(d) determining the level of specific binding as the difference between the level of binding determined in step (c) and the level of binding determined in step (b).

In a second aspect, the invention provides a method for quantitative and/or qualitative detection of a microorganism or nucleic acid sequence in a first sample, comprising the steps of:

(a) adding to the first sample a labelled oligonucleotide probe that specifically binds to a target sequence under high stringency conditions,

(b) adding to a second sample labelled and unlabelled oligonucleotide probes that specifically bind to the target sequence under high stringency conditions,

(c) determining the level of binding of the labelled oligonucleotide probe in step (a),

(d) determining the level of binding of the labelled oligonucleotide probe in step (b),

(e) determining the level of specific binding as the difference between the levels of binding determined in steps (c) and (d),

wherein the level of specific binding is the difference between the levels determined in the absence and presence of unlabelled oligonucleotide probe.

In a third aspect, the invention provides a method for quantitative and/or qualitative detection of a microorganism or nucleic acid sequence in a first sample, comprising the steps of:

(a) adding to the first sample a labelled oligonucleotide probe that can specifically binds to a target sequence under high stringency conditions,

(b) adding to a second sample labelled non-

sense oligonucleotide probe that cannot specifically bind to the target sequence under high stringency conditions,

(c) determining the level of binding of the labelled oligonucleotide probe in step (a) under high stringency conditions,

(d) determining the level of binding of the labelled non-sense oligonucleotide probe in step (b) under high stringency conditions,

(e) determining the level of specific binding as the difference between the levels of binding determined in steps (c) and (d),

wherein the level of specific binding is the difference between the levels determined under high and low stringency conditions.

Preferably, the unlabelled oligonucleotide probe is present at a greater concentration than the corresponding labelled oligonucleotide probe. More preferably, the unlabelled oligonucleotide probe is present at least three times higher in concentration than the concentration of the corresponding labelled oligonucleotide probe.

The labelled oligonucleotide probe may be labelled with any suitable label known in the art. Suitable, non-limiting examples of useful labels include fluorochromes, eg. fluorescein isothiocyanate (FITC), rhodamine, Texas Red, phycoerythrin, allophycocyanin, 6-carboxyfluorescein (6-FAM), 2', 7'-dimethoxy-4',5'-dichloro-6-carboxyfluorescein (JOE), 6-carboxy-X-rhodamine (ROX), 6-carboxy-2',4',7',4,7-hexachlorofluorescein (HEX), 5-carboxyfluorescein (5-FAM) or N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), tetrachlorofluorescein (TET), radioactive labels, eg. ^{32}P, ^{35}S, ^{3}H; as well as others. The label may be a two stage system, where the amplified DNA is conjugated to biotin, haptens, or the like having a high affinity binding partner, eg. avidin, specific antibodies, etc., where the binding partner is conjugated

to a detectable label. The label may be conjugated to one or both of the oligonucleotide probes. Alternatively, the pool of oligonucleotide probes used in the hybridisation is labelled, so as to incorporate the label into the hybridisation product.

Preferably, the label is either TAMRA or TET.

It will be appreciated by those skilled in the art that numerous oligonucleotide probes can be utilised in the method of the invention.

Preferably, the oligonucleotide probe is Eub338, Arc915 or Nsm156.

Most preferably, the oligonucleotide probe is either 5'-GCTGCCTCCCGTAGGAGT-3', 5'-GTGCTCCCCCGCCAATTCCT-3', 5'-TATTAGCACATCTTTCGAT-3' or a combination of these probes.

In a fourth aspect, the invention provides a method according to either the first, second or third aspect of the invention, further comprising the step of adding a labelled nonsense oligonucleotide probe to the sample, wherein said nonsense oligonucleotide probe does not bind to the target sequence, but binds non-specifically to cell components. The level of non-specific binding of the nonsense oligonucleotide probe is subtracted from the level of specific binding.

Preferably, the nonsense oligonucleotide probe is added in step (b) of the first, second or third aspect of the invention.

More preferably, the nonsense oligonucleotide probe is added in step (b) of the second or third aspect of the

invention.

Preferably, the nonsense oligonucleotide probe is labelled with the same fluorochrome as the oligonucleotide probe that specifically binds to the target sequence.

More preferably, the nonsense oligonucleotide probe is ns-Eub and ns-Arc.

Most preferably, the nonsense probe is either
5'-CGACGGAGGGCATCCTCA-3' and/or
5'-CACGAGGGGGCGGTTAAGGA-3'.

While the detection method described herein may be used to identify any nucleic acid including cDNA, RNA, or genomic DNA, it preferably detects RNA or genomic DNA. More preferably, the nucleic acid is RNA. Even more preferably, the nucleic acid is rRNA. Most preferably, the nucleic acid is 16S rRNA.

The samples to be tested may be pretreated before analysis by any technique known in the art. For example the cells of the sample can be killed with disinfectant such as paraformaldehyde, and treated with organic solvents, surfactants, or lytic enzymes to enhance the permeability of the cell walls so as to facilitate the passage of probe(s) into the organism(s). The cells of the sample can be purified and concentrated by centrifugation, filtration, sedimentation, flotation, electrophoresis and other methods.

In a fifth aspect, the invention provides a method according to the first, second, third or fourth aspect of the invention comprising the further step of determining the level of label in the supernatant or filtrate of the sample, and subtracting the level of label in the supernatant from the level of specific binding of the

labelled oligonucleotide probe.

Preferably, the solid residues in the sample are removed by centrifugation or filtration and the fluorescence, luminescence or radioactivity in the supernatant or filtrate is measured.

Persons skilled in the art will appreciate that the techniques disclosed herein may be used on any type of sample, for example, an environmental, medical, veterinary, agricultural or biotechnological sample. Preferably, the sample is from an environmental source. More preferably, the sample is anaerobic sludge.

It will further be appreciated by those skilled in the art that the techniques disclosed herein may be used for the detection of a plurality of microorganisms and nucleic acid sequences in a sample.

Preferably, the microorganisms include:
(a) common representatives of the microflora of the intestine, rumen, oral cavity, and vagina such as bifidobacteria, Bacteroides spp., Clostridium spp., Bacillus spp., Lactobacillus spp. and like organisms;
(b) indicator microorganisms, which are used in the detection of water quality, such as Escherichia coli, coliforms, enterobacteria, Clostridium spp. and like organisms;
(c) microorganisms indicating soil fertility or the risk of biodeterioration or corrosion of soil materials such as heterotrophs, nitrifying bacteria, sulfate-reducing bacteria, sulfur-oxidising bacteria and like organisms; and
(d) microorganisms utilised in environmental engineering systems such as methogens, eubacteria, nitrifying bacteria, iron-reducing and sulfate-reducing bacteria, filamentous bacteria, phosphate-accumulating bacteria and

like organisms.

For the purposes of this specification it will be clearly understood that the word "comprising" means "including but not limited to", and that the word "comprises" has a corresponding meaning.

DETAILED DESCRIPTION OF THE INVENTION

The practice of the present invention employs, unless otherwise indicated, conventional molecular biology, cellular biology, and recombinant DNA techniques within the skill of the art. Such techniques are well known to the skilled worker, and are explained fully in the literature. *See*, e.g., Sambrook and Russell "Molecular Cloning: A Laboratory Manual" (2001); Cloning: A Practical Approach," Volumes I and II (D.N. Glover, ed., 1985); Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); Nucleic Acid Hybridisation" (B.D. Hames & S.J. Higgins, eds., 1985); B. Perbal, "A Practical Guide to Molecular Cloning" (1984), and Sambrook, et al., "Molecular Cloning; A Laboratory Manual" (1989). Ausubel, F. et al., 1989-1999, "Current Protocols in Molecular Biology" (Green Publishing, New York).

Before the present methods are described, it is understood that this invention is not limited to the particular materials and methods described, as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims. It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a microorganism" includes one or more microorganisms, and a

reference to " a nucleic acid sequence" is a reference to one or more sequences and equivalents thereof known to those skilled in the art, and so forth. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any materials and methods similar or equivalent to those described herein can be used to practice or test the present invention, the preferred materials and methods are now described.

All publications mentioned herein are cited for the purpose of describing and disclosing the cell lines, protocols, reagents and vectors which are reported in the publications and which might be used in conjunction with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

In describing the present invention, the following terminology is used in accordance with the definitions set out below.

ABBREVIATIONS

FISH	Fluorescence in situ hybridisation
TAMRA	Carboxytetramethyl rhodamine
TET	tetrachlorofluorescein
PCT	Polymerase chain reaction
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
Tm	melting temperature

DETAILED DESCRIPTION OF THE INVENTION

In one preferred embodiment of the invention, a sample is

taken from a source containing microorganisms or target nucleic acid sequences. While the source may include an environmental, veterinary, agricultural, biotechnological or medical source, it is preferably anaerobic sludge from a waste water treatment plant. This sample is then tested for the presence of a microorganism.

The term "microorganism" includes any microscopic organism or taxonomically related macroscopic organism within the categories algae, bacteria, fungi, protozoa, viruses and subviral agents or the like. Although, the preferable microorganism is those found in sources described above. For example those microorganisms found in anaerobic sludge such as methanogens, eubacteria or nitrifying bacteria.

In one particularly preferred embodiment, the sample is pre-treated before testing by fixing with paraformaldehyde. There are other pretreatments that can be used including fixing the sample on a solid support or in suspension using an agent such as paraformaldehyde, formalin and the like.

Other pretreatment methods include treatment with organic solvents, surfactants, or lytic enzymes to enhance the permeability of cell walls and facilitate the passage of probes into the organisms. Lysing buffers are known in the art. EP 199,439; Potts, T.V. and Berry, Em. Internat. J. Sys. Bacter., 33:765-771 (1983); Bonta, Y., et al., J. Dent. Res., 64:793-798 (1985). Generally, these buffers are between pH 7.0 and 8.0, and contain both chelating agents and surfactants. Typically, a lysing solution is a buffered detergent solution having a divalent metal chelator or a buffered chaotrophic salt solution containing a detergent (such as SDS), a reducing agent and a divalent metal chelator (EDTA). The use of enzymes such as N-acetyl-muramidase (lysozyme) or proteases (such as Protease K) will facilitate lysis and offer high quality

results. The cells of the sample can be also purified and concentrated by centrifugation, filtration, sedimentation, flotation, electrophoresis and other methods.

In order to detect and quantify the target microorganism or nucleic acid sequence, oligonucleotide probes that are sufficiently complementary to the above-described nucleic acid are utilised to hybridise thereto, preferably under high stringency conditions. The term "nucleic acid" refers to deoxyribonucleic acid and ribonucleic acid in all their forms, i.e., single and double-stranded DNA, cDNA, rRNA, and the like.

The person skilled in the art will appreciate that oligonucleotides for use as hybridisation probes may be prepared by any suitable method, such as by purification of a naturally-occurring DNA or by *in vitro* synthesis. For example, oligonucleotides are readily synthesised using various techniques in organic chemistry, such as those described by Narang, et al., *Meth. Enzymol.* 68:90-98 (1979); Brown, et al., *Meth. Enzymol.* 68:109-151 (1979); Caruther, et al., *Meth. Enzymol.* 154:287-313 (1985). The general approach to selecting a suitable hybridisation probe or primer is well known; see Keller, et al., DNA Probes, pp. 11-18 (Stockton Press, 1989).

Fortunately, many of the criteria for selection of oligonucleotides suitable for use as probes in the invention are the same as the criteria for selecting oligonucleotides for performing the polymerase chain reaction ("PCR"). Thus, selection of oligonucleotides for use as probes can begin with a software program for selecting oligonucleotide primers for PCR. A number of such programs are available commercially and on-line, including programs found at the web sites maintained by: chemie.uni-marburg.de; genome.wi.mit.edu; alces.med.umn.edu; and gcg.com.

Probe design can also be performed manually. As with the programs for selecting PCR primers, probe design typically begins with the selection of a "seed" set of target nucleic acid sequences or genes.

That is, a group of target nucleic acid sequences or genes, usually selected to be different, is chosen for consideration as to whether they contain sequences useable as probes. Conveniently, genes or sequences known to be different can be selected simply by selecting genes from the known microorganism libraries. For example, the Eub338 and Arc915 probes are often used for detecting bacteria and Archaea, respectively, while the Nsm156 probe is often used for detecting bacteria of the *Nitrosomonas* group, respectively.

Once a set of genes has been selected as a seed set, the sequences of the genes are first examined in the same manner as for selecting PCR primers. For example, standard texts teach with respect to PCR that typical primers are 15 to 28 nucleotides in length and have 50 to 60% G and C composition. See, e. g., Innis, et al. eds., PCR Protocols, Academic Press. San Diego (1990) at page 9 (the entirety of Innis et al. is hereby incorporated by reference). Further, Innis et al. teach that complementarity within primer pairs should be avoided as this promotes the formation of primer-dimer artefacts, as should palindromic sequences within primers. All of these considerations can be used in designing probes for use in the invention.

Oligonucleotides can be tested for self-complementarity and for complementarity among themselves using annealing tests described by Hillier and Green (PCR Methods and Applications. 1. 124-128 (1991)), with slight modifications. For tests of self complementarity, a

sequence in the 5' to 3' orientation is compared with the same sequence in the 3' to 5' orientation. For tests of complementarity between two different oligonucleotides, the two sequences are placed in opposing orientations (that is, one is placed in the 5' to 3' orientation and the other in the 3' to 5' orientation).

The sequences are compared in every register of comparison using a scoring matrix containing values of complementarity for every pair of nucleotide symbols. For each register of comparison, the score of each base pair comparison is determined. The scores of contiguous base pairs with positive comparison values are summed. The maximum score of all such contiguous segments, taken over all registers of comparison between the sequences, determines the total "oligo-oligo" annealing score. Complementarity at the 3' ends of the oligonucleotide sequences has a particularly large influence on PCR-induced oligonucleotide-dimer formation. Therefore, the maximum score of all contiguous segments that include the position of either oligonucleotide sequence, taken over all registers of comparison, is separately determined as the 3' oligo-oligo annealing score. The lower the score, the more desirable the oligonucleotide for use as a probe. All other factors being equal, the oligonucleotides are ranked for use as probes in order of their score, from lowest to highest.

Once an initial group of oligonucleotides for potential use of probes has been generated, either manually or by use of a program for selecting PCR primers, and has met the criteria set forth above, they can be further tested to select a group of oligonucleotides particularly suitable for use in the invention. First, it is desirable that the oligonucleotides selected for the probes have melting temperatures within a few degrees of one another.

Thus, it is preferable that the oligonucleotides have melting temperatures within about 8°C of one another, and more preferable that the melting temperatures be within about 6°C of each other. Even more preferably, the oligonucleotides should have melting temperatures within about 4°C of each other. Most preferably, the melting temperatures will be within about 2°C of each other. It will often occur that it is difficult to obtain sets of oligonucleotides which meet all the other criteria and in which all of the Tms are within 2°C or 4°C of each other. Typically, however, the Tms of the oligonucleotides under consideration will cluster in two or more groups, with each group consisting of oligonucleotides with Tms which are close to each other. These groups of oligonucleotides with closely related Tms will frequently be employed.

Melting temperatures can be calculated using the nearest-neighbour model of Borer, et al. (J. Mol. Biol. 86: 843-853 (1974)) as modified slightly by Rychlik, et al. (Nucleic Acids Res. 18: 6409-6412 (1990)) and the thermodynamic parameters for DNA nearest neighbour interactions determined by Breslauer, et al. (Proc. Natl. Acad. Sci. USA. 83: 3746-3750 (1986)): T ("-AH/ (AS+Rx In (c/4))-273.15 + 16.6 x log [K (+)] where AH is the enthalpy of helix formation, AS is the entropy of helix formation (including helix initiation), R is the molar gas constant (1.987 cal/degree Celsius/mol), and c is the oligo concentration. These calculations are also an integral part of most common PCR oligo primer selection programs, including one or more of those discussed above.

Second, once a group of potential probes has been selected (either by a program or manually), the potential probes should be prioritised by similarity analysis for oligonucleotides that are similar to many (5-5000) cDNAs, allowing for a mismatch of 1-5 nucleotides. Similarity analysis is a standard method in the art and is routinely

performed using any of several widely available programs, such as the Basic Local Alignment Search Tool, or "BLAST" program, which is made available by the U. S. National Institutes of Health and can be used online at, or downloaded from ncbi.nlm.nih.gov/BLAST. Other such programs include FASTA, (see. Pearson and Lipman. Proc Natl Acad Sci (USA) 85: 2444-2448 (1988) and Pearson, Meth Enzym 183: 63-98 (1990) and BLITZ.

Third, each of the potential probes is preferably hybridized to a synthetic nucleic acid target to determine if it displays an adequate range of hybridization.

Although the sequences which ultimately become probes originate as sequences complementary to a limited set of the nucleic acid sequences or genes selected to be the "seed" sequences, they end by being able to detect the presence of virtually any nucleic acid sequence or gene. Moreover, a large number of nucleic acid sequences or genes can serve as the "seed" sequences. Accordingly, the particular sequences or genes selected to be seed sequences are not critical to the practice of the invention. Moreover, it is anticipated that numerous oligonucleotides will work in the methods of the invention.

Once the practitioner understands the concept of the invention as taught herein, a large number of probe sets can be selected by routine methods to function in the methods taught herein. Thus, practice of the invention is not limited to the selection of any one particular set of probes.

The optimum hybridisation conditions to use in the methods of the invention will vary depending on the particular set of probes chosen by the practitioner. Accordingly, once a probe set is selected, the hybridisation conditions to be

used with that probe set will have to be determined.

This determination is conveniently done empirically. Typically, the determination is conducted by hybridising the probe set multiple times to a group of dissimilar nucleic acids.

The conditions under which the hybridisations are made less stringent for each successive hybridisation, until conditions are reached under which members of the probe set hybridise to a substantial proportion of the nucleic acids, and with differing levels of intensity which are above background. The hybridisation conditions to be used need only be determined once for any particular probe set.

The determination usually commences by selecting the sample nucleic acids to which the probe set will be hybridised. Typically, the nucleic acids will be rRNAs (for ease of discussion, the text below will refer to the sample nucleic acids as rRNAs, although other nucleic acids can be used). It is desirable that the rRNAs be chosen to be dissimilar so that the ability of the probes to detect a variety of rRNAs can be confirmed.

Conveniently, the rRNAs can be chosen from the UniGene collection of libraries so that each one is known to be from a different gene. The number of cDNAs used should not be so great as to be unwieldy, but large enough so that the practitioner can observe differences in binding affinity. Usually, between about 25 and about 100 cDNAs is sufficient for these determinations. The number of cDNAs need not exceed 1000.

Typically, the cDNAs are "spotted" on a surface in an ordered fashion, to form an array with the cDNAs; in known positions. One member of the probe set is selected (this can be done at random or in an order chosen for the

practitioner's convenience) and the melting temperature of the probe determined. Melting temperatures can be estimated or calculated by standard equations based on the purine and pyrimidine composition of the nucleotides comprising the probe. Preferably, the cDNA from which the probe was derived is included in the array to provide a positive contraband to provide a signal representing 100% hybridisation to assist in quantitating hybridisations to other cDNAs.

The probes are initially hybridised to the cDNAs under fairly stringent conditions to provide a starting point at which hybridisation is not expected to a significant degree except to the rRNAs from which the probe was derived. Typically, the first hybridisation is conducted at about 5°C below the melting temperature of the probe, and the hybridisation of the probe to the cDNAs is then determined. Since it is unlikely that the probe will closely match the sequence of any of the genes (except for the rRNAs from which it was derived and related cDNAs that have identical stretches of sequence), it is expected that it will not hybridise to a significant degree to even one of the cDNAs in the array during this first hybridisation, except for the rRNAs from which it was derived.

The hybridisation is then repeated, but with the annealing temperature reduced by 2 to 5°C. and the hybridisation to the rRNAs; at the new temperature determined. The cycle of reducing temperature and determining hybridisation continues until the probe has been found to hybridise to between 5-40% (optimally, about 25%) of the cDNAs present, with a range of intensities. The same procedure is then followed for the next probe, and so on, until optimal hybridisation conditions have been determined for all the probes.

If the probes have been selected carefully, the conditions

for their optimal hybridisation should be closely matched. As noted above with respect to the Tm of the oligonucleotides, even if the optimal conditions for the probes are not identical for all of the probes, it will usually be the case that the optimal conditions for the probes will fall into two or three groups. Hybridisations for the probes falling into each individual group can be carried out at the same time, provided that the probes within the group are labelled in a way which permits them to be distinguished from each other.

In the practice of the invention, a test nucleic acid (such as a sample) is hybridised to at least three members of a set of nucleic acids (such as a probe set), including a member having some percentage of complementarity to the test nucleic acid, a member with a lesser percentage of complementarity to the test nucleic acid than does the first member of the set, and a third with a still lower degree of complementarity. The hybridisations are commenced at the higher end of the temperature range (for example, at 10°C below the calculated Tm), and repeated at successively lower temperature until satisfactory low stringency conditions are reached. One of skill knows when satisfactory low stringency conditions are reached when the three nucleic acids bind to the test nucleic acid with a degree of intensity proportional to their respective degrees of complementarity to the test nucleic acid and above background. That is, the member of the set with the highest degree of complementarity binds with the greatest intensity, the member with the intermediate degree of complementarity binds with an intensity below that of the most complementary member of the set but higher than that of a less complementary member, and the least complementary member binds with less intensity than either of these two members. The lowest intensity binding should still be distinguishable as above background so that the practitioner can confirm all three

oligonucleotides have bound to the test nucleic acid.

It is desirable both during the testing and during use of the final probe set to include as one of the test nucleic acids the cDNA from which each probe was derived. This provides a positive control for 100% hybridisation for each probe, and permits quantitation of the signal from other hybridisations.

The following are four examples of non-stringent hybridisation conditions which can be used to hybridise probes to nucleic acids of interest, illustrating four different ways of varying parameters to obtain non-stringent conditions and the high and low stringency conditions employed in the present invention.

1. The nucleic acids of interest and the probes can be incubated overnight at 42°C, in 6x SSC (that is, 0.9M NaCl, 0.1M NaCitrate). 5x Denhardt's (that is, 0.1 % each Ficoll, polyvinylpyrrolidone, and bovine serum albumin). and 0.1 % sodium dodecyl sulphate (SDS). The hybridised nucleic acids and probes are then washed 2 to 3 times with 6xSSC and 0.1% SDS for 30 minutes each time at room temperature. Finally, they are washed again for 20 min, at about 9 to 25°C, at about 10 to 25°C, at about 11 to 25°C, or at about 12 to about 25°C below the calculated Tm of the oligonucleotide, in 6xSSC, and 0. 1% SDS. The actual temperature used will take into account the salt and formamide concentrations, and can be tested empirically by the method described above to determine if a satisfactory signal is obtained from the desired hybridisation while permitting discrimination of that signal from any background which may also be present.

2. Exemplary formamide conditions. Prehybridisation and hybridisation are conducted at room temperature (considered to be from about 66 F to about 73 F) in a

solution composed 50% of formamide, 5x SSC, 20 mM Tris (pH 7.6), 1% of Denhardt's, 10% of dextran sulfate, and 0.1% SDS. The wash is conducted in 0.2x SSC, 0.1% SDS, at about 9 to 25°C, at about 10 to 25°C. at about 11 to 25°C, or at about 12 to about 25°C below the calculated Tm. The actual temperature used will take into account the salt and formamide concentrations, and can be tested empirically by the method described above to determine if a satisfactory signal is obtained from the desired hybridisation while permitting discrimination of that signal from any background which may also be present. See. e. g., Denhardt. Biophys. Res. Comm. 23: 641 (1966); Gillespie and Spigelman. J. Mol. Biol., 12: 829 (1965).

3. Modified Church's Procedure. Prehybridization and hybridization are conducted at 45 C, in a solution of 0.25mM sodium phosphate, pH 7.2, and 0.1% SDS. The wash is conducted in the same solution at about 9 to 25°C, at about 10 to 25°C, at about 11 to 25°C. or at about 12 to about 25°C below the calculated Tm. The actual temperature used will take into account the salt and formamide concentrations, and can be tested empirically by the method described above to determine if a satisfactory signal is obtained from the desired hybridisation while permitting discrimination of that signal from any background which may also be present. See, e. g.. Church and Gilbert. Proc. Natl. Acad. Sci. (USA) 81: 1991 (1984).

4. TMAC conditions. Prehybridisation and hybridisation are conducted at 45°C. in a solution of 3M tetramethylammonium chloride ("TMAC"), 0.1mM sodium phosphate, pH 6.8,1 mM EDTA, 5x Denhardt's, and 0.6% SDS. The wash is conducted in a solution containing 3M TMAC, 50 mM Tris-Cl, pH 8, and 0.2% SDS at about 9 to 25°C, at about 10 to 25°C, at about 11 to 25°C, or at about 12 to about 25°C below the calculated Tm. The actual temperature used will take into account the salt and formamide

concentrations, and can be tested empirically by the method described above to determine if a satisfactory signal is obtained from the desired hybridisation while permitting discrimination of that signal from any background which may also be present.

In this method, the calculation of Tm is based on the length of the oligonucleotide, as defined in Jacobs el al., Nucl. Acids Res. 16: 4637-4650 (1988).

Persons of skill in the art will appreciate that numerous permutations can be made in the combination of temperature and other parameters, such as salt concentration and the presence or absence of organic compounds such as formamide or DMSO, can be used to achieve non-stringent conditions for use in the present invention. The effects of changing these parameters are well known in the art. The effect on T, 1, of changes in the concentration of formamide, for example, is reduced to the following equation: T, = 81.5 +16.6 (log Na') +0.41 (% G+C)- (600/oligo length)-0.63 (% formamide). Reductions in Tm due to TMAC and the effects of changing salt concentrations are also well known.

Changing the temperature is, however, a preferred method of changing the stringency of hybridisation due to its simplicity and ease of control.

Once the labelled probes and/or unlabelled probes are subjected to low stringency conditions, these conditions are then converted to high stringency conditions so that the level of binding can be determined.

The term "stringent conditions" as used herein for hybridisation or annealing of nucleic acid molecules according to this invention are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015M NaCl/0.0015M sodium citrate/0.1% sodium

dodecyl sulfate (SDS) at 50°C, or (2) employ during hybridisation a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50mM sodium citrate at 42°C. Another example is use of 50% formamide, 5 X SSC (0.75M NaCl, 0.75M sodium citrate), 50mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 X Denhardt's solution, sonicated salmon sperm DNA (50µg/mL), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 X SSC and 0.1% SDS.

Exemplary probes include oligomers that are at least about 10 nucleic residues long and that are selected from any 10 or more contiguous residues of DNA of the present invention.

Typically, the probes will be from about 12 to about 30 nucleotides in length.

The present invention also contemplates probes that are 150 nucleic acid residues long or longer.

The person skilled in the art will appreciate that the probes of the present invention include oligonucleotide probes, single-stranded probes, double-stranded probes and RNA probes. These probes can be synthesised or are known probes available from a source.

In one particular preferred embodiment, the Eub338, Arc915 or Nsm156 probes are used.

Oligomeric probes of the present invention are labelled to render them readily detectable. In one preferred embodiment, the oligonucleotide probes are labelled with TAMRA and TET. It will be appreciated that detectable labels may be any species or moiety that may be detected either visually or with the aid of an instrument.

Commonly used detectable labels are radioactive and fluorescent labels such as, for example, ^{32}P, ^{14}C, ^{125}I, ^{3}H, ^{35}S., VIC, FAM, TET, TAMRA and the like. Biotin-labelled nucleotides can be incorporated into DNA or RNA by such techniques as nick translation, chemical and enzymatic means, and the like. The labelled probes are detected after hybridisation, using indicating means such as a radiometer, radiospectrometer, fluorometer, fluorescence spectrometer, luminometer, luminescence spectrometer, and the like. Oligonucleotide probes may also be labelled with immunodetectable fluorescent derivatives, with biotin analogues, and the like. Oligonucleotide probes may also be labelled by means of attachment to a protein. Nucleic acids cross-linked to radioactive or fluorescent histone single-stranded binding protein may also be used. Those of ordinary skill in the art will recognise that there are other suitable methods for detecting oligomeric probes and other suitable detectable labels that are available for use in the practice of the present invention.

Once hybridisation occurs, and the products of hybridisation are removed by centrifugation or filtration from the hybridisation system, the remaining radioactivity, fluorescence, or luminescence is quantified by corresponding means such as a radiometer, radiospectrometer, fluorometer, fluorescence spectrometer, luminometer, luminescence spectrometer, and the like. Other methods may be used to quantify the labelled probes including the fluorescent microscopy, autoradiography, electrophoresis and the like.

Although the invention has been described with reference to presently preferred embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention. Moreover, the following examples are offered by way of illustration only and are not intended to limit the invention in any manner.

All patent and literature references cited herein are expressly incorporated.

Example 1. Quantitative detection of bacteria and methanogens in anaerobic sludge.

Samples of anaerobic sludge were taken from the mesophilic digester in municipal wastewater treatment plant. The normal method of FISH fixation was used: the samples were fixed in 3% of paraformaldehyde in phosphate buffered saline (PBS) immediately after sampling. The concentration of dry matter was determined by the standard method for suspended solids of the sludge (American Public Health Association, 1998) was 20.3 ± 0.4 mg of dry matter/ml (mean ± s.d.).

Two 16S rRNA-targeted oligonucleotide probes were used to analyse the methanogenic microbial communities. The probes were synthesised with an aminolinker at the 5'-end and purified by HPLC (Synthegen, TX, U.S.A.). The Eub338 probe of sequence 5'-GCTGCCTCCCGTAGGAGT-3', is often used for the detection of *Bacteria* because it is specific for most organisms in this domain. This probe is also known as name S-D-Nbact-0338-a-A-18, was labelled with carboxytetramethyl rhodamine (TAMRA). The Arc915 probe of sequence 5'-GTGCTCCCCCGCCAATTCCT-3' which is also known as S-D-Arch-0915-a-A-20 is the most commonly used probe to detect the cells of Archaea, namely, methanogens, in the anaerobic digester. This probe was labelled with tetrachlorofluorescein (TET).

To ensure that the probes and hybridisation procedure maintained the binding specificity of the selected probes with the 16S rRNA of *Bacteria* or *Archaea*, control probes were used. One kind of control involved the use of an unlabelled ul-Eub338 probe, which had the same sequence as the Eub338 probe. Similarly, the ul-Arc915 probe was an

unlabelled version of the Arc915 probe. The concentration of unlabelled probe in the hybridisation solution was 10-25-fold larger than the concentration of the corresponding labelled probe. It was used for strong competitive inhibition of the hybridisation of the labelled probe with the target. This was undertaken to ensure the specificity of probe binding. If this is true and the binding is specific, in the presence of an excess of unlabelled probe no or weak binding will occur, and FISH will be absent.

Another type of control probe used was the "nonsense" probe. This probe was labelled with the same fluorochrome as the main probe, but its sequence did not target rRNA. The following "nonsense" probes were used in this study: TAMRA-labelled ns-Eub with the sequence 5'-CGACGGAGGGCATCCTCA-3' and TET-labelled ns-Arc with the sequence 5'-CACGAGGGGGCGGTTAAGGA-3'. The sequences of the nonsense probes were not matched to any bacterial 16S rRNA. The binding of these probes by the microbial biomass may be considered as the non-specific binding of the probes with the cell components.

The emission fluorescence spectra or synchronous fluorescence spectra were measured with a Luminescence Spectrometer LS-50B (Perkin-Elmer, UK) to determine the concentration of the probe after hybridisation in the experiment and in the control. The emission fluorescence spectrum of the TAMRA-labelled Eub338 probe was measured from 575 to 600 nm at an excitation wavelength of 561 nm; maximum emission was at 583 nm. Optical slits were set at 10 nm for excitation and emission wavelengths. Synchronous fluorescence spectrometry was performed by the synchronised changing of the excitation and emission wavelengths with 20 nm intervals. The excitation wavelength was increased from 440 to 620 nm with a scan speed of 240 nm/min. Correspondingly, the wavelength of the monochromator of the emitted light was increased

synchronously from 460 to 640 nm. Excitation and emission optical slits were set at 10 nm. Maximal emission for the labelled probes observed during synchronous scanning were 541-542 nm for the TET-labelled Arc915 probe and 579-580 nm for the TAMRA-labelled Eub338 probe. Due to this difference in emission wavelengths the probes may be measured individually or simultaneously in a mixture. The height of the peak fluorescence emission linearly correlated (coefficient of correlation was 0.99) with the concentration of the probe in solution, within the concentration range 1 to 100 pmol/ml.

Fluorescence *in situ* hybridisation (FISH) was performed mostly at 60°C, though the temperature was changed from 10°C to 70°C in some experiments. The final concentration of formamide in the hybridisation solution was 50% v/v. Final concentrations of the fluorescence-labelled probes in the hybridisation solution were 27 pmol/ml for the Eub338 probe and 18 pmol/ml for the Arc915 probe. A 10 µl aliquot of the probe solution was added to the mixture of 0.5 ml of fixed anaerobic sludge, 0.5 ml of hybridisation buffer (0.9 M NaCl, 0.01% sodium dodecyl sulphate, 20 mM Tris-HCl, pH 7.2) and 1.0 ml of formamide. The mixture in 2 ml polypropylene tubes was placed in a hybridisation incubator (LabLine Instruments Inc. IL, U.S.A.) with rotation speed 6 rev/min for 4 h at different temperatures. After the incubation the suspension was centrifuged at 11,000 x g for 15 min in an Eppendorf 5415C centrifuge. The supernatant was used for the measurement of the concentration of non-hybridised probes (fluorescence in the experiment) by emission fluorescence scanning. To determine the fluorescence of the probe before hybridisation two controls were made. In the control A, 0.5 ml of the suspension of the fixed anaerobic sludge was mixed with 0.5 ml of hybridisation buffer and 1.0 ml of formamide. This mixture was incubated and treated as described above. However, the solution of the

probe was added into the supernatant before the fluorescence measurement. This control was made to account for the effect of substances, which are extracted from biomass during the incubation and interfere with the probe fluorescence. Control B accounted for the self-fluorescence of these extracted substances. The quantity of bound probe (Q) was calculated from the fluorescence in control A, control B, and in experiment by the following equation:

$$Q=C(F_{cA}-F_e)/(F_{cA}-F_{cB})X \qquad (1)$$

where C is the concentration of the probe in hybridisation solution before FISH (in control A), pmol/ml; F_{cA} and F_e is the fluorescence of the probe in control A and experiment, correspondingly, in the units of fluorescence spectrometer; F_{cB} is fluorescence in control B; X is final concentration of the sludge in the incubation sample, mg of dry matter/ml.

The binding of the probes with the anaerobic sludge was analysed in triplicate under different temperatures. The binding of the Eub338 probe with the biomass was minimal between 10°C and 40°C, 0.10 ± 0.02 pmol/mg of dry matter (mean ± s.d.), and had a maximum between 60°C and 70°C, 0.16 ± 0.04 pmol/mg of dry matter (mean ± s.d.). The binding of Arc915 probe was minimal between 10°C and 30°C, 0.06 ± 0.01 pmol/mg of dry matter (mean ± s.d.), and had a maximum between 40°C and 60°C, 0.10 ± 0.02 pmol/mg of dry matter (mean ± s.d.). The mean values shown above are statistically significantly different from one another. The curves of the dissociation between the probe and the cellular matter were similar but not the same as the dissociation curves for the hybrids of the oligonucleotides with rRNA. This difference is most probably due to significant non-specific binding of the probe with the cells under temperature, which is lower

than the dissociation temperature (T_d). This non-specific binding involves the binding of the probe with cell components and the dead matter of anaerobic sludge.

The binding of labelled Arc915 probe with anaerobic sludge under the presence of corresponding unlabelled probe was low at 60°C-70°C but was not changed at 10°C-30°C. This proves that the binding of labelled Arc915 probe at 60°C-70°C is mostly specific binding but the binding of this labelled probe at 10°C-30°C is a non-specific one because it is not inhibited by the corresponding unlabelled probe.

The ratio of the binding of nonsense probe ns-Arc and labelled specific probe Arc915 at low temperature (10°C-30°C) was close to 1.0 but dropped to 0.5-0.6 at 60°C-70°C due to increased binding of labelled specific probe. This additionally proves that the binding of Arc915 at low temperature is non-specific because the binding of labelled nonsense probe can only be non-specific. The non-specific binding of labelled nonsense probe ns-Arc depended slightly on temperature.

The quantity of the probe specifically bound (hybridised) with the anaerobic biomass may be evaluated also from the dissociation curve. The binding of the probe at a temperature between 10°C and 30°C was considered to be non-specific binding. This may be due to binding of the fluorochrome or the oligonucleotide sequence with the cell surface, plasma membrane, or intracellular components. FISH involves temperature-dependent binding, and it is well known that its binding is maximal when hybridisation is performed at temperatures greater than T_d, at 60°C-70°C in our experiments. Therefore, the maximum binding, shown in Fig. 3, may be considered to be the sum of non-specific and specific binding. In such a case, the specific binding (hybridisation) may be determined as the difference between the total and the non-specific binding.

The average value of the specific binding of the Eub338 probe with the anaerobic biomass was [(0.16-0.10)/0.16]·100%=38% of the total binding, and similarly the average value of the specific binding of the Arc915 probe with the anaerobic sludge was [(0.10-0.06)/0.10]·100%=40% of total binding. The values of specific binding (hybridisation) of the probes as determined from melting curves were 0.06±0.01 pmol/mg of dry matter for the Eub338 probe and 0.04±0.01 pmol/mg of dry matter for the Arc915 probe.

The binding of labelled nonsense probe is non-specific binding. The ratio of the binding of nonsense probe and labelled specific probe at low temperature (10°C-30°C) was close to 1.0 but dropped to 0.53-0.61 at 60°C-70°C. It may be interpreted that specific binding of labelled probe was 39%-47% of total binding.

Other method for the evaluation of specific binding between oligonucleotide and cell rRNAs in the anaerobic sludge involves the inhibition of the specific binding of a labelled oligonucleotide by the same, but unlabelled oligonucleotide. The binding of the labelled probes hybridised at 60°C in our experiments was significantly inhibited when the concentration of unlabelled probe in the hybridisation solution was greater than the concentration of corresponding labelled probe. The binding of the labelled probe when present at a higher concentration than the unlabelled probe was considered to be due to non-specific binding. Therefore, specific binding (hybridisation) is represented by the difference between the total probe binding and its binding in the presence of an unlabelled probe. The specific binding of oligonucleotide probes with the anaerobic sludge was 47% of the maximal binding of the Eub338 probe, and 50% of the maximal binding of Arc915 probe. The values determined by this method were higher than ones determined by the first

method (i.e., analysis of dissociation curves), as follows: 47% against 38% for Eub338, and 50% against 40% for Arc915, respectively. The values of specific binding (hybridisation) of the labelled probes determined by competitive inhibition of FISH by the unlabelled probe were 0.08±0.01 pmol/mg of dry matter for the Eub338 probe and 0.04±0.01 pmol/mg of dry matter for the Arc915 probe.

Example 2. Quantitative detection of eubacteria and nitrifying bacteria in activated sludge (aerobic microbial biomass)

Experimental samples of activated sludge were collected from a 10-litre pilot nitrifying reactor, which was used for the simultaneous bioremoval of ammonium and organic matter from wastewater (Kim & Kim 1999). Some samples of the activated sludge were taken for microbiological analysis, and other samples were fixed in 50% ethanol (v/v, final concentration) immediately after the sampling. To determine the concentration of suspended biomass, 1.5 ml of the sample was centrifuged in an Eppendorf tube at 11,000 x g for 10 min in an Eppendorf 5415C centrifuge and dried at 60°C until it reached a constant weight.

To check the data obtained by the invented method by the traditional microbiological methods, the samples were diluted by a factor of either 10^{-4} or 10^{-5}. 0.1 ml of the suspension was spread onto a solid medium for the growth and enumeration of the colonies of ammonium- and nitrite-oxidising bacteria. The solid medium used to cultivate the ammonium-oxidising bacteria contained the following ingredients per litre of distilled water: 0.5 g $(NH_4)_2SO_4$, 0.04 g $MgSO_4 \cdot 7H_2O$, 0.04 g $CaCl_2 \cdot 2H_2O$, 0.2 g KH_2PO_4, 20 g Noble Agar (Difco), and 0.1 ml solution of metals "44". The medium was adjusted to pH 8.0 with 10N NaOH. The composition of the solution of metals "44" per 100 ml of distilled water was as follows: 1.1 g $ZnSO_4 \cdot 7H_2O$, 0.25 g

EDTA, 0.15 g $MnSO_4 \cdot 7H_2O$, 0.04 g $CuSO_4 \cdot 5H_2O$, 0.02 g $Co(NO_3)_2 \cdot 6H_2O$, 0.02 g $Na_2B_4O_7 \cdot 10H_2O$, and a pH adjusted to 4 with 10 M H_2SO_4. The inoculated Petri dishes were incubated in the dark at 28°C for at least two weeks before counting the colony-forming units (s.f.u.). The solid medium used to cultivate the nitrite-oxidising bacteria contained the following ingredients per litre of distilled water: 1.5 g $KHCO_3$, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.3 g KNO_2, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g NaCl, 0.01 g $CaCl_2 \cdot 2H_2O$, 20 g Noble Agar (Difco), and 0.2 ml solution of metals "44". The inoculated Petri dishes were incubated in the dark at 28°C for at least two weeks before counting the c.f.u.

Two 16S rRNA-targeted oligonucleotide probes were used to compare the nitrifying community in the aerobic sludge and in the biomass of a nitrifying reactor. The probes were synthesised with an aminolinker at the 5'-end and purified by HPLC. The Nsm156 probe was supplied from Synthegen, TX, U.S.A., and the Eub338 probe was supplied from MWG-Biotech AG, Germany. The sequence of the Eub338 probe is 5'-GCTGCCTCCCGTAGGAGT-3', is used to detect most eubacteria. It is also known under the name S-D-Nbact-0338-a-A-18. According to the manufacturer's data, the melting temperature for this probe is 60.5°C. The probe was labelled with rhodamine. The Nsm156 probe was used for hybridisation with the cells of the *Nitrosomonas* group of the ammonium-oxidising bacteria, namely *Nitrosomonas europea*, *Nitrosomonas eutropha*, and *Nitrosococcus mobilis*. It has the sequence 5'-TATTAGCACATCTTTCGAT, and is also known as S-G-Nsm-0156-a-A-19. The solutions of probes were stored frozen at -18°C.

The FISH was performed at temperatures from 20°C to 80°C; the optimal temperature was between 46 and 53°C. The final concentration of formamide in the hybridisation solution was 40% v/v. The final concentrations of the probes in the hybridisation solution were as follows: 27 pmol/ml

Eub338 and 4 pmol/ml Nsm156. The final concentrations in the hybridisation solution with the mixture of the probes were as follows: 27 pmol/ml Eub338 and 9 pmol/ml Nsm156. The final concentration of the biomass was 17 mg of dry weight/ml. 1.5 ml of the sample was centrifuged at 11,000 x g for 10 min in an Eppendorf 5415C centrifuge.

The solution of the one probe or the mixture of probes was added to 1.5 ml of the biomass suspension in a solution of formamide at a final concentration of 40% w/v and incubated for 2 h at different temperatures. After hybridisation, the suspension was incubated at 4°C for 0.5 h, then centrifuged, and 1.5 ml of water was added to the supernatant. This solution was used to measure the concentration of the unhybridised probes by emission scanning or synchronous scanning fluorescence spectrometry.

To determine the fluorescence of the probe before hybridisation (in the control), 1.5 ml of the biomass suspension in a solution of formamide (final concentration was 40% w/v) was incubated and treated as described above, but the solution of the probe was added to the supernatant before the fluorescence measurement. It was made to take into account the effect of the substances extracted from the biomass on the fluorescence of the probe. The quantity of the hybridised probe was calculated as the difference between the concentrations of the probe before (in the control) and after hybridisation (in the experiment).

To determine the concentration of the probe in solution before and after hybridisation, emission scanning or synchronous fluorescence spectrometry was performed with a Luminescence Spectrometer LS-50B (Perkin-Elmer). The fluorescence emission scanning of the Eub338 probe was made from 575 to 600 nm with an excitation wavelength of

561 nm, and the maximum emission was obtained at 583 nm. The fluorescence emission scanning of the Nsm156 probe fluorescence was performed from 500 to 550 nm with an excitation wavelength of 490 nm, and the maximum emission was obtained at 519 nm. The optical slits were 10nm for the excitation and emission wavelengths. Synchronous fluorescence spectrometry was performed by changing the excitation and emission wavelengths with interval of 20 nm. The excitation wavelength was increased from 380 to 580 nm with a scan speed of 240 nm/min during the synchronous scanning. Correspondingly, the wavelength of the monochometer of the emission light was increased synchronously from 400 to 600 nm. The excitation and emission optical slits were 5 nm. The maximum emissions for the labelled probes were as follows: 496-500 nm for Nsm156 and 556-558 nm for Eub338. The height of the peaks of the fluorescence emission linearly correlated (coefficient of correlation was 0.99) with the concentration of the probes in the hybridisation solution.

The binding of all the probes with the biomass of activated sludge was analysed in triplicate by emission scanning fluorescence spectrometry. There was minimal binding of the probes at temperatures lower 40°C, but when the temperature was increased to 60°C the binding was increased step-wise. This pattern of binding was similar for the curve of the hybridisation of the oligonucleotide and reflects the melting of hybrids at temperatures that are higher than the melting temperature T_m. The T_m for all probes were between 20°C and 60°C. The binding of the Eub338 probe with the suspended nitrifying biomass at temperatures lower than T_m was 0.40 ± 0.03 pmol/mg of dry biomass of activated sludge. At 1.08 ± 0.04 pmol/mg of dry biomass after the step-wise increase, the temperature was higher T_m. Similar values for the binding of the Nsm156 probe were 0.219 ± 0.002 pmol/mg of dry biomass of activated sludge and 0.035 ± 0.04 pmol/mg of dry biomass,

respectively.

To evaluate the non-specific binding of the probes, certain assumptions were made. It was considered that the stoichiometry of the non-specific binding of the oligonucleotide core and fluorescent label of the probes does not significantly depend on maintaining a temperature within the range of 20°C to 80°C. However, the specific binding (i.e. the hybridisation between the probe and sequence of rRNA) does depend on temperatures that occur in steps. It is known that the hybridisation is absent when the temperature is below T_m, but the hybridisation is maximised when the temperature is slightly higher than T_m. Therefore, the binding of the probe at a temperature lower than T_m may be conventionally considered as the non-specific binding of a probe with cell components. The values of T_m were within the range of 40°C to 60°C, so the binding of the probe at a temperature lower than 40°C was conventionally considered as a non-specific one. It was conventionally considered that the same value of non-specific binding was at a temperature higher than T_m, but the additional binding at this temperature was specific binding of the oligonucleotide probe (hybridisation). To calculate this specific binding (level of hybridisation), the value of the non-specific binding at a temperature lower than T_m was subtracted from the value of the total binding at a temperature higher than T_m.

The non-specific binding of the oligonucleotide probes with the biomass was 37% of the total binding of the Eub338 probe and 54% of the total binding of the Nsm156 probe. The specific binding of the probes was as follows: 0.68 ± 0.04 pmol of the Eub338 probe/mg of dry biomass and 0.016 ± 0.002 pmol of the Nsm156 probe/mg of dry biomass of the activated sludge. The ratio of the specific binding of the Nsm156 probe was 2.3:1.

Another technique, synchronous scanning fluorescence spectrometry, was used to measure the simultaneous FISH with some probes. Mixing the Eub338 and Nsm156 probes may be used to simultaneously characterise the ratio between the eubacteria and ammonium-oxidising bacteria in the suspended nitrifying biomass. The mixture of the Eub338 and Nsm156 probes may be used to simultaneously characterise the ratio between the eubacteria, ammonium-oxidising bacteria, and nitrite-oxidising bacteria in the suspended nitrifying biomass. The data are shown in Table 1. The ratio of the specific binding of the Nsm156 probe was 2.0:1.

Table 1. Whole-cell FISH with the mixtures of three probes measured by synchronous fluorescence spectrometry (average value ± standard deviation are shown).

Value	Probe	
	Eub338	Nsm156
Total binding pmol/mg of biomass.	0.75±0.08	0.04±0.01
Specific binding [a], pmol/mg of biomass.	0.47±0.05	0.02±0.00
Ratio of specific bindings.	47	:2

[a] specific binding of the probe was calculated as the difference between the total binding and the binding at a temperature lower than T_m.

The content of the ammonium-oxidising bacteria was (55±7) x 10^6 c.f.u./mg of dry biomass, and the content of the nitrite-oxidising bacteria was (23±2) x 10^6 c.f.u./mg of dry biomass. The ratio of the number of ammonium- and nitrite-oxidising bacteria in the biomass was 2.4:1.

The tests demonstrated the simplicity and short time of the analysis performed by the invented kits and methods. For example, quantitative detection of anaerobic methanogens was performed without complex media and anaerobic chamber. Quantitative detection of nitrifying bacteria by the invented kits and methods was performed within some hours but traditional detection requires a month for the cultivation of these bacteria. The quantification in the tests was performed by high quality fluorescence spectrometer but it can also be determined by simple portable fluorometer.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for quantitative and/or qualitative detection of a microorganism or nucleic acid sequence in a sample, comprising the steps of:

(a) adding to the sample, a labelled oligonucleotide probe that specifically binds to a target sequence,

(b) determining the level of binding of the labelled probe with the target sequence under low stringency conditions,

(c) determining the level of binding of the labelled probe with the target sequence under high stringency conditions,

(d) determining the level of specific binding as the difference between the level determined in step c and the level determined in step b.

2. A method according to claim 1, wherein the sample is an environmental, veterinary, agricultural, aquacultural, biotechnological or medical sample.

3. A method according to any one of claims 1 to 2, wherein the sample is anaerobic sludge.

4. A method according to any one of claims 1 to 3, wherein the sample is human or animal feces.

5. A method according to any one of claims 1 to 4, wherein the microorganism is selected from the group consisting of algae, archaeae, bacteria, fungi, protozoa, viruses or subviral agents.

6. A method according to any one of claims 1 to 5, wherein the microorganism is bacteria.

7. A method according to any one of claims 1 to 6, wherein the microorganism is a methanogen, a eubacteria

or a nitrifying bacteria.

8. A method according to any one of claims 1 to 7, wherein the nucleic acid detected is DNA or RNA.

9. A method according to any one of claims 1 to 8, wherein the nucleic acid detected is cDNA, genomic DNA, rRNA, mRNA or tRNA.

10. A method according to any one of claims 1 to 9, wherein said nucleic acid is rRNA.

11. A method according to any one of claims 1 to 10, wherein said nucleic acid is 16S RNA.

12. A method according to any one of claims 1 to 11, wherein the oligonucleotide probe is labelled with a fluorochrome, enzyme, chemiluminescent, paramagnetic or radioactive label.

13. A method according to any one of claims 1 to 12, wherein the oligonucleotide is labelled with TAMRA or TET.

14. A method according to any one of claims 1 to 13, wherein the oligonucleotide probe is Eub338, Arc915, Nsm156 or a combination of these probes.

15. A method according to any one of claims 1 to 14, further comprising the step of adding a labelled nonsense oligonucleotide to the sample, wherein the level of non-specific binding of the nonsense oligonucleotide probe is subtracted from the level of specific binding.

16. A method according to claim 15, wherein the nonsense oligonucleotide probe is ns-Eub and/or ns-Arc.

17. A method according to any one of claims 1 to 16, comprising the further step of determining the level of

label in the supernatant of the sample, and subtracting the level of label in the supernatant from the level of specific binding of the labelled oligonucleotide probe.