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Method of identifying hairpin DNA probes by partial fold analysis

Abstract

Method of identifying molecular beacons in which a secondary structure prediction algorithm is employed to identify oligonucleotide sequences within a target gene having the requisite hairpin structure. Isolated oligonucleotides, molecular beacons prepared from those oligonucleotides, and their use are also disclosed.

Inventors: **Miller; Benjamin L.** (Penfield, NY)**, Strohsahl; Christopher M.** (Saugerties, NY)
Assignee: **University of Rochester** (Rochester, NY)

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References Cited [Referenced By]

U.S. Patent Documents

5215899	June 1993	Dattagupta
5556749	September 1996	Mitsuhashi et al.
5925517	July 1999	Tyagi et al.
6114121	September 2000	Fujiwara et al.
6194155	February 2001	Cohen
6251588	June 2001	Shannon et al.
6277607	August 2001	Tyagi et al.
6312906	November 2001	Cass et al.
6355437	March 2002	Neri et al.
6365729	April 2002	Tyagi et al.
6380377	April 2002	Dattagupta
7070933	July 2006	Browne
2003/0013109	January 2003	Ballinger et al.
2003/0054346	March 2003	Shannon et al.
2003/0143535	July 2003	Lyamichev et al.

Foreign Patent Documents

00/43552	Jul 2000	WO

Other References

Zuker et al., Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. Nucleic Acids Research 9(1) : 133-148 (1981). cited by examiner .
Bonnet et al., "Thermodynamic Basis of the Enhanced Specificity of Structured DNA Probes," Proc. Natl. Acad. Sci. USA 96:6171-6176 (1999). cited by other .
Du et al., "Hybridization-Based Unquenching of DNA Hairpins on Au Surfaces: Prototypical "Molecular Beacon" Biosensors," J. Am. Chem. Soc. 125:4012-4013 (2003). cited by other .
Dubertret et al., "Single-Mismatch Detection Using Gold-Quenched Fluorescent Oligonucleotides," Nat. Biotech. 19:365-370 (2001). cited by other .
Broude, "Stem-loop Oligonucleotides: A Robust Tool for Molecular Biology and Biotechnology," Trends in Biotechnology 20(6):249-56 (2002). cited by other .
Elsayed et al., "Development and Validation of a Molecular Beacon Probe-Based Real-Time Polymerase Chain Reaction Assay for Rapid Detection of Methicillin Resistance in Staphylococcus aureus," Arch. Pathol. Lab. Med. 127:845-9 (2003). cited by other .
Kushon et al., "Effect of Secondary Structure on the Thermodynamics and Kinetics of PNA Hybridization to DNA Hairpins," J. Am. Chem. Soc. 123(44):10805-13 (2001). cited by other .

Park et al., "Rapid Identification of Candida dubliniensis Using a Species-Specific Molecular Beacon," Journal of Clinical Microbiology 38(8):2829-36 (2000). cited by other .
Riccelli et al., "Hybridization of Single-stranded DNA Targets to Immobilized Complementary DNA Probes: Comparison of Hairpin Versus Linear Capture Probes," Nucleic Acids Research 29(4):996-1004 (2001). cited by other.

Primary Examiner: Whisenant; Ethan
Attorney, Agent or Firm: Nixon Peabody LLP

Government Interests

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Parent Case Text

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application Ser. No. 60/533,894, filed Jan. 2, 2004, which is hereby incorporated by reference in its entirety.

Claims

The invention claimed is:

1. A method of identifying a hairpin nucleic acid probe that hybridizes over its entire length to a target nucleic acid molecule, the method comprising: providing a target nucleic acid sequence that is larger than about 100 nucleotides in length; predicting a folded structure of the target nucleic acid sequence; identifying a nucleotide sequence of a hairpin within the folded structure of the target nucleic acid sequence; and predicting a folded structure for the identified nucleotide sequence of the hairpin, in the absence of other nucleotides of the target nucleic acid sequence, wherein the folded structure of a hairpin that has a predicted E value of at most about -3 kcal/mol is a probe that hybridizes over its entire length to the target nucleic acid molecule.

2. The method according to claim 1 wherein the nucleotide sequence of the hairpin is between about 12 and about 60 nucleotides in length.

3. The method according to claim 1 wherein the folded structure of the hairpin has a predicted E value of between about -4 kcal/mol and about -12 kcal/mol.

4. The method according to claim 1 further comprising: predicting a folded structure of a duplex formed between the hairpin and its complement.

5. The method according to claim 4 further comprising: determining whether duplex formation is energetically favorable.

6. The method according to claim 1 further comprising: performing a database search for nucleotide sequences that are similar to the identified nucleotide sequence of the hairpin.

7. The method according to claim 6 further comprising: determining, from the results of the performed database search, whether a clear demarcation exists between scores for target nucleic acid sequences and scores for non-target nucleic acid sequences.

8. A method of preparing a molecular beacon comprising: providing a hairpin nucleic acid probe identified according to the method of claim 1; and tethering a fluorescent label and a quenching agent to the opposed termini of the provided hairpin nucleic acid probe to form a molecular beacon, wherein the molecular beacon is substantially non-fluorescent in the absence of a nucleic acid complementary to the hairpin nucleic acid probe.

9. The method according to claim 8, wherein said providing comprises: synthesizing a nucleic acid molecule corresponding to the nucleotide sequence of the hairpin probe.

10. The method according to claim 8, wherein the fluorescent label is tethered to the 5' terminus and the quenching agent is tethered to the 3' terminus.

11. The method according to claim 8, wherein the fluorescent label is tethered to the 3' terminus and the quenching agent is tethered to the 5' terminus.

12. The method according to claim 8, wherein the quenching agent is a solid surface.

13. The method according to claim 8, wherein the quenching agent is a micro- or nano-particle.

14. The method according to claim 8, wherein the fluorescent label is a fluorescent dye, semiconductor quantum dot, lanthanide atom-containing complex, or fluorescent protein.

15. The method according to claim 8, wherein the quenching agent is a metal or 4-([4-(Dimethylamino)phenyl]azo)benzoic acid.

16. The method according to claim 15, wherein the metal is gold, silver, platinum, copper, cobalt, iron, or iron-platinum.

17. A method of preparing a hairpin nucleic acid molecule comprising: synthesizing a hairpin nucleic acid molecule identified according to the method of claim 1.

18. A method of identifying a hairpin nucleic acid probe that hybridizes over its entire length to a target nucleic acid molecule, the method comprising: providing a target nucleic acid sequence that is larger than about 100 nucleotides in length; predicting a folded structure of the target nucleic acid sequence; identifying a nucleotide sequence of a hairpin within the folded structure of the target nucleic acid sequence, the hairpin being between about 12 and about 60 nucleotides in length; and determining whether (i) self-folding of the identified hairpin and (ii) hairpin binding over its entire length to the target nucleic acid molecule will be energetically favorable.

Description

FIELD OF THE INVENTION

The present invention generally relates to the use of DNA hairpins as molecular beacon probes. More specifically, the present invention is directed to methods of generating highly specific and highly selective molecular beacon probes by using naturally occurring DNA hairpins present in organisms of interest.

BACKGROUND OF THE INVENTION

Methods for the rapid detection and serotyping of pathogens are of high interest, due in part to the dramatic

improvement in treatment efficacy for a bacterial or viral infection diagnosed early relative to one diagnosed at a later stage (Inglesby et al., "Anthrax as a Biological Weapon: Medical and Public Health Management," J. Am. Med. Assoc. 281:1735-1745 (1999)). Unfortunately, most current methods of pathogen identification rely on some level of sample manipulation, (enrichment, fluorescent tagging, etc.) which can be costly in terms of both time and money. Thus, eliminating sample labeling will result in a significant savings and has the potential to speed diagnosis. The use of DNA hairpins as "molecular beacons" (Broude, "Stem-loop Oligonucleotides: a Robust Tool for Molecular Biology and Biotechnology," Trends Biotechnol. 20:249-256 (2002)), either in solution (Tyagi et al., "Molecular Beacons: Probes that Fluoresce upon Hybridization," Nature Biotech. 19:365-370 (2001); Dubertret et al., "Single-mismatch Detection Using Gold-quenched Fluorescent Oligonucleotides," Nature Biotech. 19:365-370 (2001)) or immobilized on a solid surface (Fang et al., "Designing a Novel Molecular Bacon for Surface-Immobilized DNA Hybridization Studies," J. Am. Chem. Soc. 121:2921-2922 (1999); Wang et al., "Label Free Hybridization Detection of Single Nucleotide Mismatch by Immobilization of Molecular Beacons on Agorose Film," Nucl. Acids. Res. 30:61 (2002); Du et al., "Hybridization-based Unquenching of DNA Hairpins on Au Surfaces: Prototypical "Molecular Beacon" Biosensors," J. Am. Chem. Soc. 125:4012:4013 (2003); Fan et al., "Electrochemical Interrogation of Conformational Changes as a Reagentless Method for the Sequence-specific Detection of DNA," Proc. Natl. Acad. Sci. USA 100:9134-9137 (2003)), has proven to be a useful method for "label-free" detection of oligonucleotides. Molecular beacons consist of DNA hairpins functionalized at one terminus with a fluorophore and at the other terminus with a quencher. In the absence of their complement, they exist in a closed, "dark" conformation. Hybridization occurs on introduction of complementary oligonucleotides, which concomitantly forces open the hairpin and allows for a fluorescent, "bright" state.

Traditionally, as illustrated in FIG. 1, molecular beacons have been designed by supplementing the targeted DNA sequence at both termini with additional self-complementary nucleotides to force the formation of a hairpin (Monre et al., "Molecular Beacon Sequence Design Algorithm," Biotechniques 34:68-73 (2003)). While generally successful, the addition of non target-derived nucleotides increases the potential for non-specific binding, thus potentially reducing both the sensitivity and selectivity of the probe beacon. Modifications of this discovery protocol, such as the "shared stem" methodology of Bao and coworkers (Tsourkas et al., "Structure-function Relationships of Shared-Stem and Conventional Molecular Beacons," Nucl. Acids Res. 30:4208-4215 (2002)), still incorporate several bases unrelated to the target sequence. Thus, the latter approach potentially suffers from the same deficiencies. It would be desirable to identify a reliable approach for identifying DNA hairpins that overcomes the above-noted deficiencies.

The present invention is directed to achieving these objectives and otherwise overcoming the above-noted deficiencies in the art.

SUMMARY OF THE INVENTION

A first aspect of the present invention relates to a method of identifying hairpin nucleic acid probes. The method includes the steps of: providing a target nucleic acid sequence that is larger than about 100 nucleotides in length; predicting a folded structure of the target nucleic acid sequence; identifying the nucleotide sequence of a hairpin within the folded structure of the target nucleic acid sequence; and predicting a folded structure of the identified nucleotide sequence of the hairpin, in the absence of other nucleotides of the target nucleic acid sequence, wherein the folded structure of the hairpin has a predicted E value of at most about -3 kcal/mol.

A second aspect of the present invention relates to a method of preparing a molecular beacon. The method includes the steps of: providing a hairpin nucleic acid probe identified according to the first aspect of the present invention; and tethering a fluorescent label and a quenching agent to the opposed termini of the provided hairpin nucleic acid probe to form a molecular beacon, wherein the molecular beacon is substantially non-fluorescent in the absence of a nucleic acid complementary to the hairpin nucleic acid probe.

A third aspect of the present invention relates to a method of preparing a hairpin nucleic acid molecule. This method includes the steps of identifying the nucleotide sequence of a hairpin in accordance with the first aspect of the present invention; and synthesizing the identified hairpin nucleic acid molecule.

A fourth aspect of the present invention relates to an isolated nucleic acid molecule prepared according to the third aspect of the present invention.

A fifth aspect of the present invention relates to an isolated molecular beacon that includes a nucleic acid molecule according to the fourth aspect of the present invention; a fluorescent label tethered to one terminus of the nucleic acid molecule; and a quenching agent tethered to the other terminus of the nucleic acid molecule.

Additional aspects of the present invention relate to the use of the hairpin nucleic acid molecules and molecular beacons as probes in the detection of target nucleic acid molecules, according to any of a variety of hybridization-based detection procedures.

The ability to rapidly detect the presence of biological agents in the environment is of keen interest to the civilian and military health communities. The use of DNA hairpins as "molecular beacons" has proven a useful method for the detection of bacterial oligonucleotides. The present invention affords a significant improvement over previously employed molecular beacons by using naturally occurring DNA hairpins as molecular beacon probes. This circumvents the need for supplementation with additional bases; as noted in the Examples, supplementation or modification of the naturally occurring hairpins is likely to result in energetically less favorable complementation.

The working examples of the present invention demonstrate the significant specificity and energetically stable target/hairpin dimerizations, thus producing viable molecular beacons for varying experimental conditions, probes and fluorophores. By selecting probes based on their predicted structures and free energy, and by controlling probe length, the present invention affords a systematic approach for preparing nucleic acid probes and molecular beacons that can be used to selectively and sensitively discriminate between target and non-target molecules.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the prior art method of DNA hairpin probe design demonstrating the section of non-pairing sequences present in the final complex.

FIG. 2 shows the final probe/target complex of the present invention when the probe is selected based on total sequence complementarity.

FIG. 3 shows the predicted secondary structure and hairpin regions selected from Bacillus anthracis. A partial gene sequence of the Bacillus anthracis pag gene (isolate IT-Carb3-6254) (Adone et al., J. Appl. Microbio. 92:1-5 (2002), which is hereby incorporated by reference in its entirety) was obtained from GenBank Accession AJ413936, which is hereby incorporated by reference in its entirety. The secondary structure of .about.1000 nucleotide fragment (SEQ ID NO: 1) of the aforementioned sequence was then computationally predicted using RNAstructure v. 3.7 (Mathews et al., J. Mol. Biol. 288:911-940 (1999), which is hereby incorporated by reference in its entirety).

FIGS. 4A-B show structural predictions for two excised sequences: BaPag668-706 (Pag668-706) (SEQ ID NO: 2) and BaPag1208-1241(Pag1208-1241) (SEQ ID NO: 3). The sequences are isolated from the full sequence and subjected to secondary structure predictions. The number of nucleotides is indicated by nt count.

FIG. 5 demonstrates that the specificity of the BaPag 668-706 hairpin for its target is supported by a BLAST search of the GenBank database using the BaPag 668-706 sequence. A clear demarcation exists between target scores (of 78) and non-target scores (of 42 and lower) indicating that only sequences from Bacillus anthracis, the target organism, have high scores; whereas other "matching sequences" from non-target organisms have significantly lower scores.

FIG. 6 shows the predicted secondary structure and hairpin regions selected from the Staphylococcus aureus

genome, a segment of which (SEQ ID NO: 4) was obtained from Genbank Accession AP003131, which is hereby incorporated by reference in its entirety. The secondary structure of the obtained segment was predicted using computer program RNAStructure version 3.7 (Mathews et al., J. Mol. Biol. 288:911-940 (1999), which is hereby incorporated by reference in its entirety). From this predicted structure, two naturally occurring hairpins were identified, one corresponding to AH2 and the other corresponding to BH2.

FIGS. 7A-B show structural predictions for two excised sequences: AH2 (SEQ ID NO: 5) and BH2 (SEQ ID NO: 6). The sequences were isolated from the full sequence and subjected to second structure predictions. The AH2 sequence appears primarily to target an intergenic region between ORFID:SA0529 and ORFID:SA0530, and the BH2 sequence appears to target an intergenic region between ORFID:SA0529 and ORFID:SA0530 but also includes several bases within the latter open reading frame.

FIGS. 8A-D show the final structural prediction of BaPag 668-706 (SEQ ID NO: 2), BaPag 1208-1241 (SEQ ID NO: 3), AH2 (SEQ ID NO: 5), and BH2 (SEQ ID NO: 6) in duplex with their corresponding complements (SEQ ID NOS: 7-10, respectively). Having confirmed that the selected hairpin(s) satisfy initial selected criteria, a final structural prediction of the sequence in duplex with its complement was computed. Each of these duplexes have a predicted E value that is about nine to ten-fold greater than the predicted E value (or .DELTA..DELTA.G value) for the hairpin alone, and therefore they are expected to favorably form a duplex with their targets.

FIG. 9 demonstrates that hairpins favorably hybridize with their target DNA. Samples of BaPag668 (BaPag668-706) and BaPag1208 (BaPag1208-1241), both alone and mixed with equal amount of complement, were run on a native polyacrylamide gel. The presence of single bands in Lanes 1 and 3 is evidence that the hairpins preferentially adopt one structure because any variations from the predicted structure would either enhance or retard the variant's migration through the gel, thus creating multiple bands. The upward shift seen in Lanes 2 and 4 is indicative of the addition of mass that occurs during the hybridization of the hairpins with their targets. The increased contrast of the bands in Lanes 2 and 4 also gives indication that the hairpins are successfully forming double-stranded duplexes with their targets, as the dye used preferentially binds double-stranded regions of DNA.

FIGS. 10A-H show the thermal melting curves for DNA hairpin probes. Unmodified versions of BaPag668-706, BaPag1208-1241, AH2, BH2, and their complements were purchased from Invitrogen (cartridge purity). All thermal melts were conducted on a Gilford spectrophotometer, with the oligonucleotides dissolved in 0.5 M NaCl Buffer (20 mM cacodylic acid, 0.5 mM EDTA, and 0.5 M NaCl, pH=7.28). Samples were warmed to 90.degree. C. and subsequently cooled to 10.degree. C. prior to running melts. Solution temperatures were raised by 1.degree. C. per minute over a range of 15.degree. C. to 90.degree. C. and data points were collected approximately every 30 s (FIGS. 10A-D). All melting temperatures (x-axis) of BaPag668-706, BaPag1208-1241, AH2, and BH2 were found to be concentration independent (absorbance is indicated on the y-axis). The unmodified hairpins were then mixed with a ten-fold excess of complementary DNA and a second series of melting profiles were obtained (FIGS. 10E-H). As was expected, introduction of complement to the hairpins produced a biphasic transition curve, with the first transition corresponding to the linearization of the target DNA, which is also believed to possess ordered secondary structure, and the second, higher temperature, transition corresponding to the melting point of the duplex DNA.

FIG. 11 shows the solution phase performance of the BaPag668-706 probe. BaPag668-706 was purchased from Integrated DNA Technologies, Inc. as a molecular beacon using 5'-fluorescein and 3'-dabcyl as the fluorophore and quencher, respectively. BaPag668-706 was diluted to a concentration of 300 nM in 0.5 M NaCl Buffer (20 mM Cacodylic acid, 0.5 mM EDTA, and 0.5 M NaCl, pH=7.28), to which target DNA was then added such that the final ratio of target to beacon ranged from 1:1 to 4:1. Samples were allowed to incubate 5 hours at room temperature and were kept out of direct light as much as possible prior to excitation to prevent photobleaching. Samples were transferred to a Starna Cells 23-Q-10 Quartz fluorometer cell (10 mm pathlength) and placed on an Acton Research Instruments Fluorometer System. The fluorophore was excited at 490 nm and the resulting emission was monitored from 500 to 620 nm (x-axis). BaPag668-706 exhibits minimal fluorescence alone, and, as expected, addition of the target complementary oligonucleotide causes fluorescence to increase in a concentration-dependent manner.

FIGS. 12A-F show the performance of the BaPag1208-1241 probe immobilized in a 1:10 ratio with mercaptopropanol on an Au-film. The 5'-thiol terminated version of BaPag1208-1241 was immobilized on a thin Au film in the presence of mercaptopropanol as described previously (Du et al., "Hybridization-based Unquenching of DNA Hairpins on Au Surfaces: Prototypical "Molecular Beacon" Biosensors," J. Am. Chem. Soc., 125:4012:4013 (2003), which is hereby incorporated by reference in its entirety) with the only major change being the use of 0.5 M NaCl buffer as the diluent as opposed to deionized water. When immobilized on an Au-film in a 1:10 ratio with mercaptopropanol, BaPag1208-1241 shows greater than an 18-fold increase in fluorescence intensity (y-axis) in response to incubation in a 2.5 .mu.M target solution (FIGS. 12A-C). When the concentration of the target solution is lowered to 1.0 .mu.M, the observed response drops to about 10-fold, which is still significant (FIGS. 12D-F).

FIGS. 13A-F show the performance of the BaPag1208-1241 probe immobilized in a 1:1 ratio with mercaptopropanol on an Au-film. BaPag1208-1241 was immobilized onto an Au-film with mercaptopropanol in a 1:1 ratio and subjected to the same target concentrations as described previously. As seen in FIGS. 13A-C, when immobilized in a 1:1 ratio with mercaptopropanol, BaPag1208 shows a superior response, as measured by fluorescence intensity (y-axis), to target over that observed when the immobilization ratio is 1:10. This increased response is especially significant at lower concentrations as is evidenced by the greater than 20-fold intensity increase observed after incubation in 1.0 .mu.M target (FIGS. 13D-F).

FIG. 14 summarizes the sensitivity of BaPag1208-1241 to a target sequence when immobilized onto an Au-film. The nanomolar target concentration is depicted on the x-axis. The fold increase in binding of BaPag1208-1241 to a target sequence is depicted on the y-axis.

FIGS. 15A-F show the use of Au-immobilized AH2 and BH2 beacons to detect complementary DNA sequences. The AH2 and BH2 hairpins were 3'-modified with tetramethylrhodamine ("TAMRA") and Cy5, respectively. Each probe was dissolved separately in a solution of mercaptopropanol and water (1:10 molar ratio of probe and mercaptopropanol). The resulting probe solutions were then mixed in a 1:1 ratio, added to Au-chips and measurements of baseline fluorescence made (FIGS. 15B and 15E). The Au-films were then separately incubated in their appropriate complementary target solutions and fluorescence measured (FIGS. 15C and 15F). The fluorescence demonstrated in FIGS. 15C and 15F indicate that AH2 and BH2 probe beacons effectively detect complementary DNA sequences.

FIGS. 16A-C show the calculated hybridization energies for folding-derived and modified BaPag668-706 beacons. FIG. 16A shows the same secondary structure as in FIG. 4A (SEQ ID NO: 2). The termini of probe BaPag668-706 was extended by the self-complementary sequence [d(CGACG)].sub.2 (SEQ ID NO: 11), then the hybridization energy calculated (FIG. 16B). Five bases were removed from each end of BaPag668-706, replaced with [d(CGACG)].sub.2, and the hybridization energy again calculated (FIG. 16C). BaPag673 corresponds to BaPag668 with 5 bases removed from each end (SEQ ID NO: 12). The complementary sequence of BaPag668-706 is also shown (SEQ ID NO: 7). In each case, calculated .DELTA..DELTA.G was less favorable for the modified beacons than for the probes derived directly from folding.

FIGS. 17A-C show the calculated hybridization energies for folding-derived and modified BaPag1208-1241 beacons. FIG. 17A shows the same secondary structure as in FIG. 4B (SEQ ID NO: 3). The termini of probe BaPag1208-1241 was extended by the self-complementary sequence [d(CGACG)].sub.2 (SEQ ID NO: 13), then the hybridization energy calculated (FIG. 17B). The complementary sequence of BaPag1208-1241 is also shown (SEQ ID NO: 8). Five bases were removed from each end of BaPag1208-1241, replaced with [d(CGACG)].sub.2 (SEQ ID NO: 14), and the hybridization energy again calculated (FIG. 17C). BaPag1213 corresponds to BaPag1208 with 5 bases removed from each end. In each case, calculated .DELTA..DELTA.G was less favorable for the modified beacons than for the probes derived directly from folding.

DETAILED DESCRIPTION OF THE INVENTION

The method of the invention involves obtaining or providing a probe nucleotide sequence from a molecular

target. The target nucleotide sequence can be sequenced from an isolated cDNA or obtained from an online database such as GenBank. Regardless of the source of the target nucleotide sequence, a partial fold analysis is performed on the nucleotide sequence using any of a variety of suitable folding software such as, e.g., RNAStructure program (available from D. Turner at the University of Rochester, Rochester, N.Y.), Mfold software package (available from M. Zucker at the Rensselear Polytechnic Institute, Rensselear, N.Y.), and Vienna RNA software package, including RNAfold, RNAeval, and RNAsubopt (available from I. Hofacker at the Institute for Theoretical Chemistry, Vienna, Austria). With respect to the RNAStructure program, applicants have discovered that segments larger than approximately 1000 bases would crash the program RNAstructure v. 3.7. Thus, it may or may not be possible to predict the secondary structure of an entire nucleic acid molecule depending on the length thereof. Ideally, the secondary structure of the entire sequence would be predicted, but as demonstrated in the examples that is not necessary.

The resulting folded structure may or may not be the true active conformation of the RNA molecule in a cellular environment; however, it represents the lowest free energy state as predicted using such software. It is believed that more often than not, the predicted lowest free energy state of the nucleic acid molecule sufficiently resembles the true active conformation. Nonetheless, the resulting folded structure is analyzed to identify hairpin regions thereof.

Having identified hairpin structures within the folded structure of the prospective target nucleic acid molecule, the hairpin sequences are isolated from the larger sequence (i.e., that was used as input to the folding software). The isolation can be performed in silico. Once isolated, the hairpin sequence is subjected to a second structural prediction as was performed on the prospective target nucleic acid molecule.

The overall length of the selected hairpin is preferably between about 12 and about 60 nucleotides, more preferably between about 20 and about 50 nucleotides, most preferably between about 30 and about 40 nucleotides. It should be appreciated, however, that longer or shorter nucleic acids can certainly be used. According to the preferred hairpins, the regions forming the stem of the hairpin are preferably at least about 4 nucleotides in length and up to about 28 nucleotides in length, depending on the overall length of the nucleic acid probe and the size of a loop region present between the portions forming the stem. It is believed that a loop region of at least about 4 or 5 nucleotides is needed to form a stable hairpin. The regions forming the stem can be perfectly matched (i.e., having 100 percent complementary sequences that form a perfect stem structure of the hairpin conformation) or less than perfectly matched (i.e., having non-complementary portions that form bulges within a non-perfect stem structure of the hairpin conformation). When the first and second regions are not perfectly matched, the regions forming the stem structure can be the same length or they can be different in length.

Importantly, applicants have found that the predicted E value for the hairpin should preferably be at most about -3 kcal/mol, more preferably at most about -3.5 kcal/mol, most preferably between about -4 kcal/mol and about -12 kcal/mol. It should be appreciated, however, that identified hairpins can still function as molecular probes if their predicted E value falls outside these ranges.

Once the structure of the hairpin itself has been predicted, the duplex formed between the hairpin and its complement is subjected to a structural prediction as was performed on the prospective target nucleic acid molecule and the hairpin. This step, not necessary for identification of the hairpin per se, is performed primarily to ensure that the hybridization of the two sequences (hairpin and complement), and thus the disruption of the hairpin, will be an energetically favorable process. Ideally there should be an increase in the predicted E value preferably at least about a two-fold increase, more preferably at least about a five-fold increase or even more preferably at least about a ten-fold increase. This structural prediction also serves to demonstrate the primary advantage of the technique: after hybridization, there are no extraneous unhybridized nucleotides and, thus, lowered risk of nonspecific binding.

To further verify the specificity of the hairpin sequence for its complement, the hairpin sequence can be used to perform a BLAST database search (of, e.g., the GenBank database). Ideally, the resulting BLAST search will show not only high match scores for molecular targets (or target organisms), but also a sharp discrepancy (or

clear demarcation) between the high match scores of the target and any match scores of nucleic acid molecules bearing lower similarity. By sharp discrepancy and clear demarcation, it is intended that a gap of at least about 5 points, preferably at least about 10 points, more preferably at least about 15 points, most preferably at least about 20 points, exists between the target and non-target sequences. This is exemplified in Example 1 below.

Having thus identified suitable hairpin nucleic acid molecules that can be utilized for the detection of target nucleic acids and, thus, the identification of target organisms (by virtue of hybridization between the hairpin and the target), persons of skill in the art can readily synthesize hairpin nucleic acid molecules and prepare molecular beacons containing the same in accordance with known procedures.

The hairpin nucleic acid molecules can be synthesized according to standard procedures. Commercial synthesis facilities, in particular, are adept at providing this service.

Molecular beacons can be constructed by tethering to the termini of the hairpin nucleic acid molecule a fluorescent label and a quenching agent, respectively. In one embodiment, the fluorescent label is tethered to the 5' end of the hairpin nucleic acid molecule and the quenching agent is tethered to the 3' end thereof. In another embodiment, the fluorescent label is tethered to the 3' end of the hairpin nucleic acid molecule and the quenching agent is tethered to the 5' end thereof.

The fluorescent label can be any fluorophore that can be conjugated to a nucleic acid and preferably has a photoluminescent property that can be detected and easily identified with appropriate detection equipment. Exemplary fluorescent labels include, without limitation, fluorescent dyes, semiconductor quantum dots, lanthanide atom-containing complexes, and fluorescent proteins. The fluorophore used in the present invention is characterized by a fluorescent emission maxima that is detectable either visually or using optical detectors of the type known in the art. Fluorophores having fluorescent emission maxima in the visible spectrum are preferred.

Exemplary dyes include, without limitation, Cy2.TM., YO-PRO.TM.-1, YOYO.TM.-1, Calcein, FITC, FluorX.TM., Alexa.TM., Rhodamine 110, 5-FAM, Oregon Green.TM. 500, Oregon Green.TM. 488, RiboGreen.TM., Rhodamine Green.TM., Rhodamine 123, Magnesium Green.TM., Calcium Green.TM., TO-PRO.TM.-1, TOTO.RTM.-1, JOE, BODIPY.RTM. 530/550, Dil, BODIPY.RTM. TMR, BODIPY.RTM. 558/568, BODIPY.RTM. 564/570, Cy3.TM., Alexa.TM. 546, TRITC, Magnesium Orange.TM., Phycoerythrin R&B, Rhodamine Phalloidin, Calcium Orange.TM., Pyronin Y, Rhodamine B, TAMRA, Rhodamine Red.TM., Cy3.5.TM., ROX, Calcium Crimson.TM., Alexa.TM. 594, Texas Red.RTM., Nile Red, YO-PRO.TM.-3, YOYO.TM.-3, R-phycocyanin, C-Phycocyanin, TO-PRO.TM.-3, TOTO.RTM.-3, DiD DilC(5), Cy5.TM., Thiadicarbocyanine, and Cy5.5.TM.. Other dyes now known or hereafter developed can similarly be used as long as their excitation and emission characteristics are compatible with a light source and non-interfering with other fluorescent labels that may be tethered to different hairpin nucleic acid molecules (i.e., not capable of participating in fluorescence resonant energy transfer or FRET).

Attachment of dyes to the oligonucleotide probe can be carried out using any of a variety of known techniques allowing, for example, either a terminal base or another base near the terminal base to be bound to the dye. For example, 3'-tetramethylrhodamine (TAMRA) may be attached using commercially available reagents, such as 3'-TAMRA-CPG, according to manufacturer's instructions (Glen Research, Sterling, Va.). Other exemplary procedures are described in, e.g., Dubertret et al., Nature Biotech. 19:365-370 (2001); Wang et al., J. Am. Chem. Soc., 125:3214-3215 (2003); Bioconjugate Techniques, Hermanson, ed. (Academic Press) (1996), each of which is hereby incorporated by reference in its entirety.

Exemplary proteins include, without limitation, both naturally occurring and modified (i.e., mutant) green fluorescent proteins (Prasher et al., Gene 111:229-233 (1992); PCT Application WO 95/07463, each of which is hereby incorporated by reference in its entirety) from various sources such as Aequorea and Renilla; both naturally occurring and modified blue fluorescent proteins (Karatani et al., Photochem. Photobiol. 55(2):293-299 (1992); Lee et al., Methods Enzymol. (Biolumin. Chemilumin.) 57:226-234 (1978); Gast et al., Biochem. Biophys. Res. Commun. 80(1):14-21 (1978), each of which is hereby incorporated by reference in its entirety)

from various sources such as Vibrio and Photobacterium; and phycobiliproteins of the type derived from cyanobacteria and eukaryotic algae (Apt et al., J. Mol. Biol. 238:79-96 (1995); Glazer, Ann. Rev. Microbiol. 36:173-198 (1982); Fairchild et al., J. Biol. Chem. 269:8686-8694 (1994); Pilot et al., Proc. Natl. Acad. Sci. USA 81:6983-6987 (1984); Lui et al., Plant Physiol. 103:293-294 (1993); Hournard et al., J. Bacteriol. 170:5512-5521 (1988), each of which is hereby incorporated by reference in its entirety), several of which are commercially available from ProZyme, Inc. (San Leandro, Calif.). Other fluorescent proteins now known or hereafter developed can similarly be used as long as their excitation and emission characteristics are compatible with the light source and non-interfering with other fluorescent labels that may be present.

Attachment of fluorescent proteins to the oligonucleotide probe can be carried out using substantially the same procedures used for tethering dyes to the nucleic acids, see, e.g., Bioconjugate Techniques, Hermanson, ed. (Academic Press) (1996), which is hereby incorporated by reference in its entirety.

Nanocrystal particles or semiconductor nanocrystals (also known as Quantum Dot.TM. particles), whose radii are smaller than the bulk exciton Bohr radius, constitute a class of materials intermediate between molecular and bulk forms of matter. Quantum confinement of both the electron and hole in all three dimensions leads to an increase in the effective band gap of the material with decreasing crystallite size. Consequently, both the optical absorption and emission of semiconductor nanocrystals shift to the blue (higher energies) as the size of the nanocrystals gets smaller. When capped nanocrystal particles of the invention are illuminated with a primary light source, a secondary emission of light occurs at a frequency that corresponds to the band gap of the semiconductor material used in the nanocrystal particles. The band gap is a function of the size of the nanocrystal particle. As a result of the narrow size distribution of the capped nanocrystal particles, the illuminated nanocrystal particles emit light of a narrow spectral range resulting in high purity light. Particles size can be between about 1 nm and about 1000 nm in diameter, preferably between about 2 nm and about 50 nm, more preferably about 5 nm to about 20 nm.

Fluorescent emissions of the resulting nanocrystal particles can be controlled based on the selection of materials and controlling the size distribution of the particles. For example, ZnSe and ZnS particles exhibit fluorescent emission in the blue or ultraviolet range (.about.400 nm or less); Au, Ag, CdSe, CdS, and CdTe exhibit fluorescent emission in the visible spectrum (between about 440 and about 700 nm); InAs and GaAs exhibit fluorescent emission in the near infrared range (.about.1000 nm), and PbS, PbSe, and PbTe exhibit fluorescent emission in the near infrared range (i.e., between about 700-2500 nm). By controlling growth of the nanocrystal particles it is possible to produce particles that will fluoresce at desired wavelengths. As noted above, smaller particles will afford a shift to the blue (higher energies) as compared to larger particles of the same material(s).

Preparation of the nanocrystal particles can be carried out according to known procedures, e.g., Murray et al., MRS Bulletin 26(12):985-991 (2001); Murray et al., IBM J. Res. Dev. 45(1):47-56 (2001); Sun et al., J. Appl. Phys. 85(8, Pt. 2A): 4325-4330 (1999); Peng et al., J. Am. Chem. Soc. 124(13):3343-3353 (2002); Peng et al., J. Am. Chem. Soc. 124(9):2049-2055 (2002); Qu et al., Nano Lett. 1(6):333-337 (2001); Peng et al., Nature 404(6773):59-61 (2000); Talapin et al., J. Am. Chem. Soc. 124(20):5782-5790 (2002); Shevenko et al., Advanced Materials 14(4):287-290 (2002); Talapin et al., Colloids and Surfaces, A: Physiochemical and Engineering Aspects 202(2-3):145-154 (2002); Talapin et al., Nano Lett. 1(4):207-211 (2001), each of which is hereby incorporated by reference in its entirety. Alternatively, nanocrystal particles can be purchased from commercial sources, such as Evident Technologies.

Attachment of a nanocrystal particle to the oligonucleotide probe can be carried out using substantially the same procedures used for tethering dyes thereto. Details on these procedures are described in, e.g., Bioconjugate Techniques, Hermanson, ed. (Academic Press) (1996), which is hereby incorporated by reference in its entirety.

Exemplary lanthanide atoms include, without limitation, Ce, Pr, Nd, Pm, Sm, Eu, Gd, Th, Dy, Ho, Er, Tm, Yb, and Lv. Of these, Nd, Er, and Th are preferred because they are commonly used in fluorescence applications. Attachment of a lanthanide atom (or a complex containing the lanthanide atom) to the oligonucleotide probe can be carried out using substantially the same procedures used for tethering dyes thereto. Details on these procedures are described in, e.g., Bioconjugate Techniques, Hermanson, ed. (Academic Press) (1996), which is

hereby incorporated by reference in its entirety.

The quenching agent can be any agent that can be conjugated to a nucleic acid and preferably is characterized by an absorbance pattern that is matched to cause complete or substantially complete quenching of fluorescence emitted by the fluorescent label. The quenching agent can be another fluorophore that absorbs emissions by the fluorescent label and emits a different fluorescent emission pattern (i.e., during FRET) or the quenching agent can be formed of a material that absorbs fluorescent emissions by the fluorescent label but without a corresponding emission pattern. Examples of the former materials are those described above with respect to the fluorescent label and whose absorption and emission patterns are well suited to achieve FRET. Examples of the latter materials include, without limitation, dyes, such as 4-([4-(Dimethylamino)phenyl]azo)benzoic acid (dabcyl); and metals such as gold, silver, platinum, copper, cobalt, iron, iron-platinum, etc. Of these, the dye dabcyl and the metals gold, silver, and platinum are typically preferred.

The quenching agent can either be in the form of a small molecule such as a dye, a particle such as a micro- or submicron-sized (i.e., nano-) particle, or in the form of a substrate that contains thereon a sufficient density of the quenching agents such that the surface thereof is effectively a quenching surface. In one embodiment, the quenching agent is a dye or a metal nanoparticle. In another embodiment, a substrate have a quenching metal surface is utilized, such as a substrate bearing a gold film thereon.

Assembly of the hairpin probe, e.g., on the metal surface, is carried out in the presence of a spacing agent. Preferred spacing agents are non-nucleic acid thiols. Exemplary spacing agents include, without limitation, 3-mercapto-1-propanol, 1-mercapto-2-propanol, 2-mercaptoethanol, 1-propanethiol, 1-butanethiol, 1-pentanethiol, 3-mercapto-1,2-propanediol, 1-heptanethiol, 1-octanethiol, and 1-nonanethiol. Ratios of non-nucleic acid thiol:DNA hairpin employed in the assembly process are typically about 1:1 or greater, more preferably about 5:1 or greater. It is believed that the spacing agent provides spacing between individual molecules of DNA hairpin on the metal surface. Chips assembled in the absence of spacing agent are, at best, poorly functional.

When multiple molecular beacons are used (e.g., in a microarray or other similar format) and each is conjugated to a fluorescent label, it is preferable that the fluorescent labels can be distinguished from one another using appropriate detection equipment. That is, the fluorescent emissions of one fluorescent label should not overlap or interfere with the fluorescent emissions of another fluorescent label being utilized. Likewise, the absorption spectra of any one fluorescent label should not overlap with the emission spectra of another fluorescent label (which may result in undesired FRET that can mask emissions by the other label).

The probes and molecular beacons identified in accordance with the present invention can be used in any of a variety of hybridization-based applications, typically though not exclusively detection procedures for identifying the presence in a sample of a target nucleic acid molecule. By way of example, uses of the probes and molecular beacons are described in greater detail in PCT Patent Application to Miller et al., entitled "Hybridization-Based Biosensor Containing Hairpin Probes and Use Thereof," filed Jan. 2, 2003, now WO 2004/061127, which is hereby incorporated by reference in its entirety.

EXAMPLES

The Examples set forth below are for illustrative purposes only and are not intended to limit, in any way, the scope of the present invention.

Example 1

Hairpins Targeted to Bacillus anthracis pag Gene

A large sequence structure prediction from Bacillus anthracis is shown in FIG. 3 and depicts the "folding" of large sequences of DNA revealing several naturally occurring hairpins. The sequences are then isolated from the full sequence and subjected to second structure prediction. FIGS. 4A-B show structural predictions for two of these excised sequences.

These natural hairpins, BaPag668-706 (Pag 668) and BaPag1208-1241 (Pag 1208), both appear to be good candidates for use as a molecular beacon, because each contains between about 30 to about 40 nucleotides and each has a E.sub.predict between about -4 kcal/mol and about -12 kcal/mol.

Having confirmed that the selected hairpin(s) satisfy initial selection criteria, a final structural prediction of the sequence in duplex with its complement was computed (FIGS. 8A-B). This last prediction was done primarily to ensure that the hybridization of the two DNA sequences, and thus the disruption of the hairpin will be an energetically favorable process. Each of these duplexes have a predicted .DELTA..DELTA.G value that is about nine to ten-fold greater than the predicted E (.DELTA.G) value for the hairpin alone, and therefore they are expected to favorably form a duplex with their targets.

The specificity of the hairpin of FIG. 4 for its target was supported by a BLAST search of the GenBank database using the BaPag 668-704 sequence. The results of this BLAST search are shown below in FIG. 5. In particular, the BLAST results indicate that only sequences from Bacillus anthracis, the target organism, have high scores; whereas other "matching sequences from non-target organisms have significantly lower scores. In this instance, a clear demarcation exists between target scores (of 78) and non-target scores (of 42 and lower). This demonstrates that this hairpin will be specific for its target.

Example 2

Hairpins Targeted to Staphylococcus aureus Genome

Two DNA hairpins, AH2 and BH2, were designed to incorporate portions of the Staphylococcus aureus genome (Genbank Accession AP003131, which is hereby incorporated by reference in its entirety). The AH2 sequence appears to target an intergenic region between ORFID:SA0529 and ORFID:SA0530, and the BH2 sequence appears to target an intergenic region between ORFID:SA0529 and ORFID:SA0530 but also includes several bases within the latter open reading frame.

A segment of the complete Staphylococcus aureus genome was obtained from the GenBank database and the secondary structure of the obtained segment was predicted using computer program RNAStructure version 3.7 (Mathews et al., J. Mol. Biol. 288:911-940 (1999), which is hereby incorporated by reference in its entirety), as shown in FIG. 6. From this predicted structure, two naturally occurring hairpins were identified, one designated AH2 and the other designated BH2 (FIG. 6).

Having identified these two sequences, these sequences were isolated from the larger sequence and subjected to a second structure prediction as described above. The predicted structure of AH2 is characterized by a predicted free energy value of about -6.1 kcal/mol (FIG. 7A) and the predicted structure of BH2 is characterized by a predicted free energy value of about -3.5 kcal/mol (FIG. 7B). Both are within the size range of about 30-40 nucleotides.

Having selected AH2 and BH2, a final structural prediction of the duplexes (AH2 and BH2 with their respective complements) was carried out to determine their .DELTA..DELTA.G value. The duplex containing AH2 was predicted to have a free energy value of -32.2 kcal/mol and the duplex containing BH2 was predicted to have a free energy value of -35.5 kcal/mol (FIGS. 8C-D). These values indicate that the hybridization between the hairpin and its target will be an energetically favorable process. A BLAST search was independently performed using the AH2 and BH2 sequences, the results indicating that only segments of the Staphylococcus aureus genome contain highly related nucleotide sequences.

Example 3

Hairpins Targeted to Other Pathogen

This process described above and exemplified in Examples 1-2 has also been performed using Exophiala

dermatitidis 18S ribosomal RNA gene sequences to identify hairpin probes that can be used to identify the target gene (and organism); Trichophyton tonsurans strain 18S ribosomal RNA gene sequences to identify hairpin probes that can be used to identify the target gene (and organism); and Bacillus cereus genomic DNA to identify hairpin probes that can be used to identify the target DNA (and organism). These sequences have been reported in PCT Patent Application to Miller et al., entitled "Hybridization-Based Biosensor Containing Hairpin Probes and Use Thereof," filed Jan. 2, 2003, now WO 2004/061127, which is hereby incorporated by reference in its entirety.

Example 4

Hairpins Favorably Hybridize with their Target DNA

Samples of BaPag668 and BaPag1208, both alone and mixed with equal amount of complement, were run on a native polyacrylamide gel. The purpose of this experiment was two-fold: (1) to demonstrate that, as predicted, the designed hairpins form only one preferred structure, and (2) to provide another example that the hairpins will favorably hybridize with their target DNA. The results are shown in FIG. 9.

The presence of single bands in Lanes 1 and 3 is evidence that the hairpins preferentially adopt one structure. This claim can be made confidently because the distance non-duplexed DNA migrates through a polyacrylamide gel is based on both the size (molecular weight) and shape of the molecule in question. Any variations from the predicted structure would either enhance or retard the variant's migration through the gel, thus creating multiple bands.

The upward shift seen in Lanes 2 and 4 is indicative of the addition of mass that occurs during the hybridization of the hairpins with their targets. The increased contrast of the bands in Lanes 2 and 4 also gives indication that the hairpins are successfully forming double-stranded duplexes with their targets, as the dye used preferentially binds double-stranded regions of DNA.

Example 5

Thermal Melting Curves for DNA Hairpin Probes

Determination of the presence of an ordered secondary structure was accomplished via the procurement of thermal melting profiles. All melting temperatures of BaPag668-706, BaPag1208-1241, AH2, and BH2 were found to be concentration independent. As discussed by others (Inglesby et al., "Anthrax as a Biological Weapon: Medical and Public Health Management," J. Am. Med. Assoc. 281:1735-1745 (1999), which is hereby incorporated by reference in its entirety), the observed concentration independence is a strong indicator of the presence of an ordered secondary structure, presumed to be the desired hairpins. The unmodified hairpins were then mixed with a ten-fold excess of complementary DNA and a second series of melting profiles were obtained (FIGS. 10E-H). As was expected, introduction of complement to the hairpins produced a biphasic transition curve, with the first transition corresponding to the linearization of the target DNA, which is also believed to possess ordered secondary structure, and the second, higher temperature, transition corresponding to the melting point of the duplex DNA.

Example 6

Solution-Phase Performance of Beacon BaPag668-706

To provide an initial indication of the ability of the BaPag668-706 probe to function as a molecular beacon, the response to target DNA of BaPag668-706 when modified with a 5'-fluorescein and a 3'-dabcyl. The modified BaPag668-706 beacon was mixed with increasing concentrations of target DNA in aluminum foil covered eppendorf tubes. After approximately one hour at room temperature, fluorescence measurements were procured to determine the efficacy of the beacon. As shown in FIG. 11, BaPag668-706 exhibits minimal fluorescence alone, and, as expected, addition of the target complementary oligonucleotide causes fluorescence to increase in

a concentration-dependent manner.

Example 7

Performance of BaPag1208 Immobilized on an Au-Film

The performance of the functionalized hairpins as Au-immobilized DNA sensors was examined. BaPag1208 was immobilized onto an Au film in much the same manner as has previously been reported (Du et al., "Hybridization-based Unquenching of DNA Hairpins on Au Surfaces: Prototypical "Molecular Beacon" Biosensors," J. Am. Chem. Soc. 125:4012:4013 (2003), which is hereby incorporated by reference in its entirety), with the only major change being the use of 0.5 M NaCl buffer as the diluent as opposed to deionized water. BaPag1208 was initially immobilized in a 1:10 ratio with mercapto propanol, the results of which are shown in FIGS. 12A-C.

When immobilized on an Au-film in a 1:10 ratio with mercaptopropanol, BaPag1208 shows greater than an 18-fold increase (FIGS. 12A-C) in fluorescence intensity in response to incubation in a 2.5 .mu.M target solution. When the concentration of the target solution is lowered to 1.0 .mu.M, the observed response drops to about 10-fold, which is still significant (FIGS. 12D-F).

Despite reports that 1:10 ratio of beacon to mercaptopropanol provided for the best signal to noise ratio for more traditional beacons, additional studies suggested that for BaPag1208, a 1:1 ratio may provide a more effective beacon. As such, BaPag1208 was immobilized onto an Au-film with mercaptopropanol in a 1:1 ratio and subjected to the same target concentrations as described previously. As can be clearly seen in FIGS. 13A-C, when immobilized in a 1:1 ratio with mercaptopropanol, BaPag1208 shows a superior response to target over that observed when the immobilization ratio is 1:10. This increased response is especially significant at lower concentrations as is evidenced by the greater than 20-fold intensity increase observed after incubation in 1.0 .mu.M target. (FIG. 13D-F).

Initial studies as to the sensitivity of BaPag1208-1241 when immobilized onto an Au-film have been started and are summarized in FIG. 14. BaPag1208-1241 immobilized beacons have shown a nearly a 10-fold response to a 1.0 mL solution of 1.0 nM target (1.0 pmol). Studies planned for the very near future should elucidate the absolute limit of detection for a solution of synthetic and native targets.

Example 8

Performance of AH2 and BH2 Immobilized on an Au-Film

To examine the suitability of the "partial gene folding" derived beacons in such a scheme, the Staphylococcus aureus probes AH2 and BH2 were obtained modified with a 5'-thiol (allowing for attachment to Au film using standard chemistry), and either a 3'-rhodamine (AH2) or a 3'-Cy5 (BH2). These two probes were concurrently assembled in a 1:1 ratio in the presence of mercaptopropanol on two Au films. Individual films were then treated with solutions of either AH2-complement or BH2-complement. Addition of 1.0 .mu.M AH2-complement yielded a chip with significant fluorescence around 585 nm (FIG. 15C), while addition of 1.0 .mu.M BH2-complement produced weak, but still observable Cy5 fluorescence (675 nm) (FIG. 15F). A partial reason for the weak signal observed from the Cy5 is due to the small absorption cross section for Cy5 in green wavelengths. Indeed, using an AH2-Cy5 functionalized surface, excitation at 633 nm (cross section 8 times greater than at 514 nm) produced twice as much fluorescence intensity from Cy5. These results suggest that although differentiating multiple targets with only a single light source is not yet optimized, co-immobilization of two probes produces a functional chip.

Example 9

Calculated Hybridization Energies for Folding-Derived and Modified Beacons

It is difficult to rigorously compare folding-derived to modified beacons, since changing the sequence obviously alters more than one experimental parameter. However, the effects of modification can be predicted, as shown by the calculations in FIGS. 16 and 17. The termini of probes BaPag668-706 and BaPag1208-1241 were first extended by the self-complementary sequence [d(CGACG)].sub.2, then the hybridization energy calculated (FIGS. 16B and 17B, respectively). Second, five bases were removed from each end of BaPag668-706 and BaPag1208-1241, replaced with [d(CGACG)].sub.2, and the hybridization energy again calculated (FIGS. 16C and 17C, respectively). In each case, calculated .DELTA..DELTA.G values were less favorable for the modified beacons than for the probes derived directly from folding.

The fact that the hybridization product of the new beacon is energetically superior to that of the traditional design should lead the new beacon to have a higher sensitivity. The binding free energy for hybridization .DELTA.G.sub.bind is related to the observed equilibrium association constant K.sub.A by: -.DELTA.G.sub.bind=-RTlnK.sub.A, where T is the temperature and R the universal gas constant (Riccelli et al., "Hybridization of Single-stranded DNA Targets to Immobilized Complementary DNA Probes: Comparison of Hairpin Versus Linear Capture Probes," Nucl. Acids Res. 29: 996-1004 (2001), which is hereby incorporated by reference in its entirety). The use of hairpins that have 100% sequence participation in duplex formation allows for a more energetically favorable duplex than would exist for a hairpin that contains non-specific termini. Thus, the duplex that forms the more energetically favorable dimer will be expected to bind much more tightly, and therefore is expected to be more sensitive. Highly sensitive detection schemes are preferred for rapid detection and identification of pathogens in a clinical sample.

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

SEQUENCE LISTINGS

1

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