



(86) Date de dépôt PCT/PCT Filing Date: 2008/04/23  
(87) Date publication PCT/PCT Publication Date: 2008/11/06  
(85) Entrée phase nationale/National Entry: 2009/10/22  
(86) N° demande PCT/PCT Application No.: US 2008/005245  
(87) N° publication PCT/PCT Publication No.: 2008/133917  
(30) Priorité/Priority: 2007/04/25 (US11/740,112)

(51) Cl.Int./Int.Cl. *C07H 21/00* (2006.01),  
*C12Q 1/68* (2006.01), *C40B 30/04* (2006.01),  
*C40B 40/06* (2006.01), *C12N 15/11* (2006.01),  
*C12N 15/31* (2006.01)  
(71) Demandeur/Applicant:  
ADVANDX, INC., US  
(72) Inventeurs/Inventors:  
STENDER, HENRIK, DK;  
FIANDACA, MARK, US;  
OLIVEIRA, KENNETH, US;  
BERGERON, ELIZABETH, US  
(74) Agent: BORDEN LADNER GERVAIS LLP

(54) Titre : **SONDES D'ACIDE NUCLEIQUE PEPTIDIQUE POUR ANALYSE DE CERTAINES ESPECES DE STAPHYLOCOQUE**  
(54) Title: **PEPTIDE NUCLEIC ACID PROBES FOR ANALYSIS OF CERTAIN STAPHYLOCOCCUS SPECIES**

(57) **Abrégé/Abstract:**

The present invention relates to peptide nucleic acid (PNA) probes, PNA probe sets and methods for the analysis of certain Staphylococcus species optionally present in a sample. The invention further relates to diagnostic kits comprising such PNA probes or PNA probe sets.

## (12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
6 November 2008 (06.11.2008)

PCT

(10) International Publication Number  
**WO 2008/133917 A1**

(51) International Patent Classification:  
*C12Q 1/68* (2006.01)

(21) International Application Number:  
PCT/US2008/005245

(22) International Filing Date: 23 April 2008 (23.04.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
11/740,112 25 April 2007 (25.04.2007) US

(71) Applicant (for all designated States except US): **ADVANDX, INC.** [US/US]; 10A Roessler Road, Woburn, MA 01801 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **STENDER, Henrik** [DK/DK]; Fasanhaven 5, DK-2820 Gentofte (DK). **FIANDACA, Mark** [US/US]; 80 Main Street, Princeton, MA 01541 (US). **OLIVEIRA, Kenneth** [US/US]; 843 East Second Street, Apt. #2, Boston, MA 02127 (US). **BERGERON, Elizabeth** [US/US]; 900h Brookside Drive, Andover, MA (US).

(74) Agents: **SPARKS, Jonathan, M.** et al.; Edwards Angell Palmer & Dodge LLP, P.O. Box 55874, Boston, MA 02205 (US).

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

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

## Published:

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

(54) Title: PEPTIDE NUCLEIC ACID PROBES FOR ANALYSIS OF CERTAIN STAPHYLOCOCCUS SPECIES

(57) Abstract: The present invention relates to peptide nucleic acid (PNA) probes, PNA probe sets and methods for the analysis of certain Staphylococcus species optionally present in a sample. The invention further relates to diagnostic kits comprising such PNA probes or PNA probe sets.

WO 2008/133917 A1

5

## PEPTIDE NUCLEIC ACID PROBES FOR ANALYSIS OF CERTAIN STAPHYLOCOCCUS SPECIES

### RELATED APPLICATIONS

10 This application claims the benefit of U.S. Patent Application Serial No. 11/740,112, filed April 25, 2007. The entire content of this application is incorporated herein by this reference in their entirety.

### FIELD OF THE INVENTION

15 The present invention relates to peptide nucleic acid (PNA) probes, PNA probe sets and methods for the analysis of certain *Staphylococcus* species optionally present in a sample. The invention further relates to diagnostic kits comprising such PNA probes or PNA probe sets. The methods and kits of the invention are particularly useful for simultaneous analysis of one or more of  
20 *Staphylococcus* species other than *S. aureus* and of *S. aureus*.

### BACKGROUND

*Staphylococcus aureus* is a well-known human pathogen associated with surgical site infections, bloodstream infections (BSI) and other serious  
25 infections. In contrast, other *Staphylococcus* species, such as particularly *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus lugdenensis*, *Staphylococcus saprophyticus* and *Staphylococcus simulans* commonly found on the skin, are with the exception of catheter-related infections rarely of clinical significance. Furthermore, in  
30 those instances where a BSI is caused by a *Staphylococcus* species other than *S. aureus* there is often a difference in antibiotic susceptibility or viability between those organisms and *S. aureus*. Differentiation between *Staphylococcus* species other than *S. aureus* and *S. aureus* is therefore extremely important for directing appropriate patient therapy and patient  
35 management.



The presence of *Staphylococcus* species in clinical specimens is routinely determined by the presence of Gram-positive cocci in clusters (GPCC) by Gram staining and microscopic analysis, however; differentiation between *S. aureus* and other *Staphylococcus* species must await subculture, overnight incubation followed by biochemical analysis, such as coagulase and latex agglutination testing, or more recently by molecular testing. The presence of other *Staphylococcus* species is often determined on the basis of a negative tube coagulase test result and is typically reported as coagulase-negative staphylococci (CNS). This is particularly problematic for analysis of GPCC positive blood culture bottles as only 20-30% of GPCC positive blood bottles are due to *S. aureus* (Karlowsky et al., *Ann Clin Microbiol Antimicrob* 3:7 (2004)). In the majority of cases physicians therefore have to make decisions based on negative test results, i.e. absence of *S. aureus*.

DNA probes for analysis of *Staphylococcus aureus* and all *Staphylococcus* species (genus-specific probes) have been described (WO0066788, Kempf et al., *J. Clin. Microbiol* 38:830-838 (2000)) as well as PNA probes for the analysis of *S. aureus* (US6664045, Oliveira et al., *J. Clin. Microbiol.* 40:247-251 (2002)). These probes all target sequences that are either species-specific or genus-specific.

Comparative analysis of ribosomal RNA (rRNA) sequences or genomic DNA sequences corresponding to said rRNA (rDNA) has become a widely accepted method for establishing phylogenetic relationships between bacterial species (Woese, *Microbiol. Rev.* 51:221-271 (1987)). Consequently, Bergey's Manual of systematic bacteriology has been revised based on rRNA or rDNA sequence comparisons. Ribosomal RNA or rDNA sequence differences between closely related species enable design of species-specific probes for microbial identification thus enabling diagnostic microbiology to be based on a single genetic marker rather than a series of phenotypic markers as in traditional microbiology (DeLong et al., *Science* 342:1360-1363 (1989)). However, the design of probes targeting a cohort of species is particularly problematic and requires a combination of highly specific probe constructs and unique target sequences.

PNAs are useful candidates for investigation when developing probes targeting a subset of species because they hybridize to nucleic acids with increased sequence specificity as compared to DNA probes. Prior art  
5 therefore also comprises examples of PNA probes targeting mycobacteria other than *Mycobacterium tuberculosis* (US6753421) and enterococci other than *Enterococcus faecalis* (Oliveira et al., Abstract #D-2003, Interscience Conference on Antimicrobial Agents and Chemotherapy, September 27-30, 2002, San Diego, CA).

10 Despite its name, Peptide Nucleic Acid (PNA) is neither a peptide nor a nucleic acid, it is not even an acid. PNA is a non-naturally occurring polyamide that can hybridize to nucleic acids (DNA and RNA) with sequence specificity according to Watson-Crick base pairing rules (See: U.S. Pat. No.  
15 5,539,082) and Egholm et al., *Nature* 365:566-568 (1993)). However, whereas nucleic acids are biological materials that play a central role in the life of living species as agents of genetic transmission and expression, PNA is a recently developed totally artificial molecule, conceived in the minds of chemists and made using synthetic organic chemistry. PNA also differs  
20 structurally from nucleic acid. Although both can employ common nucleobases (A, C, G, T, and U), the backbones of these molecules are structurally diverse. The backbones of RNA and DNA are composed of repeating phosphodiester ribose and 2-deoxyribose units. In contrast, the backbones of the most common PNAs are composed on (aminoethyl)-glycine  
25 subunits. Additionally, in PNA the nucleobases are connected to the backbone by an additional methylene carbonyl moiety. PNA is therefore not an acid and therefore contains no charged acidic groups such as those present in DNA and RNA. The non-charged backbone allows PNA probes to hybridize under conditions that are destabilizing to DNA and RNA. Such  
30 attributes enable PNA probes to access targets, such as highly structured rRNA and double stranded DNA, known to be inaccessible to DNA probes (See: Stephano & Hyldig-Nielsen, IBC Library Series Publication #948. International Business Communication, Southborough, MA, pp. 19-37 (1997)).



PNA probes are not the equivalent of nucleic acid probes in structure or function.

Positive identification of *Staphylococcus* species other than *S. aureus* would be advantageous in many cases and simultaneous analysis of both *S. aureus* and other *Staphylococcus* species would be ideal as treatment decisions for the presence of either *S. aureus* or other *Staphylococcus* species would always be based on a positive test results. This feature would also offer a significant advantage when a mixture of *S. aureus* and other *Staphylococcus* species are present.

#### SUMMARY OF THE INVENTION

This invention is directed to PNA probes, or PNA probe sets and their use as well as kits useful for the analysis of certain *Staphylococcus* species, preferably those other than *Staphylococcus aureus* optionally present in a sample of interest. In accordance with the claims, the PNA probes are directed to ribosomal RNA (rRNA) or the genomic sequences corresponding to said rRNA (rDNA) or its complement.

In one embodiment, this invention is directed to PNA probes for analysis of one *Staphylococcus* species other than *S. aureus*, such as but not limited to *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus lugdenensis*, *Staphylococcus saprophyticus* and *Staphylococcus simulans*.

In another embodiment, this invention is directed to PNA probes for analysis of two or more *Staphylococcus* species other than *S. aureus*.

In one aspect, provided herein are PNA probes comprising a nucleobase sequence suitable for the analysis of one or more *Staphylococcus* species other than *S. aureus*.

In one aspect, provided herein are PNA probes comprising a nucleobase sequence suitable for the analysis of two or more *Staphylococcus* species other than *S. aureus*.

- 5 In another embodiment, a target sequence of the *Staphylococcus* species comprises rRNA, rDNA or a complement of rRNA or rDNA.

In one embodiment, the nucleobase sequence suitable for the analysis of *Staphylococcus epidermidis*, comprises a PNA probe complementary to a  
10 target sequence of *Staphylococcus epidermidis* rRNA or rDNA or its complement.

In another embodiment, the nucleobase sequence suitable for the analysis of *Staphylococcus simulans*, comprises a PNA probe complementary to a target  
15 sequence of *Staphylococcus simulans* rRNA or rDNA or its complement.

In one embodiment, the nucleobase sequence suitable for the analysis of *Staphylococcus haemolyticus*, comprises a PNA probe complementary to a  
20 target sequence of *Staphylococcus haemolyticus* rRNA or rDNA or its complement.

In another embodiment, the nucleobase sequence suitable for the analysis of *Staphylococcus lugdunensis*, comprises a PNA probe being complementary  
25 to a target sequence of *Staphylococcus lugdunensis* rRNA or rDNA or its complement.

In one embodiment, the nucleobase sequence suitable for the analysis of *Staphylococcus saprophyticus* comprises a PNA probe complementary to a  
30 target sequence of *Staphylococcus saprophyticus* rRNA or rDNA or its complement.

In one embodiment, the nucleobase sequence suitable for the analysis of *Staphylococcus epidermidis* and one or more other *Staphylococcus* species other than *Staphylococcus aureus*, comprises a PNA probe complementary to



a target sequence of *Staphylococcus epidermidis* rRNA or rDNA or its complement.

In another embodiment, the nucleobase sequence suitable for the analysis of  
5 *Staphylococcus simulans* and one or more other *Staphylococcus* species other than *Staphylococcus aureus*, comprises a PNA probe complementary to a target sequence of *Staphylococcus simulans* rRNA or rDNA or its complement.

10 In one embodiment, the nucleobase sequence suitable for the analysis of *Staphylococcus haemolyticus* and one or more other *Staphylococcus* species other than *Staphylococcus aureus*, comprises a PNA probe complementary to a target sequence of *Staphylococcus haemolyticus* rRNA or rDNA or its complement.

15 In one embodiment, the nucleobase sequence suitable for the analysis of *Staphylococcus lugdunensis* and one or more other *Staphylococcus* species other than *Staphylococcus aureus*, comprises a PNA probe complementary to a target sequence of *Staphylococcus lugdunensis* rRNA or rDNA or its  
20 complement.

In another embodiment, the nucleobase sequence suitable for the analysis of *Staphylococcus saprophyticus* and one or more other *Staphylococcus* species other than *Staphylococcus aureus*, comprises a PNA probe complementary to  
25 a target sequence of *Staphylococcus saprophyticus* rRNA or rDNA or its complement.

In one embodiment, at least a portion of the probe is at least about 86% identical to the nucleobase sequence or complement thereof selected from  
30 the following sequences: AGA-CGT-GCA-TAG-T (Seq. Id. No. 1), GCT-AAT-ACG-GCG (Seq. Id. No. 2), GCT-AAT-ACG-CCG-C (Seq. Id. No. 3).



In another embodiment, at least a portion of the probe is selected from the following sequences: AGA-CGT-GCA-TAG-T (Seq. Id. No. 1), GCT-AAT-ACG-GCG (Seq. Id. No. 2), GCT-AAT-ACG-CCG-C (Seq. Id. No. 3).

- 5 In one embodiment, the probe sequence is between about 8-17 subunits in length.

In one embodiment, the probe is labeled with at least one detectable moiety.

- 10 In one embodiment, the detectable moiety or moieties are selected from the group consisting of: a conjugate, a branched detection system, a chromophore, a fluorophore, a spin label, a radioisotope, an enzyme, a hapten, an acridinium ester and a luminescent compound.
- 15 In one embodiment, the probe is self-reporting. In another embodiment, the probe is a PNA Linear Beacon. In one embodiment, the probe is unlabeled. In one embodiment, the probe is bound to a support. In another embodiment, the probe further comprises a spacer or a linker.
- 20 In one embodiment, in situ hybridization is used for analysis of one or more *Staphylococcus* species other than *S. aureus*.

In one aspect, provided herein are PNA probe sets one or more PNA probes of claims 1-24 and at least one PNA probe for the analysis of *S. aureus*.

25

In one embodiment, the probes are differently labeled for independent analysis of two or more *Staphylococcus* species.

- In one aspect, provided herein are PNA probe sets comprising one or more
- 30 PNA probes of claims 1-24 and at least one PNA blocking probe for the analysis of one or more *Staphylococcus* species other than *S. aureus*.

In another embodiment, the probe sets further comprise a PNA blocking probe for analysis of *S. aureus*.

In one aspect, provided herein are methods for the analysis of Staphylococcus species other than *S. aureus* in a sample, comprising: a) contacting at least one of the PNA probes of claims 1-18 to the sample, b) hybridizing the PNA probe to a target sequence of Staphylococcus species other than *S. aureus* in the sample; and c) detecting the hybridization, wherein the detection of hybridization is indicative of the presence, identity and/or amount of Staphylococcus species other than *S. aureus* in the sample.

- 10 In one aspect, provided herein are methods for the analysis of two or more Staphylococcus species, said method comprising: a) contacting a PNA probe set of claims 1-18 to the sample, b) hybridizing the PNA probes to a target sequence of Staphylococcus species in the sample; and c) detecting the hybridization, wherein the detection of hybridization is indicative of the  
15 presence, identity and/or amount of Staphylococcus species in the sample.

In one embodiment, two or more Staphylococcus species are *S. aureus* and one or more Staphylococcus species other than *S. aureus*.

- 20 In one embodiment the probe sets are used for analysis of a cohort of Staphylococcus species other than *S. aureus*.

In one embodiment, the cohort of Staphylococcus species other than *S. aureus* detected is Coagulase-Negative Staphylococci, clinically significant  
25 Coagulase-Negative Staphylococci, or a subset of clinically significant Coagulase-Negative Staphylococci.

In another embodiment, the probes are independently detectable non-independently detectable, or a combination of independently and non-  
30 independently detectable, wherein the probes differ from one another by as little as a single base, and are complementary or substantially complementary to partially conserved target regions of phylogenetically related organisms.

In one embodiment, probes or probe sets are used to eliminate or reduce cross hybridization between a *Staphylococcus aureus* specific probe, and a *Staphylococcus scheiferi* target.

- 5 In one embodiment, *S. aureus* and one or more *Staphylococcus* species other than *S. aureus* are simultaneously and independently detected.

In one embodiment, the analysis takes place in situ. In another embodiment, the analysis takes place by fluorescence in situ hybridization.

10

In one embodiment, the methods are used to detect a nucleic acid comprising a target sequence wherein said nucleic acid has been synthesized or amplified in a reaction.

- 15 In one embodiment, preferred nucleic acid synthesis or nucleic acid amplification reactions are selected from the group consisting of: Polymerase Chain Reaction (PCR), Ligase Chain Reaction (LCR), Strand Displacement Amplification (SDA), Transcription-Mediated Amplification (TMA), Rolling Circle Amplification (RCA) and Q beta replicase.

20

In one embodiment, the methods further comprise adding at least one blocking probe to reduce or eliminate hybridization of the PNA probe to non-target sequence.

- 25 In another embodiment, the target sequence is immobilized to a surface. In one embodiment, the PNA probe is immobilized to a surface. In one embodiment, the PNA probe is one component of an array. In one embodiment, the sample is a biological sample. In another embodiment, the biological sample is blood, urine, secretion, sweat, sputum, stool, mucous, or  
30 cultures thereof.

In one aspect, provided herein are kits suitable for performing an assay for analysis of one or more *Staphylococcus* species other than *S. aureus* in a



sample, wherein said kit comprises: a) a PNA probe according to claim 1 to 24 and b) other reagents or compositions necessary to perform the assay.

In one embodiment, one or more *Staphylococcus* species other than *S. aureus* and at least one other microorganism optionally present in a sample are independently detected, identified and/or quantitated.

In one embodiment, one or more *Staphylococcus* species other than *S. aureus* and *S. aureus* optionally present in a sample are independently detected, identified and/or quantitated.

In another embodiment, one or more *Staphylococcus* species other than *S. aureus* optionally present in a sample is detected, identified and/or quantitated and its susceptibility to antimicrobial agents is determined.

In one embodiment, one or more *Staphylococcus* species other than *S. aureus* detected is Coagulase-Negative *Staphylococci*, clinically significant Coagulase-Negative *Staphylococci*, or a subset of clinically significant Coagulase-Negative *Staphylococci*.

In another embodiment, the kit is used in an in situ hybridization assay.

In one embodiment, the kit is a fluorescence in situ hybridization assay for simultaneous, independent (multiplex) identification of one more *Staphylococcus* species other than *S. aureus* and of *S. aureus*.

In one embodiment, the kit is used for a real-time PCR assay.

In one embodiment, the kit is used to examine clinical samples such as clinical specimens or cultures thereof.

Other embodiments are disclosed *infra*.

## DETAILED DESCRIPTION OF THE INVENTION

## I. Definitions:

As used herein, the term "nucleobase" means those naturally occurring and those non-naturally occurring heterocyclic moieties commonly known to those who utilize nucleic acid technology or utilize peptide nucleic acid technology to thereby generate polymers that can sequence specifically bind to nucleic acids.

As used herein, the term "nucleobase sequence" means any segment of a polymer that comprises nucleobase-containing subunits. Non-limiting examples of suitable polymers or polymer segments include oligodeoxynucleotides, oligoribonucleotides, peptide nucleic acids, nucleic acid analogs, nucleic acid mimics, and/or chimeras.

As used herein, the term "target sequence" means the nucleobase sequence that is to be detected in an assay.

As used herein, the term "probe" means a polymer (e. g. a DNA, RNA, PNA, chimera or linked polymer) having a probing nucleobase sequence that is designed to sequence-specifically hybridize to a target sequence of a target molecule of an organism of interest.

As used herein, the term "peptide nucleic acid" or "PNA" means any oligomer, linked polymer or chimeric oligomer, comprising two or more PNA subunits (residues), including any of the polymers referred to or claimed as peptide nucleic acids in United States Patent Nos. 5,539,082, 5,527,675, 5,623,049, 5,714,331, 5,736,336, 5,773,571, 5,786,461, 5,837,459, 5,891,625, 5,972,610, 5,986,053, 6,107,470 and 6,357,163. In the most preferred embodiment, a PNA subunit consists of a naturally occurring or non-naturally occurring nucleobase attached to the aza nitrogen of the N- [2- (aminoethyl)] glycine backbone through a methylene carbonyl linkage.

As used herein, the terms "label" and "detectable moiety" are interchangeable and shall refer to moieties that can be attached to a probe to thereby render



the probe detectable by an instrument or method.

Reference herein to "*Staphylococcus* species other than *S. aureus*," or a related phrase means essentially one or more *Staphylococcus* species of the *Staphylococcus* genus except for *S. aureus*. With a few exceptions, *Staphylococcus* species other than *S. aureus* is equivalent to coagulase-negative staphylococci, which is a medical expression for *Staphylococcus* species other than *S. aureus*. A cohort of *Staphylococcus* species other than *S. aureus* essentially means two or more species of *Staphylococcus* species other than *S. aureus* and may comprise all coagulase-negative staphylococci, clinically significant coagulase-negative staphylococci or a subset of these. *Staphylococcus* species other than *Staphylococcus aureus*, may include, for example *S. capitis*, *S. cohnii*, *S. epidermidis*, *S. saprophyticus*, *S. intermedius*, *S. hyicus*, *S. haemolyticus*, *S. hominis*, *S. lugdunensis*, *S. saccharolyticus*, *S. schleiferi*, *S. sciuri*, *S. simulans*, *S. warneri* and/or *S. xylosus*.

The term "sample" as used herein refers to any biological sample or clinical sample that could contain an analyte for detection. Preferably the biological sample is in liquid form or as a tissue sample. Most preferably, the sample is from blood culture. Liquid samples include clinical samples, e.g. urine, blood, wounds, sputum, laryngeal swabs, gastric lavage, bronchial washings, aspirates, serum, nasal discharge, sweat, plasma, semen, cerebrospinal fluid, tears, pus, amniotic fluid, saliva, lung aspirate, gastrointestinal contents, vaginal discharge, urethral discharge, expectorates and cultures thereof. Preferred tissue samples or cultures thereof include chorionic villi specimens, skin epithelials, genitalia epithelials, gum epithelials, throat epithelials, hair and biopsies. Tissue samples may be either freshly prepared, or preserved for some time in a fixative such as but not limited to ethanol, methanol, paraformaldehyde, glutaraldehyde, formalin, paraffin, formaldehyde, formamide, or mixtures thereof. Non-clinical samples include food, beverages, water, pharmaceutical products, personal care products, dairy products or environmental samples and cultures thereof.

As used herein, "multiplex assay" includes assays in which multiple targets can potentially be detected, and identified. Identification can be specific, e.g., a particular species of microorganism, or generic, e.g., a particular genus of microorganism. Generic identification may also include the identification of a cohort of species of microorganisms, which includes one or more members of



a defined group of species. One example of a defined group of species is the coagulase-negative staphylococci.

As used herein, independent and simultaneous detection includes an assay  
5 that can at once yield identification results for more than one target. For example, the detection of one or species of *Staphylococcus* other than *aureus*, and *Staphylococcus aureus* may be done at the same time with unique labels for each probe, thus resulting in independent and simultaneous detection.

10 Other reagents or compositions necessary to perform the assay, may include, for example, wash solutions, slides, coverslips, one or more PNA probes according to the invention, culture vessels, slide warmer, incubator, mounting fluid, and PCR components, including enzymes and buffers.

15 PNA probes (originally described in United States Patent No. 5,539,082 and Egholm et al., Nature 365:566-568 (1993), herein attached as reference) have inherent physico/chemical characteristics as compared to naturally occurring nucleic acid probes, which allow the design of rapid and accurate assays.

20 PNA probes offer another advantage over nucleic acid probes when applied in fluorescence *in situ* hybridization (FISH) assays due to their improved cellular penetration of the rigid cell wall of Gram-positive bacteria such as *Staphylococcus* species. Where nucleic acid probes require fixation and permeabilization with cross-linking agents and/or enzymes (for example see  
25 Kempf et al., *J. Clin. Microbiol* 38:830-838 (2000)), PNA probes can be applied directly following smear preparation as exemplified in Example 1.

In preferred embodiments, PNA probes have relatively short nucleobase sequences, such as 15 nucleobases as described in Example 1. Naturally  
30 occurring nucleic acid probes due to their weaker stabilities and lower melting temperatures ( $T_m$ ) are typically at least 18 nucleobases in length (For example see Kempf et al., *J. Clin. Microbiol* 38:830-838 (2000)). The greater specificity of PNA probes provides better discrimination to closely related non-target sequences with a single or just a few nucleobase difference(s) as

required for analysis of rRNA or rDNA of closely related *Staphylococcus* species.

Exemplary PNA probe nucleobase sequences according to the invention  
5 include: AGA-CGT-GCA-TAG-T (Seq. Id. No. 1), GCT-AAT-ACG-GCG (Seq. Id. No. 2) and GCT-AAT-ACG-CCG-C (Seq. Id. No. 3).

In yet another embodiment, the PNA probes may be part of a PNA probe set comprising either two or more PNA probes for analysis of two or more species  
10 of *Staphylococcus* species other than *S. aureus* or at least one PNA probe for analysis of *Staphylococcus* species other than *S. aureus* and a PNA probe for analysis of *S. aureus*. That is, some PNA probes of the invention are specific for two or more species of *Staphylococcus* species other than *S. aureus*. Preferably, PNA probes within a PNA probe set are differently labeled for  
15 independent analysis of two or more *Staphylococcus* species. In a modification of this embodiment, and as exemplified in Examples 1 and 2, multiple probes may be identically labeled to detect a particular *Staphylococcus* species or cohort of *Staphylococcus* species, while an optional other probe or probes is differently labeled for analysis of a second  
20 species or cohort of species.

The method according to the invention comprises contacting a sample with one or more of the PNA probes described above. According to the method, the presence, absence and/or number of *Staphylococcus* species other than  
25 *Staphylococcus aureus* are detected, identified and/or quantitated by correlating the hybridization, under suitable hybridization conditions, of the probing nucleobase sequence of the probe to the target sequence. Consequently, the analysis is based on a single assay with a definitive outcome. In contrast, current routine methods for definitive analysis of  
30 *Staphylococcus* species other than *S. aureus* are based on multiple phenotypic characteristics involving multiple tests.

In another embodiment, the PNA probes are applied simultaneously with previously published PNA probes for analysis of *Staphylococcus aureus* (see



Oliveira et al., J. Clin. Microbiol. 40:247-251 (2002)) either in parallel reactions or in the same reaction (multiplex) for simultaneous analysis of *Staphylococcus aureus* and *Staphylococcus* species other than *S. aureus*.

This way the presence of *Staphylococcus* species other than *S. aureus* are further supported by the absence of *S. aureus* specific signal or visa versa, such final test results are interpreted on the basis on both a positive and a negative reaction. The PNA probe set therefore provides internal controls which eliminate the need to perform separate control experiments. Preferably, the two PNA probes are independently labeled such that the analysis is performed in one reaction (multiplex). Simultaneous analysis is also an advantage for specimens containing a mixture of both *S. aureus* and *Staphylococcus* species other than *S. aureus*. In such cases the use of the PNA probes for FISH offers the advantage of single cell detection, such that cells of both *Staphylococcus* species other than *S. aureus*, and *S. aureus* can be viewed simultaneously and differentiated by specific labels as exemplified below. In contrast other technologies are not able to distinguish mixed cultures from false-positive reactions without performing additional control experiments.

In still another embodiment, this invention is directed to kits suitable for performing an assay that detects, identifies and/or quantitates *Staphylococcus* species other than *Staphylococcus aureus* optionally present in a sample and/or determination of antibiotic resistance. The kits of this invention comprise one or more PNA probes and other reagents or compositions that are selected to perform an assay or otherwise simplify the performance of an assay. In particular, the combined analysis of *Staphylococcus* species other than *S. aureus* and *Staphylococcus aureus* is well-suited for routine testing of GPCC positive blood culture bottles where the use of multiple PNA probes serve secondarily as internal controls.

Another benefit derived from the use of multiple PNA probes is the use of blocking probes. In this preferred embodiment, the blocking probe strategy is employed in the design of probes for use in a multiplex assay where probes are directed against similar regions of conserved target molecules in closely



related organisms. For example, a pair of independently detectable probes which are substantially similar in nucleobase sequence and  $T_m$  may be designed to hybridize to highly conserved regions of two targets in which the targeted nucleobase sequences differ by only one base. In this case, the tendency of the first probe to hybridize non-specifically to the complementary target of the second probe is discouraged by the presence and relative stability of the second probe/target hybrid. Similarly, multiple probes may be designed which are either independently detectable or non-independently detectable, or a combination of independently and non-independently detectable, which all differ from one another by as little as a single base, and which are complementary, or at least substantially complementary to partially conserved target regions of phylogenetically related organisms. The competition for target sites can result in higher probe specificities, while lowering the likelihood of cross hybridization.

Those of ordinary skill in the art will appreciate that a suitable PNA probe need not have exactly these probing nucleobase sequences described herein to be operative but may be modified according to the particular assay conditions. For example, shorter PNA probes can be prepared by truncation of the nucleobase sequence if the stability of the hybrid needs to be modified to thereby lower the  $T_m$  and/or adjust for stringency. Similarly, the nucleobase sequence may be truncated at one end and extended at the other end as long as the discriminating nucleobases remain within the sequence of the PNA probe. Such variations of the probing nucleobase sequences within the parameters described herein are considered to be embodiments of this invention.

Those of ordinary skill in the art will also appreciate that the complement probing sequence is equally suitable for assays, such as but not limited to real-time PCR, that are directed against rDNA as a target sequence.

## 2. Description

### I. General:

#### PNA Synthesis:

Methods for the chemical assembly of PNAs are well known (see: Patent Nos.

5 5,539,082, 5,527,675, 5,623,049, 5,714,331, 5,736,336, 5,773,571, 5,786,461, 5,837,459, 5,891,625, 5,972,610, 5,986,053 and 6,107,470).

#### PNA Labeling:

10 Preferred non-limiting methods for labeling PNAs are described in US 6,110,676, 6,361,942, and 6,355,421, the examples section of these specifications or are otherwise well known in the art of PNA synthesis and peptide synthesis.

#### 15 Labels:

Non-limiting examples of detectable moieties (labels) suitable for labeling PNA probes used in the practice of this invention would include a dextran conjugate, a branched nucleic acid detection system, a chromophore, a fluorophore, a spin label, a radioisotope, an enzyme, a hapten, an acridinium  
20 ester and a chemiluminescent compound.

Other suitable labeling reagents and preferred methods of attachment would be recognized by those of ordinary skill in the art of PNA, peptide or nucleic acid synthesis.

25

Preferred haptens include 5 (6)-carboxyfluorescein, 2,4-dinitrophenyl, digoxigenin, and biotin.

Preferred fluorochromes (fluorophores) include 5 (6)-carboxyfluorescein (Flu),  
30 tetramethyl-6-carboxyrhodamine (tamra), 6- ((7- amino-4-methylcoumarin-3-acetyl) amino) hexanoic acid (Cou), 5 (and 6)-carboxy-X- rhodamine (Rox), Cyanine 2 (Cy2) Dye, Cyanine 3 (Cy3) Dye, Cyanine 3.5 (Cy3.5) Dye, Cyanine 5 (Cy5) Dye, Cyanine 5.5 (Cy5.5) Dye Cyanine 7 (Cy7) Dye, Cyanine 9 (Cy9) Dye (Cyanine dyes 2, 3, 3.5, 5 and 5.5 are available as NHS esters

from Amersham, Arlington Heights, IL), JOE, Tamara or the Alexa dye series (Molecular Probes, Eugene, OR).

Preferred enzymes include polymerases (e. g. Taq polymerase, Klenow PNA  
5 polymerase, T7 DNA polymerase, Sequenase, DNA polymerase 1 and phi29  
polymerase), alkaline phosphatase (AP), horseradish peroxidase (HRP) and  
most preferably, soy bean peroxidase (SBP).

#### Unlabeled Probes:

10 The probes that are used for the practice of this invention need not be labeled  
with a detectable moiety to be operable within the methods of this invention,  
for example, the probes of the invention may be attached to a solid support,  
which renders them detectable by known array technologies.

#### 15 Self-Indicating Probes:

Beacon probes are examples of self-indicating probes which include a donor  
moiety and an acceptor moiety. The donor and acceptor moieties operate  
such that the acceptor moieties accept energy transferred from the donor  
moieties or otherwise quench signal from the donor moiety. Though the  
20 previously listed fluorophores (with suitable spectral properties) might also  
operate as energy transfer acceptors, preferably, the acceptor moiety is a  
quencher moiety. Preferably, the quencher moiety is a non-fluorescent  
aromatic or heteroaromatic moiety. The preferred quencher moiety is 4-((-4-  
(dimethylamino) phenyl) azo) benzoic acid (dabcyl). In a preferred  
25 embodiment, the self-indicating Beacon probe is a PNA Linear Beacon as  
more fully described in US 6,485,901.

In another embodiment, the self-indicating probes of this invention are of the  
type described in WIPO patent application W097/45539. These self-indicating  
30 probes differ as compared with Beacon probes primarily in that the reporter  
must interact with the nucleic acid to produce signal.

#### Spacer/Linker Moieties:



Generally, spacers are used to minimize the adverse effects that bulky labeling reagents might have on hybridization properties of probes. Preferred spacer/linker moieties for the nucleobase polymers of this invention consist of one or more aminoalkyl carboxylic acids (e.g., aminocaproic acid), the side chain of an amino acid (e.g., the side chain of lysine or ornithine), natural amino acids (e.g., glycine), aminooxyalkylacids (e.g., 8-amino-3,6-dioxaoctanoic acid), alkyl diacids (e.g., succinic acid), alkyloxy diacids (e.g., diglycolic acid) or alkyldiamines (e.g., 1, 8-diamino-3, 6-dioxaoctane).

#### 10 Hybridization Conditions/Stringency:

Those of ordinary skill in the art of nucleic acid hybridization will recognize that factors commonly used to impose or control stringency of hybridization include formamide concentration (or other chemical denaturant reagent), salt concentration (i.e., ionic strength), hybridization temperature, detergent concentration, pH and the presence or absence of chaotropes. Optimal stringency for a probe/target sequence combination is often found by the well-known technique of fixing several of the aforementioned stringency factors and then determining the effect of varying a single stringency factor. The same stringency factors can be modulated to thereby control the stringency of hybridization of a PNA to a nucleic acid, except that the hybridization of a PNA is fairly independent of ionic strength. Optimal stringency for an assay may be experimentally determined by examination of each stringency factor until the desired degree of discrimination is achieved.

#### 25 Suitable Hybridization Conditions:

Generally, the more closely related the background causing nucleic acid contaminants are to the target sequence, the more carefully stringency must be controlled. Blocking probes may also be used as a means to improve discrimination beyond the limits possible by optimization of stringency factors. Suitable hybridization conditions will thus comprise conditions under which the desired degree of discrimination is achieved such that an assay generates an accurate (within the tolerance desired for the assay) and reproducible result.

Aided by no more than routine experimentation and the disclosure provided herein, those of skill in the art will easily be able to determine suitable hybridization conditions for performing assays utilizing the methods and compositions described herein. Suitable *in situ* hybridization or PCR

5 conditions comprise conditions suitable for performing an *in situ* hybridization or PCR procedure. Thus, suitable *in situ* hybridization or PCR conditions will become apparent to those of skill in the art using the disclosure provided herein, with or without additional routine experimentation.

#### 10 Blocking Probes:

Blocking probes are nucleic acid or non-nucleic acid probes that can be used to suppress the binding of the probing nucleobase sequence of the probing polymer to a non-target sequence. Preferred blocking probes are PNA probes (see: US 6,110,676). It is believed that blocking probes operate by

15 hybridization to the non-target sequence to thereby form a more thermodynamically stable complex than is formed by hybridization between the probing nucleobase sequence and the non-target sequence. Through formation of the more stable and preferred complex, the less stable non-preferred complex between the probing nucleobase sequence and the non-

20 target sequence is prevented from forming. Thus, blocking probes can be used with the methods, kits and compositions of this invention to suppress the binding of the probes to a non-target sequence that might be present and interfere with the performance of the assay.

#### 25 Probing Nucleobase Sequence:

The probing nucleobase sequence of a probe of this invention is the specific sequence recognition portion of the construct. Therefore, the probing nucleobase sequence is a nucleobase sequence designed to hybridize to a specific target sequence wherein the presence, absence or amount of the

30 target sequence can be used to directly or indirectly detect the presence, absence or number of organisms of interest in a sample. Consequently, with due consideration to the requirements of a probe for the assay format chosen, the length and sequence composition of the probing nucleobase sequence of



the probe will generally be chosen such that a stable complex is formed with the target sequence under suitable hybridization conditions.

The preferred nucleobase sequences of the probes of this invention for  
5 analysis of *Staphylococcus* species other than *S. aureus* are: AGA-CGT-  
GCA-TAG-T (Seq. ID. No. 1), GCT-AAT-ACG-GCG (Seq. ID. No. 2), GCT-  
AAT-ACG-CCG-C (Seq. ID. No. 3), and the complements thereof.

This invention contemplates that variation in these identified probing  
10 nucleobase sequences shall also provide probes that are suitable for the  
analysis of *Staphylococcus* species other than *S. aureus*. Such variation of  
the probing nucleobase sequences within the parameters described herein  
are considered to be an embodiment of this invention. Common variations  
include, deletions, insertions and frame shifts. Additionally, a shorter probing  
15 nucleobase sequence can be generated by truncation of the sequences  
identified above.

A probe of this invention will generally have a probing nucleobase sequence  
that is exactly complementary to the target sequence. Alternatively, a  
20 substantially complementary probing nucleobase sequence might be used  
since it has been demonstrated that greater sequence discrimination can be  
obtained when utilizing probes wherein there exists one or more point  
mutations (base mismatch) between the probe and the target sequence (See:  
Guo et al., Nature Biotechnology 15: 331-335 (1997)). Consequently, the  
25 probing nucleobase sequence may be only as much as 86% homologous to  
the probing nucleobase sequences identified above. Substantially  
complementary probing nucleobase sequences within the parameters  
described above are considered to be an embodiment of this invention.

30 Complements of the probing nucleobase sequence are considered to be an  
embodiment of this invention, since it is possible to generate a suitable probe  
if the target sequence to be detected has been amplified or copied to thereby  
generate the complement to the identified target sequence.

Detection, identification and/or enumeration:

By detection is meant analysis for the presence or absence of the organism optionally present in the sample. By identification is meant establishment of the identity of the organism by genus name, by genus and species name, or by other suitable category which serves to classify the organism(s) of interest. By quantitation is meant enumeration of the organisms in a sample. Some assay formats provide simultaneous detection, identification and enumeration (for example see Stender, H. et al., *J. Microbiol. Methods*. 45:31-39 (2001), others provide detection and identification (for example see Stender, H. et al., *Int. J. Tuberc. Lung Dis.* 3:830-837 (1999) and yet other assay formats just provide identification (for example see Oliveira, K et al. *J. Clin. Microbiol.* 40:247-251 (2002)).

Independent and Simultaneous Detection:

In a preferred embodiment of this invention, a multiplex assay is designed to detect one target with a labeled probe, while simultaneously detecting another target with a differently labeled probe. In a more preferred embodiment of the invention, the targets constitute rRNA or rDNA from two or more *Staphylococcus* species. In the most preferred embodiment, the assay is a PNA FISH assay designed to detect one or species of *Staphylococcus* other than *aureus*, and *Staphylococcus aureus*.

Antibiotic resistance

By determination of resistance to antibiotics is meant analysis of an organism's susceptibility to antibiotics based on specific genes or gene products, or mutations associated with resistance or susceptibility to antimicrobial agents.

II. Preferred Embodiments of the Invention:

a. PNA Probes:

In one embodiment, this invention is directed to PNA probes. The PNA probes of this invention are suitable for detecting, identifying and/or quantitating *Staphylococcus* species other than *S. aureus* optionally present in a sample.



General characteristics (e.g., length, labels, nucleobase sequences, linkers, etc.) of PNA probes suitable for the analysis have been previously described herein. The preferred probing nucleobase sequence of PNA probes of this invention are listed in Table 1.

5

Table 1

	1	2
<b>A</b>	Sequence ID	Nucleobase sequence
<b>B</b>	Seq. ID. No. 1	AGA-CGT-GCA-TAG-T
<b>C</b>	Seq. ID. No. 2	GCT-AAT-ACG-GCG
<b>D</b>	Seq. ID. No. 3	GCT-AAT-ACG-CCG-C

The PNA probes of this invention may comprise only a probing nucleobase sequence (as previously described herein) or may comprise additional  
 10 moieties. Non-limiting examples of additional moieties include detectable moieties (labels), linkers, spacers, natural or non-natural amino acids, or other subunits of PNA, DNA or RNA. Additional moieties may be functional or non-functional in an assay. Generally however, additional moieties will be selected to be functional within the design of the assay in which the PNA  
 15 probe is to be used. The preferred PNA probes of this invention are labeled with one or more detectable moieties selected from the group consisting of fluorophores, enzymes and haptens.

In preferred embodiments, the probes of this invention are used in *in situ*  
 20 hybridization (ISH) and fluorescence *in situ* hybridization assays. Excess probe used in an ISH or FISH assay typically must be removed so that the detectable moiety of the specifically bound probe can be detected above the background signal that results from still present but unhybridized probe. Generally, the excess probe is washed away after the sample has been  
 25 incubated with probe for a period of time. However, the use of self-indicating probes is a preferred embodiment of this invention, since there is no requirement that excess self-indicating probe be completely removed (washed away) from the sample since it generates little or no detectable background.

In addition to ISH or FISH assays, self-indicating probes comprising the selected probing nucleobase sequence described herein are particularly useful in all kinds of homogeneous assays such as in real-time PCR or useful with self-indicating devices (e. g. lateral flow assay) or self-indicating arrays.

5

b. PNA probe sets

Probe sets of this invention comprise two or more PNAs. In one embodiment, some of the PNA probes of the set can be blocking probes. In other embodiments, the probe set can be used to analyze two or more

10 *Staphylococcus* species other than *S. aureus*, or for the analysis of one or more *Staphylococcus* species other than *S. aureus*, and *S. aureus*.

c. Methods:

In another embodiment, this invention is directed to a method suitable for  
15 analysis of *Staphylococcus* species other than *S. aureus* optionally in a sample. The general and specific characteristics of PNA probes suitable for the analysis of *Staphylococcus* species other than *S. aureus* have been previously described herein. Preferred probing nucleobase sequences are listed in Table 1.

20

The method for analysis of *Staphylococcus* species other than *S. aureus* in a sample comprises contacting the sample with one or more PNA probes suitable for hybridization to a target sequence which is specific to *Staphylococcus* species other than *S. aureus*. According to the method,  
25 *Staphylococcus* species other than *S. aureus* in the sample is then detected, identified and/or quantitated or its resistance to antibiotics is determined. This is made possible by correlating hybridization, under suitable hybridization conditions or suitable *in situ* hybridization conditions, of the probing nucleobase sequence of a PNA probe to the target sequence of  
30 *Staphylococcus* species other than *S. aureus* sought to be detected with the presence, absence or number of the *Staphylococcus* species other than *S. aureus* organisms in the sample. Typically, this correlation is made possible by direct or indirect detection of the probe/target sequence hybrid. In a preferred embodiment, a PNA probe set is used for simultaneous analysis of



*Staphylococcus* species other than *S. aureus* and *S. aureus* using differently labeled PNA probes.

Fluorescence *in situ* Hybridization and Real-time PCR:

5 The PNA probes, methods, kits and compositions of this invention are particularly useful for the rapid probe-based analysis of *Staphylococcus* species other than *S. aureus*, preferably using PNA probe sets for simultaneous analysis of two or more *Staphylococcus* species. In preferred embodiments, *in situ* hybridization or PCR is used as the assay format for  
10 analysis of *Staphylococcus* species other than *S. aureus*. Most preferably, fluorescence *in situ* hybridization (PNA FISH) or real-time PCR is the assay format (Reviewed by Stender et al. J. Microbiol. Methods 48:1-17 (2002)). Preferably, smears for PNA FISH analysis are not treated with cross-linking agents or enzymes prior to hybridization.

15

In one embodiment, the method includes synthesizing a nucleic acid from a sample, for example by nucleic acid amplification. Preferred nucleic acid amplification reactions are selected from the group consisting of: Polymerase Chain Reaction (PCR), Ligase Chain Reaction (LCR), Strand Displacement  
20 Amplification (SDA), Transcription-Mediated Amplification (TMA), Rolling Circle Amplification (RCA) and Q beta replicase.

The practice of the present invention may employ, unless otherwise indicated, conventional techniques and descriptions of organic chemistry, polymer  
25 technology, molecular biology (including recombinant techniques), cell biology, biochemistry, and immunology, which are within the skill of the art. Such conventional techniques include polymer array synthesis, hybridization, ligation, and detection of hybridization using a label. Specific illustrations of suitable techniques can be had by reference to the example herein below.  
30 However, other equivalent conventional procedures can, of course, also be used. Such conventional techniques and descriptions can be found in standard laboratory manuals such as Genome Analysis: A Laboratory Manual Series (Vols. I-IV), Using Antibodies: A Laboratory Manual, Cells: A Laboratory Manual, PCR Primer: A Laboratory Manual, and Molecular



Cloning: A Laboratory Manual (all from Cold Spring Harbor Laboratory Press),  
Stryer, L. (1995) Biochemistry (4th Ed.). Methods and techniques applicable  
to polymer (including protein) array synthesis have been described in U.S.

Ser. No. 09/536,841, WO 00/58516, U.S. Pat. Nos. 5,143,854, 5,242,974,

5 5,252,743, 5,324,633, 5,384,261, 5,405,783, 5,424,186, 5,451,683,

5,482,867, 5,491,074, 5,527,681, 5,550,215, 5,571,639, 5,578,832,

5,593,839, 5,599,695, 5,624,711, 5,631,734, 5,795,716, 5,831,070,

5,837,832, 5,856,101, 5,858,659, 5,936,324, 5,968,740, 5,974,164,

5,981,185, 5,981,956, 6,025,601, 6,033,860, 6,040,193, 6,090,555,

10 6,136,269, 6,269,846 and 6,428,752, in PCT Applications Nos.

PCT/US99/00730 (International Publication Number WO 99/36760) and

PCT/USO1/04285, which are all incorporated herein by reference in their

entirety for all purposes. Patents that describe synthesis techniques in

specific embodiments include U.S. Pat. Nos. 5,412,087, 6,147,205,

15 6,262,216, 6,310,189, 5,889,165, and 5,959,098. For PCR methods see,

e.g., PCR Technology: Principles and Applications for DNA Amplification (Ed.

H. A. Erlich, Freeman Press, NY, N.Y., 1992); PCR Protocols: A Guide to

Methods and Applications (Eds. Innis, et al., Academic Press, San Diego,

Calif., 1990); Mattila et al., Nucleic Acids Res. 19, 4967 (1991); Eckert et al.,

20 PCR Methods and Applications 1, 17 (1991); PCR (Eds. McPherson et al.,

IRL Press, Oxford); and U.S. Pat. Nos. 4,683,202, 4,683,195, 4,800,159

4,965,188, and 5,333,675, and each of which is incorporated herein by

reference in their entireties for all purposes. The sample may be amplified on

the array. See, for example, U.S. Pat. No. 6,300,070 and U.S. patent

25 application Ser. No. 09/513,300, which are incorporated herein by reference.

Nucleic acid arrays that are useful in the present invention include those that

are commercially available from Affymetrix (Santa Clara, Calif.). Other

suitable amplification methods include the ligase chain reaction (LCR) (e.g.,

Wu and Wallace, Genomics 4, 560 (1989), Landegren et al., Science 241,

30 1077 (1988) and Barringer et al. Gene 89:117 (1990)), transcription

amplification (Kwoh et al., Proc. Natl. Acad. Sci. USA 86, 1173 (1989) and

WO88/10315), self-sustained sequence replication (Guatelli et al., Proc. Nat.

Acad. Sci. USA, 87, 1874 (1990) and WO90/06995), selective amplification of

target polynucleotide sequences (U.S. Pat. No. 6,410,276), consensus

sequence primed polymerase chain reaction (CP-PCR) (U.S. Pat. No. 4,437,975), arbitrarily primed polymerase chain reaction (AP-PCR) (U.S. Pat. No. 5,413,909, 5,861,245) and nucleic acid based sequence amplification (NABSA, see, U.S. Pat. Nos. 5,409,818, 5,554,517, and 6,063,603, each of which is incorporated herein by reference). Other amplification methods that may be used are described in, U.S. Pat. Nos. 5,242,794, 5,494,810, 4,988,617 and in U.S. Ser. No. 09/854,317, each of which is incorporated herein by reference.

Additional methods of sample preparation and techniques for reducing the complexity of a nucleic sample are described in Dong et al., Genome Research 11, 1418 (2001), in U.S. Pat. Nos. 6,361,947, 6,391,592 and U.S. patent application Ser. Nos. 09/916,135, 09/920,491, 09/910,292, and 10/013,598.

Methods for conducting polynucleotide hybridization assays have been well developed in the art. Hybridization assay procedures and conditions will vary depending on the application and are selected in accordance with the general binding methods known including those referred to in: Maniatis et al.

Molecular Cloning: A Laboratory Manual (2<sup>sup</sup>.nd Ed. Cold Spring Harbor, N.Y, 1989); Berger and Kimmel Methods in Enzymology, Vol. 152, Guide to Molecular Cloning Techniques (Academic Press, Inc., San Diego, Calif., 1987); Young and Davism, P.N.A.S, 80: 1194 (1983). Methods and apparatus for carrying out repeated and controlled hybridization reactions have been described in U.S. Pat. Nos. 5,871,928, 5,874,219, 6,045,996 and 6,386,749, 6,391,623 each of which are incorporated herein by reference.

#### Exemplary Assay Formats:

Exemplary methods for performing PNA FISH can be found in: Oliveira et al., J. Clin. Microbiol 40:247-251 (2002), Rigby et al., J. Clin. Microbiol. 40:2182-2186 (2002), Stender et al., J. Clin. Microbiol. 37:2760-2765 (1999), Perry-O'Keefe et al., J. Microbiol. Methods 47:281-292 (2001). According to one method, a smear of the sample, such as, but not limited to, a positive blood culture, is prepared on microscope slides and covered with one drop of the



fluorescent-labeled PNA probe in hybridization buffer. A coverslip is placed on the smear to ensure even coverage, and the slide is subsequently placed on a slide warmer or incubator at 55 °C for 90 minutes. Following hybridization, the coverslip is removed by submerging the slide into a pre-warmed stringent wash solution and the slide is washed for 30 minutes. The smear is finally mounted with one drop of mounting fluid, covered with a coverslip and examined by fluorescence microscopy.

*Staphylococcus* species other than *S. aureus* optimally present in a sample which may be analyzed with the PNA probes contained in the kits of this invention can be evaluated by several instruments, such as but not limited to the following examples: microscope (for example see Oliveira et al., J. Clin. Microbiol 40:247-251 (2002)), radiation sensitive film (for example see Perry-O'Keefe et al., J. Appl. Microbiol. 90:180-189) (2001), camera and instant film (for example see Stender et al., J. Microbiol. Methods 42:245-253 (2000)), luminometer (for example see Stender et al., J. Microbiol. Methods. 46:69-75 (2001), laser scanning device (for example see Stender et al., J. Microbiol. Methods. 45: 31-39 (2001) or flow cytometer (for example see Wordon et al., Appl. Environ. Microbiol. 66:284-289 (2000)). Automated slide scanners and flow cytometers are particularly useful for rapidly quantitating the number of microorganisms present in a sample of interest.

Exemplary methods for performing real-time PCR using self-reporting PNA probes can be found in: Fiandaca et al., Abstract, Nucleic Acid-Based technologies, DNA/RNA/PNA Diagnostics, Washington, DC, May 14-16, 2001, and Perry-O'Keefe et al., Abstract, International Conference on Emerging Infectious Diseases, Atlanta, 2002.

d. Kits:

In yet another embodiment, this invention is directed to kits suitable for performing an assay, which analyzes *Staphylococcus* species other than *S. aureus* optionally present in a sample. The general and preferred characteristics of PNA probes suitable for the analysis of *Staphylococcus*

species other than *S. aureus* have been previously described herein.

Preferred probing nucleobase sequences are listed in Table 1. Furthermore, methods suitable for using PNA probes to analyze *Staphylococcus* species other than *S. aureus* in a sample have been previously described herein.

5

The kits of this invention comprise one or more PNA probes and other reagents or compositions which are selected to perform an assay or otherwise simplify the performance of an assay used to analyze *Staphylococcus* species other than *S. aureus* in a sample. In preferred embodiments, the kit

10

comprises a PNA probe set for simultaneous analysis of *Staphylococcus* species other than *S. aureus* and *S. aureus* using independently detectable PNA probes.

e. Exemplary Applications for Using the Invention:

15

The PNA probes, methods and kits of this invention are particularly useful for the analysis of *Staphylococcus* species other than *S. aureus* in clinical samples, e.g., urine, blood, wounds, sputum, laryngeal swabs, gastric lavage, bronchial washings, biopsies, aspirates, expectorates as well as in food, beverages, water, pharmaceutical products, personal care products, dairy

20

products or environmental samples and cultures thereof. In preferred embodiments, the PNA probes are applied in a PNA probe set also containing a PNA probe for analysis of *S. aureus*.

Having described the preferred embodiments of the invention, it will now

25

become apparent to one of skill in the art that other embodiments incorporating the concepts described herein may be used. It is felt, therefore, that these embodiments should not be limited to disclosed embodiments but rather should be limited only by the spirit and scope of the following claims.

30



**Example 1: Evaluation of probe Sets.**

<b>Set</b>	<b>PNA probe</b>	<b>(Seq. ID. No.)</b>	<b>Sequence</b>
A.	CNS16S09b/txr	(Seq. ID. No. 1)	TXR-OO-AGA-CGT-GCA-
5	TAG-T		
B.	CNS16S16g/txr	(Seq. ID. No. 2)	TXR-OO-GCT-AAT-ACG-
	GCG		

(Note: Conventional nomenclature used to illustrate the termini of the PNA probe; O = 8-amino-3,6-dioxaoctanoic acids; TXR = Texas Red.)

10

**Bacterial strains**

Overnight cultures of reference strains (American Type Culture Collection, (ATCC) Manassas, VA, or Agricultural Research Service Culture Collection (NRRL) Peoria, IL) of *Staphylococcus* species were prepared, including

15 *Staphylococcus epidermidis*, *Staphylococcus aureus* and other relevant species were prepared.

**Preparation of smears.**

For each strain, smears were prepared on a 8-mm diameter well of a teflon-coated microscope slide (AdvanDx, Woburn, MA) by mixing one drop of

20 culture with one drop of phosphate-buffered saline containing 1% (v/v) Triton X-100 . The slide was then placed on a 55°C slide warmer for 20 min at which point the smears were dry. Subsequently, the smears were disinfected by immersion into 96% (v/v) ethanol for 5-10 minutes and air-dried.

25

**Fluorescence *in situ* hybridization (FISH).**

Smears were covered with a drop of hybridization solution containing 10% (w/v) dextran sulfate, 10 mM NaCl, 30% (v/v) formamide, 0.1% (w/v) sodium pyrophosphate, 0.2% (w/v) polyvinylpyrrolidone, 0.2% (w/v) ficoll, 5 mM

30 Na<sub>2</sub>EDTA, 1% (v/v) Triton X-100, 50 mM Tris/HCl pH 7.5 and 250 nM CNS16S09b/txr (Set A) or CNS16S16g/txr (Set B). Coverslips were placed on the smears to ensure even coverage with hybridization solution, and the slides were subsequently placed on a slide warmer (Slidemoat, Boekel,

Feasterville, PA) and incubated for 90 min at 55 °C. Following hybridization, the coverslips were removed by submerging the slides into approximately 20 ml/slide pre-warmed 25 mM Tris, pH 10, 137 mM NaCl, 3 mM KCl in a water bath at 55 °C and washed for 30 min. Each smear was finally mounted using one drop of Mounting medium (AdvanDx, Woburn, MA) and covered with a coverslip. Microscopic examination was conducted using a fluorescence microscope equipped with a FITC/Texas Red dual band filter set.

*Staphylococcus* species other than *S. aureus* was identified by red fluorescent cocci.

Table 2

	1	2	3	4
A	Species	ID#	Set A	Set B
B	<i>Candida albicans</i>	NRRL Y-17968	-	-
C	<i>Candida glabrata</i>	ATCC 15126	-	-
D	<i>Escherichia coli</i>	ATCC 43888	-	-
E	<i>Klebsiella oxytoca</i>	ATCC 43086	-	-
F	<i>Klebsiella pneumoniae</i>	ATCC 10031	-	-
G	<i>Pseudomonas aeruginosa</i>	ATCC 10145	-	-
H	<i>Enterococcus faecalis</i>	ATCC 19433	-	-
I	<i>Enterococcus faecium</i>	ATCC 27270	-	-
J	<i>Micrococcus luteus</i>	ATCC 4698	-	-
K	<i>Staphylococcus aureus</i>	ATCC 11632	-	-
L	<i>Staphylococcus epidermidis</i>	ATCC 14990	Red +	-
M	<i>Staphylococcus gallinarum</i>	ATCC 700401	-	Red +
N	<i>Staphylococcus haemolyticus</i>	ATCC 29970	Red +	Red +
O	<i>Staphylococcus lugdunensis</i>	ATCC 49576	-	Red +
P	<i>Staphylococcus saprophyticus</i>	ATCC 15305	Weak Red +	Red +
Q	<i>Staphylococcus simulans</i>	ATCC 27851	-	-
R	<i>Staphylococcus schleiferi</i>	ATCC 43808	Weak Red +	Red +
S	<i>Streptococcus agalactiae</i>	ATCC 13813	-	-
T	<i>Streptococcus pyogenes</i>	ATCC 12384	-	-

With reference to Table 2, the table displays the data from Example 1, with species identification in column 1, species ID# in column 2, probe Set A results in column 3, and probe Set B results in column 4, as indicated by the header row, row A. The symbol “-” indicates a negative result, the symbol “+” indicates a positive result. Positive results are scored by observation of fluorescent cells in the sample, either fluorescent Red or Green. With reference to Table 2, column 3, results for probe Set A, all species tested negative (-), except *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* (rows L and N), which tested positive, with Red fluorescence



(“Red positive”), and *Staphylococcus saprophyticus* and *Staphylococcus schleiferi* (rows P and R) which were both weakly positive with red fluorescence. With reference to Table 2, column 4, results for probe Set B, all species tested negative (-), except *Staphylococcus gallinarum*,  
 5 *Staphylococcus haemolyticus*, *Staphylococcus lugdunensis*, *Staphylococcus saprophyticus*, and *Staphylococcus schleiferi* (rows M-P and R), which tested Red positive. Thus, the example demonstrates that probe Sets A and B are both useful for detection of certain *Staphylococcus* species other than *S. aureus*, although weak signals are observed with certain species, and neither  
 10 probe set is useful for detection of all *Staphylococcus* species other than *S. aureus*. It is worthwhile to note that *Staphylococcus aureus*, and several other Gram-positive cocci, such as *Enterococcus faecalis*, and *Streptococcus pyogenes* had negative results with both probe sets.

## 15 Example 2: Evaluation of probe Sets.

Set	PNA probe	(Seq. ID. No.)	Sequence
C.	CNS16S09b/txr	(Seq. ID. No. 1)	TXR-OO-AGA-CGT-GCA-TAG-T
20	CNS16S16g/txr	(Seq. ID. No. 2)	TXR-OO-GCT-AAT-ACG-GCG
	Sau16S03/flu	N/A	Flu-OO-GCT-TCT-CGT-CCGTTC
D.	Ssim16S01/txr	(Seq. ID. No. 3)	TXR-OO- GCT-AAT-ACG-CCG-C
25	E. CNS16S09b/txr	(Seq. ID. No. 1)	TXR-OO-AGA-CGT-GCA-TAG-T
	CNS16S16g/txr	(Seq. ID. No. 2)	TXR-OO-GCT-AAT-ACG-GCG
	Sau16S03/flu	N/A	Flu-OO-GCT-TCT-CGT-CCGTTC
30	Ssim16S01/txr	(Seq. ID. No. 3)	TXR-OO- GCT-AAT-ACG-CCG-C

(Note: Conventional nomenclature used to illustrate the termini of the PNA probe; O = 8-amino-3,6-dioxaoctanoic acids; TXR = Texas Red; Flu = carboxy-fluorescein.)

- 5 The probe Sets were tested exactly as described in Example 1, though testing was performed on a partially different group of strains.

10 Table 3

	1	2	3	4	5
A	<b>Species</b>	<b>ATCC#</b>	<b>Set C</b>	<b>Set D</b>	<b>Set E</b>
B	<i>Enterococcus faecalis</i>	29212	-	-	-
C	<i>Enterococcus faecium</i>	27270	-	-	-
D	<i>Escherichia coli</i>	11229	-	-	-
E	<i>Klebsiella pneumoniae</i>	10031	-	-	-
F	<i>Pseudomonas aeruginosa</i>	10145	-	-	-
G	<i>Staphylococcus aureus</i>	11632	Green +	-	Green +
H	<i>Staphylococcus capitis</i>	35661	Red +	-	Red +
I	<i>Staphylococcus epidermidis</i>	14990	Red +	-	Red +
J	<i>Staphylococcus gallinarum</i>	700401	Red +	-	Red +
K	<i>Staphylococcus haemolyticus</i>	29970	Red +	-	Red +
L	<i>Staphylococcus lugdunensis</i>	49576	Red +	-	Red +
M	<i>Staphylococcus saprophyticus</i>	15305	Red +	-	Red +
N	<i>Staphylococcus schleiferi</i>	43808	Red +	-	Red +
O	<i>Staphylococcus sciuri</i>	29061	Red +	-	Red +
P	<i>Staphylococcus simulans</i>	27851	-	Red +	Red +
Q	<i>Staphylococcus warneri</i>	49454	Red +	-	Red +
R	<i>Streptococcus agalactiae</i>	13813	-	-	-
S	<i>Streptococcus pyogenes</i>	29212	-	-	-

Probe Set C is a mixture of the probes from probe Sets A and B of Example 1, combined with a third *S. aureus* specific probe, labeled with fluorescein. With reference to Table 3, the table displays the data from Example 2, with species identification in column 1, species ATCC# in column 2, probe Set C results in column 3, probe Set D results in column 4, and probe Set E results in column 5, as indicated by the header row, row A. The symbol "-" indicates a negative result, the symbol "+" indicates a positive result. Positive results are scored by observation of fluorescent cells in the sample, either fluorescent Red or Green. With reference to Table 3, column 3, results for probe Set C, *S. aureus* tested Green positive. All other samples were negative for Green fluorescence. All other *Staphylococcus* strains except *S. simulans* tested Red



positive with probe Set C. No non-staphylococci tested Red positive, or Green positive with probe Set C. With reference to column 4, probe Set D, the only positive result was observed with *S. simulans* which was Red positive. Probe Set D contains a single Texas Red labeled probe species, and thus it is not surprising that no green fluorescence was observed. Probe Set E seen in Table 3, column 5 is a mixture of the probes from probe Set C and D. In column 5, all *Staphylococcus* species are detected with either Green (*S. aureus* only) or Red (*Staphylococcus* species other than *S. aureus*) fluorescence, while no non-staphylococci are detected with fluorescent signals.

It has been demonstrated in previous experiments that the Sau16S03 probe forms a stable hybrid with *S. schleiferi* even though the probe and target sequence are not perfectly complementary (Oliveira et al., J. Clin. Microbiol. 40:247-251 (2002)). In this example, the signal from *S. schleiferi* with probe Sets C and E is a Red positive. Thus, the use of probe Sets C or E provides a more specific detection of *S. aureus* with the Sau16S03 probe, than use of the Sau16S03 probe on its own, as previously reported.

These data demonstrate that a probe mixture can be made which detects a cohort of species by one fluorescent label, and a single species with a second fluorescent label. Though the *Staphylococcus* species tested in this experiment are considered to be the most clinically relevant, other experiments not included here demonstrated that many other *Staphylococcus* species can be detected with probe Sets A, B, C and E.

All references cited herein, whether in print, electronic, computer readable storage media or other form, are expressly incorporated by reference in their entirety, including but not limited to, abstracts, articles, journals, publications, texts, treatises, technical data sheets, internet web sites, databases, patents, patent applications, and patent publications.

A number of embodiments of the invention have been described.

Nevertheless, it will be understood that various modifications may be made

without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.



We claim

1. A PNA probe comprising a nucleobase sequence suitable for the analysis of one or more *Staphylococcus* species other than *S. aureus*.
- 5 2. A PNA probe comprising a nucleobase sequence suitable for the analysis of two or more *Staphylococcus* species other than *S. aureus*.
3. The PNA probe of claims 1-2, wherein a target sequence of the *Staphylococcus* species comprises rRNA, rDNA or a complement of rRNA or  
10 rDNA.
4. The PNA probe of claim 1-2, wherein the nucleobase sequence suitable for the analysis of *Staphylococcus epidermidis*, *simulans*, *haemolyticus*, *lugdunensis*, or *saprophyticus*, comprises one or more of a  
15 PNA probe complementary to a target sequence of the *Staphylococcus epidermidis simulans*, *haemolyticus*, *lugdunensis*, or *saprophyticus* rRNA or rDNA or a complement thereof.
5. The PNA probe of claim 1, wherein the nucleobase sequence suitable  
20 for the analysis of *Staphylococcus epidermidis*, *simulans*, *haemolyticus*, *lugdunensis*, or *saprophyticus* and one or more other *Staphylococcus* species other than *Staphylococcus aureus*, comprises a PNA probe complementary to a target sequence of *Staphylococcus epidermidis*, *simulans*, *haemolyticus*, *lugdunensis*, or *saprophyticus* rRNA or rDNA or a complement thereof.  
25
6. The PNA probe of claims 1, 2 or 5, wherein at least a portion of the probe is at least about 86% identical to the nucleobase sequence or complement thereof selected from the following sequences: AGA-CGT-GCA-TAG-T (Seq. Id. No. 1), GCT-AAT-ACG-GCG (Seq. Id. No. 2), GCT-AAT-  
30 ACG-CCG-C (Seq. Id. No. 3).
7. The PNA probe of claims 1, 2 or 5, wherein at least a portion of the probe is selected from the following sequences: AGA-CGT-GCA-TAG-T (Seq.

Id. No. 1), GCT-AAT-ACG-GCG (Seq. Id. No. 2), GCT-AAT-ACG-CCG-C (Seq. Id. No. 3).

8. The PNA probe of claims 1, 2, or 5, wherein the probe sequence is  
5 between about 8 and about 17 subunits in length.
9. The PNA probe of claims 1, 2, or 5, wherein the probe is labeled with at least one detectable moiety.
- 10 10. A PNA probe set comprising one or more PNA probes of claims 1 or 2, and at least one PNA probe for the analysis of *S. aureus*.
11. A PNA probe set comprising one or more PNA probes of claims 1 or 2, and at least one PNA blocking probe for the analysis of one or more  
15 *Staphylococcus* species other than *S. aureus*.
12. A method for the analysis of *Staphylococcus* species other than *S. aureus* in a sample, comprising:
- a) contacting at least one of the PNA probes of claims 1-9 to the  
20 sample,
- b) hybridizing the PNA probe to a target sequence of *Staphylococcus* species other than *S. aureus* in the sample; and
- c) detecting the hybridization, wherein the detection of hybridization is indicative of the presence, identity and/or amount of *Staphylococcus* species  
25 other than *S. aureus* in the sample.
13. A method for the analysis of two or more *Staphylococcus* species, said method comprising:
- a) contacting a PNA probe set to the sample,
- 30 b) hybridizing the PNA probes to a target sequence of *Staphylococcus* species in the sample; and
- c) detecting the hybridization, wherein the detection of hybridization is indicative of the presence, identity and/or amount of *Staphylococcus* species in the sample.



14. The method of claim 12 or 13, wherein the probes are independently detectable non-independently detectable, or a combination of independently and non-independently detectable, wherein the probes differ from one another  
5 by as little as a single base, and are complementary or substantially complementary to partially conserved target regions of phylogenetically related organisms.
15. The method of claim 12 or 13, wherein *S. aureus* and one or more  
10 *Staphylococcus* species other than *S. aureus* are simultaneously and independently detected.
16. A kit suitable for performing an assay for analysis of one or more *Staphylococcus* species other than *S. aureus* in a sample, wherein said kit  
15 comprises: a) a PNA probe according to claim 1 to 9 and b) other reagents or compositions necessary to perform the assay.