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(54) **METHODS, KITS AND COMPOSITIONS FOR SUPPRESSING THE BINDING OF DETECTABLE PROBES TO NON-TARGET SEQUENCES IN HYBRIDIZATION ASSAYS**

VERFAHREN, REAGENTIENSÄTZE UND ZUSAMMENSETZUNGEN ZUR UNTERDRÜCKUNG DER BINDUNG VON DETEKTIERBAREN SONDEN AN NICHT-ZIELSEQUENZEN IN HYBRIDISIERUNGSTESTS

PROCEDES, KITS ET COMPOSITIONS PERMETTANT DE SUPPRIMER LA FIXATION DE SONDES DETECTABLES A DES SEQUENCES NON-CIBLES D'ACIDE NUCLEIQUE DANS DES DOSAGES D'HYBRIDATION

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## Description

### Background:

1. Technical Field

**[0001]** This invention is related to the field of probe based nucleic acid sequence detection, quantitation and analysis. More specifically, this invention relates to methods, kits and compositions suitable for suppressing the binding of detectable nucleic acid probes or detectable PNA probes to non-target sequences in an assay for detecting a target sequence of a nucleic acid molecule of interest.

2. Background Art

**[0002]** Probe-based assays are useful in the detection, quantitation and analysis of nucleic acids. Nucleic acid probes have long been used to analyze samples for the presence of nucleic add from a bacteria, fungi, virus or other organism (See for example; US patents: 4,851,330,5,288,611, 5,567,587, 5,601,984 and 5,612,183). Probe-based assays are also useful in examining genetically-based disease states or clinical conditions of interest. Nonetheless, probe-based assays have been slow to achieve commercial success. This lack of commercial success is, at least partially, the result of difficulties associated with specificity, sensitivity and reliability.

**[0003]** Nucleic add hybridization is a fundamental process in molecular biology. Sequence differences as subtle as a single base (point mutation) in very short oligomers (< 10 base pairs "bp") can be sufficient to enable the discrimination of the hybridization to complementary nucleic acid target sequences as compared with non-target sequences. Nonetheless, nucleic acid probes of greater than 10 bp in length are generally required to obtain the sequence diversity necessary to correctly identify a unique organism, disease state or clinical condition of interest. However, the ability to discriminate between closely related sequences is inversely proportional to the length of the hybridization probe because the difference in thermal stability decreases between wild type and mutant complexes as the probe length increases. Consequently, the power of probe based hybridization to correctly identify the target sequence of interest from closely related (e.g. point mutations) non-target sequences can be very limited.

**[0004]** An extensive review of the "Prinaples and Practices of Nucleic Acid Hybridization" is available (See: David E Kennell, Prinaples and Practices of Nucleic Add Hybridization, pp. 259-301). In the manuscript, the author discusses the "Use of Competitor RNA to Estimate Specifiaty". This process is based on the principle that two identical molecules will compete with each other for a common binding site. This principle is applied to assess similarities between two RNA populations competing for a common DNA. Typically, one population of RNA is labeled and the competitor population of RNA is unlabeled. The competition assay is used to estimate the degree of relation between the through the two RNA species. A process called "presaturation competition", wherein the unlabeled competitor RNA is hybridized to the DNA before hybridization of the labeled RNA, has been reported to be useful in improving the results of this type of assay (See: p 297). However, the author warns that "great caution should be exercised " in interpreting the data from these assays (See: p.291 and p. 298 first full paragraph). No data is provided which quantitates the benefits associated with the application of this methodology.

**[0005]** Gray et al. describe *in-situ* methods for chromosome-spedfic staining wherein the hybridization of labeled nucleic acid fragments to repetitive sequences of chromosomal DNA is disabled (See: Gray et al. US Pat. No. 5,447,841). In one embodiment of the invention, disabling of the hybridization capacity of the repetitive DNA sequences within nucleic acid fragments involves blocking the repetitive sequences by pre-reassociation of fragments with fragments of repetitive-sequence-rich DNA, by pre-reassociation of target DNA with fragments of repetitive-sequence-rich DNA, or pre-reassociation of both the fragments of the heterogeneous mixture and the target DNA with repetitive-sequence-rich DNA (See: col. 9, Ins. 58-68). The pre-reassociation procedure may be performed in a number of differing formats (See: claims 2-5). This method provides blocking sufficient to permit detection of large labeled nucleic add (greater than 1000 bp) hybridized to chromosomal DNA (See: Claim 1). No data is provided which quantitates the benefits associated with the application of this methodology. Moreover, this treatment merely results in nucleic acid fragments whose repetitive sequences are blocked by complementary fragments such that sufficient unique sequence regions remain free for attachment to chromosomal DNA during the *in-situ* hybridization step (See: col. 10, Ins. 3-13).

**[0006]** Hybridization assays hold promise as a means to screen large numbers of patient samples for a large number of mutations. In practice, however, it is often difficult to multiplex an assay given the requirement that each of the many very different probes in the assay must exhibit a very high degree of specificity for a specific target nucleic acid under the same or similar conditions of stringency. Recently however, a probe based assay has been shown to be effective at selectively detecting up to twelve cystic fibrosis transmembrane conductance regulator (CFTR) mutations using pools of allele specific oligonucleotides "ASOs" (See: Shuber et al., Human Mol. Gen., (1993) 2, 153-158). The authors utilized a tetramethylammonium chloride (TMAC) buffer to eliminate variability in the affinity of the nudeic acid probes

for their complementary target nucleic acid sequences. Interestingly, the authors describe the use of labeled and unlabeled nucleic acid probes in the hybridization cocktail. However, there is no discussion of the rational for applying this methodology and there is no data provided which quantitates the benefits associated with application of this technology.

**[0007]** More recently, Shuber and his coworkers introduced a technique they coined MASDA (multiplex allele specific diagnostic assay). See: Shuber et al. Human Mol. Gen. (1997) **6**, 337-347. In this assay, a single hybridization is performed with a pool of allele specific oligonucleotide probes. The ASOs are affinity purified from the pool by hybridization to the target nucleic add (patient sample) which has been immobilized to a surface. Probes, which hybridize to the target nucleic acid, are thereafter eluted from the surface and analyzed to thereby determine the presence or absence of one or more clinical conditions of interest. The authors report that they observe such a high degree of specificity of hybridization of the component labeled ASOs of the pool that, in a single assay, the method is capable of analyzing greater than 500 samples for greater than 100 known mutations. As in the prior Shuber publication, the authors describe the use of a hybridization cocktail containing both labeled and unlabeled probes. This cocktail is prepared to achieve uniform hybridization signals in the assay. However, no data is provided which quantitates the benefits associated with the application of this methodology.

**[0008]** It has been reported that oligonudeotides can be used to reduce or eliminate the hybridization of target probes to non-target sequences (See: Arnold, L.J. Jr.; European Patent Application, EP-A-304,184). However, despite recognizing the difficulty of nucleic acid point mutation analysis, Arnold does not expressly describe, suggest or teach that his invention is suitable for point mutation analysis. More importantly, the model systems used as Examples to illustrate his invention have a two base pair mismatch between the target probe and the non-target probe. Additionally, an important recognized limitation of the Arnold invention is the potential for reduction in specific signal caused by the cross reaction between the target sequence and non-target probe to thereby form a target sequence/non-target probe hybrid (See: p. 3, Ins. 27-31 and lns 48-50). This may explain why Arnold specifically expresses a preference for lower ratios of non-target probe to target probe (See: p. 7, Ins. 53-54). The data in Tables 1-4 clearly demonstrates that as the ratio of non-target probe to target probe increases, there is a substantial decrease in the specific signal.

**[0009]** In a similar fashion, the use of "blocking oligonucleotides" to suppress the capture of amplified nucleic acids of non-target organisms has also been described (See: Nycz et al, European Patent Application EP-A-725,148). Unlike Arnold, Nycz et al. did utilize "blocking oligonucleotides" which were single point mutations of the capture probes used to immobilize the amplicons to a support and thereby achieve species specific detection of nucleic acid samples. However, Nycz et al. used capture probe to blocker probes ratios of only 1 to 1 (See: discussion of Examples 2-5). Like Arnold, Nycz et al. observed as much as a thirty percent reduction in specific signal in one assay (See: The discussion of Example 3 on page 11) and careful analysis of the data provided in the Tables likewise revealed significant signal reductions in almost all cases for which comparative data was shown.

**[0010]** The background art thus far discussed does not disclose, suggest or teach anything about Peptide Nucleic Acids (PNAs).

**[0011]** Peptide Nucleic Acids (PNAs) are non-naturally occurring polyamides which can hybridize to nucleic acids (DNA and RNA) with sequence specificity. (See United States Patent No. 5,539,082 and Egholm et al., Nature (1993) 365, 566 568 as well as Pluskal et al., Faseb Journal, Abstract No. 35, 1994)). PNA's are candidates for investigation as alternatives/substitutes to nucleic acid probes in probe-based hybridization assays because they exhibit several desirable properties. PNA's are achiral polymers which hybridize to nucleic acids to form hybrids which are more thermodynamically stable than a corresponding nucleic acid/nucleic acid complex (See: Egholm et. al., Nature (1993) **365**, 566-568 as well as Pluskal et al., Faseb Journal, Abstract No. 35, 1994)). Being non-naturally occurring molecules, they are not known to be substrates for the enzymes which are known to degrade peptides or nucleic acids. Therefore, PNA's should be stable in biological samples, as well as, have a long shelf-life. Unlike nucleic acid hybridization which is very dependent on ionic strength, the hybridization of a PNA with a nucleic acid is fairly independent of ionic strength and is favored at low ionic strength under conditions which strongly disfavor the hybridization of nucleic acid to nucleic acid (See: Egholm et. al., Nature, p. 567). The effect of ionic strength on the stability and conformation of PNA complexes has been extensively investigated (See: Tomac et al. J. Am. Chem. Soc. (1996) 118, 5544-5552). Sequence discrimination is more effiaent for PNA recognizing DNA than for DNA recognizing DNA (See: Egholm et al., Nature, p. 566). However, the advantages in point mutation discrimination with PNA probes, as compared with DNA probes, in a hybridization assay appears to be somewhat sequence dependent (See: Nielsen et al. Anti-Cancer Drug Design (1993) **8**, 53 65). As an additional advantage, PNA's hybridize to nucleic add in both a parallel and antiparallel orientation, though the antiparallel orientation is preferred (See: Egholm et al., Nature, p. 566).

**[0012]** PNAs are synthesized by adaptation of standard peptide synthesis procedures in a format which is now commercially available. (For a general review of the preparation of PNA monomers and oligomers please see: Dueholm et al., New J. Chem. (1997), **21**, 19-31 or Hyrup et. aL, Bioorganic & Med. Chem. (1996) 4, 5-23). Labeled and unlabeled PNA oligomers can be purchased (See: PerSeptive Biosystems Promotional Literature: BioConcepts, Publication No. NL612, Practical PNA, *Review* and Practical PNA, Vol. 1, Iss. 2) or prepared using the commercially available products.

**[0013]** Labeled PNA probes have been hybridized to target nucleic acid subsequences of denatured dsDNA as a means to detect the presence and amount of the DNA of interest in an assay coined "pre-gel hybridization" (See: O'Keefe et al. Proc. Natl. Acad. Sci. USA (1996) 93,14670-14675). This assay relies on the rapid kinetics of PNA/DNA hybrid formation and the relatively slow rate of reannealing of the dsDNA. Thus, under conditions of low salt, the sample is analyzed for the presence of the PNA/DNA hybrid before the PNA/nucleic add complex is dissociated by the reannealing/reformation of the dsDNA. "Pre-gel hybridization" is reported to provide very good discrimination of point mutations in a DNA sample (See: figure 4 of the O'Keefe manuscript and the associated description).

**[0014]** In a similar manner, unlabeled PNAs have been shown to be effective at blocking the interstrand and intrastrand interactions of dsDNA to thereby enhance the PCR amplification of variable numbers of tandem repeat (VNTR) loci (See: Demers et al. Nucl. Acids Res., (1995) **23**, 3050-3055 and US Pat. No. 5,656,461). For this application, the unlabeled PNAs need to be designed such that they form PNA/nucleic add hybrids which are stable enough to disrupt the interstrand and intrastrand interactions of dsDNA. However, the PNA/nucleic acid complex must be susceptible to dissociation by the operation of the polymerase during primer extension.

**[0015]** In still another related application, a process coined "PCR clamping" can be used to obtain point mutation discrimination when directing unlabeled PNAs of defined sequence to interfere with the PCR process (See: Ørum et al. Nucl. Acids Res. (1993), **21**, 5332-5336). In one embodiment of PCR clamping, an unlabeled PNA, which is identical in nucleobase composition to the PCR primer, competes with the PCR primer for binding to the common recognition site. In another embodiment, the target site for the unlabeled PNA is located within the PCR amplicon region. In this embodiment, clamping operates if the PNA/nucleic acid hybrid is stable enough to prevent read through by the polymerase. In yet another embodiment, the target site for the unlabeled PNA is located adjacent to the PCR priming site. In this embodiment, PCR clamping may operate either by preventing read through of the polymerase or by preventing (blocking) primer annealing. To obtain point mutation discrimination using PCR clamping, longer mutant and wild type nucleic acid PCR primers are designed such that amplification proceeds only if the longer PCR primer is a perfect complement to the recognition site and thereby out competes the unlabeled PNA for binding within that site. PCR damping has recently been directed to analysis of the Ki-ras mutations of codon 12 and 13 (See: Thiede et al. Nucl. Acids Res. (1996) **24**, 983-984).

**[0016]** Very recently, the "Hybridization based screening on peptide nucleic acid (PNA) oligomer arrays" has been described wherein arrays of some 1000 PNA oligomers. of individual sequence were synthesized on polymer membranes (See: Weller et al. Nucl. Acids Res. (1997) **25**, 2792-2799). Arrays are generally used, in a single assay, to generate affinity binding (hybridization) information about a specific sequence or sample to numerous probes of defined composition. Thus, PNA arrays may be useful in diagnostic or antisence applications. However, in the present study, the authors note that the affinity and spedficity of DNA hybridization to immobilized PNA oligomers depended on hybridization conditions more than was expected. Moreover, there was a tendency toward nonspecific binding at lower ionic strength. Furthermore, certain very strong binding mismatches were identified which could not be eliminated by more stringent washing conditions. These results demonstrate the need for improved methods of suppressing the binding of nucleic acids to non-complementary PNAs. Moreover, these unexplained results are also illustrative of the lack of complete understanding of these newly discovered molecules (i.e. PNA)

**[0017]** There are indeed many differences between PNA probes and standard nucleic acid probes. These differences can be conveniently broken down into biological, structural, and physico-chemical differences. As discussed above and below, these biological, structural, and physico-chemical differences may lead to unpredictable results when attempting to use PNA probes in applications were nucleic acids have typically been employed. This non-equivalency of differing compositions is often observed in the chemical arts.

**[0018]** With regard to biological differences, nucleic acids, are biological materials that play a central role in the life of living species as agents of genetic transmission and expression. Their *in vivo* properties are fairly well understood. PNA, on the other hand is recently developed totally artificial molecule, conceived in the minds of chemists and made using synthetic organic chemistry. It has no known biological function.

**[0019]** Structurally, PNA also differs dramatically 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 PNA are composed on N-(2-aminoethyl)glycine units. Additionally, in PNA the nucleobases are connected to the backbone by an additional methylene carbonyl unit.

**[0020]** Despite its name, PNA is not an acid and contains no charged acidic groups such as those present in DNA and RNA. Because they lack formal charge, PNAs are generally more hydrophobic than their equivalent nucleic acid molecules. The hydrophobic character of PNA allows for the possibility of non-specific (hydrophobic/hydrophobic interactions) interactions not observed with nucleic acids. Further, PNA is achiral, providing it with the capability of adopting structural conformations the equivalent of which do not exist in the RNA/DNA realm.

**[0021]** The physico/chemical differences between PNA and DNA or RNA are also substantial. PNA binds to its complementary nucleic acid more rapidly than nucleic add probes bind to the same target sequence. This behavior is

believed to be, at least partially, due to the fact that PNA lacks charge on its backbone. Additionally, recent publications demonstrate that the incorporation of positively charged groups into PNAs will improve the kinetics of hybridization (See: Iyer et al. J. BioL Chem. (1995) **270**, 14712-14717). Because it lacks charge on the backbone, the stability of the PNA/nucleic acid complex is higher than that of an analogous DNA/DNA or RNA/DNA complex. In certain situations, PNA will form highly stable triple helical complexes or form small loops through a process called "strand displacement". No equivalent strand displacement processes or structures are known in the DNA/RNA world.

**[0022]** In summary, because PNAs hybridize to nucleic acids with sequence speafiaty, PNAs are useful candidates for developing probe-based assays. However, PNA probes are not the equivalent of nucleic acid probes. Nonetheless, even under the most stringent conditions both the exact target sequence and a closely related sequence (e.g. a non-target sequence having a single point mutation (a.k.a. single base pair mismatch)) will often exhibit detectable interaction with a labeled nucleic acid or labeled PNA probe (See: Nielsen et al. Anti-Cancer Drug Design at p. 56-57 and Weller et al. at p. 2798, second full paragraph). Any hybridization to a closely related non-target sequence will result in the generation of undesired background signal. Because the sequences are so closely related, point mutations are the some of the most difficult of all nucleic acid modifications to detect using a probe based assay. Numerous diseases, such as sickle cell anemia and cystic fibrosis, are caused by a single point mutation of genomic nucleic acid. Consequently, any method, kits or compositions which could improve the specificity, sensitivity and reliability of probe-based assays would be useful in the detection, analysis and quantitation of nucleic acid containing samples and particularly useful for nucleic add point mutation analysis.

**Disclosure Of The Invention:**

1. Summary:

**[0023]** This invention relates to methods, kits and compositions suitable for the improved detection, quantitation and analysis of nucleic acid target sequences using probe-based hybridization assays. The invention is more specifically directed to methods, kits and compositions suitable for suppressing the binding of detectable probes to non-target sequences in an assay for a target sequence of a nucleic acid target molecule. Suppression of the nonspecific binding of detectable probe directly improves the sensitivity of the assay thereby improving the signal to noise ratio of the assay. Suppression of nonspecific binding will also result in improvements in reliability since the incidence of false positives and false negative should also be reduced. Because the methods, kits and compositions of this invention are directed to the suppression of nonspecific binding of probes to nudeic acids, they are particularly well suited for the development of sensitive and reliable probe-based hybridization assays designed to analyze for point mutations. The methods, kits and compositions of this invention should also find utility for the detection, quantitation or analysis of organisms (micro-organisms), viruses, fungi and genetically based clinical conditions of interest.

**[0024]** It has been surprisingly observed that the signal caused by the nonspecific binding of detectable probes to one or more non-target nudeic add sequences can be dramatically suppressed by the addition of one or more unlabeled probes wherein the sequence of the one or more unlabeled probes is complementary to one or more non-target sequences to which the detectable probe binds in a nonspecific manner. For example, it has been observed that the addition of 25 equivalents of unlabeled PNA probe, having a single mismatch as compared with the labeled PNA probe, does not substantially alter the detection linut of the assay. However, the presence of the unlabeled PNA probe resulted in at least a 10 fold suppression in the binding of labeled PNA probe to the non-target sequence (point mutation) and a correlating improvement of approximately 30 fold, in the signal to noise ratio of the assay (see Example 4A and Figure 1).

**[0025]** When the unlabeled PNA probe was present at 500 equivalents, there was very little loss of detectable signal (approximately 3 to 10 fold). However, suppression of binding of the labeled probe to a non-target sequence (point mutation) is substantially improved as compared with the experiment wherein only 25 equivalents of unlabeled PNA probe was present (Compare: Examples 4A and 4B of this specification). The results demonstrate that point mutation discrimination improved from approximately 10 fold in the absence of the unlabeled probe to greater than 1000 fold in the presence of high levels of unlabeled (blocker) PNA probe. Consequently, when employing the methods described herein, one can achieve several logs of improvement in point mutation discrimination and similar dramatic improvements in the dynamic range of the hybridization assay.

**[0026]** The applicants are not aware of any similar method suitable for obtaining such a dramatic suppression of binding to non-target sequences and the correlating improvement in signal to noise ratio. The data presented in Example 6, demonstrates the clear superiority of PNA probes as compared with DNA probes with regard to suppression of binding to non-target sequences, improvement in signal to noise ratios and point mutation discrimination.

**[0027]** In preferred embodiments of this invention, PNA probes are used either alone or in combination with nucleic acid probes. When combined with nucleic acid probes, the preferred combination involves unlabeled PNA probes used to suppress the binding of detectable (labeled). nucleic acid probes to non-target sequences. In the most preferred

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