



(11) **EP 1 100 971 B1**

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
12.11.2008 Bulletin 2008/46

(21) Application number: **99937602.3**

(22) Date of filing: **28.07.1999**

(51) Int Cl.:
C12Q 1/68 (2006.01) C07H 21/00 (2006.01)

(86) International application number:
PCT/US1999/017145

(87) International publication number:
WO 2000/006778 (10.02.2000 Gazette 2000/06)

(54) **WAVELENGTH-SHIFTING PROBES AND PRIMERS**

WELLENLÄNGEN VERSCHIEBENDE PROBEN UND PRIMER

SONDES ET AMORCES A DECALAGE SPECTRAL

(84) Designated Contracting States:
**AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU
MC NL PT SE**

(30) Priority: **28.07.1998 US 123764**

(43) Date of publication of application:
23.05.2001 Bulletin 2001/21

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- **TYAGI SANJAY ET AL: "Wavelength-shifting molecular beacons" NATURE BIOTECHNOLOGY, NATURE PUBLISHING, US, vol. 18, no. 11, November 2000 (2000-11), pages 1191-1196, XP002215066 ISSN: 1087-0156**
- **MATTHEWS ET AL.: 'REVIEW: Analytical Strategies for the Use of DNA Probes' ANALYTICAL BIOCHEMISTRY vol. 169, February 1988, pages 1 - 25, XP002922846**

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DescriptionStatement as to Federally Sponsored Research

5 **[0001]** Some work on this invention was carried out under National Institutes of Health Grant HL-43521. The United States Government may have certain rights in this invention.

[0002] This invention relates to oligonucleotides, kits and amplification assays as defined in the claims.

Background on the Invention

10 **[0003]** Hairpin-forming oligonucleotide hybridization probes with interactive label pairs, particularly fluorescent label pairs and fluorescer-quencher label pairs, that open when the hairpin's single-stranded loop hybridizes to a target are known. Tyagi et al., PCT application No. WO95/13399; Tyagi et al., PCT application No. WO97/39008; Tyagi and Kramer (1996) Nature Biotechnology 14:303. Preferred embodiments of these probes, labeled with a fluorophore and a quencher, are "dark", that is, have relatively little or no fluorescence, when free in solution but fluoresce when hybridized to their nucleic acid targets. We refer to such embodiments as "molecular beacon probes." Some molecular beacon probes bring the fluorophore very close to a quencher when the probe is closed. Quenching is not constrained by the FRET rule of spectral overlap. They are constructed with a variety of fluorophores and are utilized in both end-point and real-time homogeneous assays, including multiplex assays. Tyagi et al PCT application No. WO98/10096; Tyagi et al. (1998) Nature Biotechnology 16:49; Kostrikis et al. (1998) Science 279:1228; Piatek et al. (1998) Nature Biotechnology 16:359. Hairpin-containing primers similarly labeled with a fluorophore and a quencher are also known. Nazarenko et al. (1997) Nucleic Acids Research 25:2516.

20 **[0004]** Also known are hairpin probes of a different structure and mode of operation, namely, short loops flanked by a first arm complementary to the target and a second arm that is partially complementary to the first and competes with the target to bind to it; and which open when the target-complementary arm hybridizes to a target and lead to FRET-based detection (Bagwell U.S. Patent No. 5,607,834).

25 **[0005]** Fluorescence assay instruments that operate with a single stimulation wavelength are much less complicated and much less expensive than instruments that operate with multiple stimulation wavelengths. Almost all assay instruments, including sophisticated instruments costing many tens of thousands of dollars (U.S.) operate with single wavelength stimulation, even if the wavelength is selectable. Because every fluorophore has an optimal excitation wavelength, the choice of fluorophores to be used with a detection instrument having such a light source is limited. Certain fluorophores will be excited poorly or essentially not at all by the source. Red fluorophores such as tetramethylrhodamine (TMR) and Texas red are only minimally excited by a blue light source. A sophisticated, expensive instrument such as the Applied BioSystems 7700 PRISM, can detect fluorescence from TMR stimulated by a blue light source, but not Texas red. Less sophisticated, less expensive instruments can detect neither one. In multiplex assays for multiple targets using multiple hairpin probes or primers having differently colored fluorophores, it is desirable to be able to use four or even more fluorophores whose emission spectra have limited overlap, but the choice of fluorophores is limited by the use of single wavelength stimulation. Also, multiplex assays suffer from the drawback that the emission intensities of some of the fluorophores are very small both in absolute terms and also relative to other fluorophores in the assay. Furthermore, because the Stokes shift (the wavelength difference between the optimal excitation wavelength and optimal emission wavelength) of fluorophores is generally only a few nanometers, hairpin probes and primers whose fluorophores have emission maxima at or very close to the wavelength of the excitation source tend to suffer from background signal resulting from the source itself being detected by the fluorometer. This tends to be more pronounced when less expensive, unsophisticated detectors are employed.

30 **[0006]** The present invention allows for the provision of probes and primers whose conformational change produces a detectable fluorescent signal having a greater Stokes shift than conventional hairpin probes and primers.

[0007] The present invention provides a wider range of hairpin-forming probes and primers that are effectively quenched ("dark") in the absence of target but are better excited in the presence of target by a monochromatic light source.

35 **[0008]** The present invention also allows for the provision of a group, or series, of hairpin-forming probes or primers containing differently colored fluorophores, all of which can be reasonably well excited by a single monochromatic light source.

[0009] The present invention provides probes and primers suitable for use with single-wavelength stimulation instruments, including unsophisticated instruments for which the current choices are extremely limited.

40 **[0010]** The present invention allows for the provision of hairpin-forming probes and primers having reduced background signal as a consequence of the source itself not being detected.

45 **[0011]** Furthermore, the present invention allows for probes that fluoresce in one color if hybridized to target but change color if digested by a nuclease.

[0012] Aspects of the invention will be apparent from the description, including the claims, which follows.

SUMMARY OF THE INVENTION

[0013] We have discovered that hairpin-forming probes and primers may be constructed such that their fluorescence when closed is suppressed, but when open, they are well excited by a monochromatic light source and emit strongly at a wavelength distanced from the wavelength of the source. Certain preferred embodiments are "dark" when closed, by which we mean that their total fluorescence when closed is less than twenty percent of their total fluorescence when open and that they do not change color upon opening. Probes according to this invention are suitable for use in end-point detection assays, and probes and primers according to this invention are suitable for use in real-time amplification assays such as, for example, polymerase chain reaction (PCR) assays. The end-point assays and real-time assays may be multiplex assays. Assay kits containing the probes and primers are also provided.

[0014] In one aspect of the invention, there is provided a fluorescently labeled hairpin-forming oligonucleotide having a closed conformation including a single-stranded loop and a stem duplex and an open conformation not including said stem duplex, wherein hybridization of said loop to a target sequence causes the oligonucleotide to assume its open conformation, said oligonucleotide containing:

- a) a fluorescent emitter moiety having an excitation spectrum and an emission spectrum including a maximum emission wavelength;
- b) a fluorescent harvester moiety having an excitation spectrum including a maximum excitation wavelength, having an emission spectrum that overlaps the excitation spectrum of the emitter moiety and including a maximum emission wavelength, the emission of the harvester moiety at its maximum emission wavelength having a first magnitude when said harvester moiety is unquenched and stimulated at its maximum excitation wavelength; and
- c) a quencher moiety capable of quenching the fluorescence of at least one of the emitter moiety and the harvester moiety, e.g., DABCYL or a variant of DABCYL

wherein when said oligonucleotide is in the closed conformation said quencher moiety is in a quenching relationship to at least one of said harvester and emitter moieties and wherein, when excited at the maximum excitation wavelength of the harvester moiety, emission at the maximum emission wavelength of the harvester moiety is substantially suppressed relative to said first magnitude and emission at the maximum emission wavelength of the emitter moiety has a second magnitude, and when said oligonucleotide is in the open conformation said quencher moiety is not in a quenching relationship with said harvester or said emitter moiety wherein, when excited at the maximum excitation wavelength of the harvester moiety, emission at the maximum emission wavelength of the harvester moiety is substantially suppressed relative to said first magnitude, energy is transferred from the harvester moiety to the emitter moiety, and emission at the maximum emission wavelength of the emitter moiety is detectably greater than said second magnitude.

[0015] For molecular beacon probes modified according to this invention, hybridization of the probes to target strands causes the probes to shift from their closed conformation to their open conformation.

[0016] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by persons of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, methods and materials are described below. reference in their entirety. In case of conflict with the publications, patents and patent applications referred to herein, the present application, including definitions will control. In addition, the materials, methods and examples described herein are illustrative only and not intended to be limiting.

Brief Description of The Drawings**[0017]**

Fig. 1 is a graph of absorbance and emission spectra of two fluorophores, fluorescein and tetramethylrhodamine (TMR).

Fig. 2 is a graph of fluorescence of a conventional fluorescein-containing molecular beacon probe during PCR thermal cycling.

Fig. 3 is a graph of fluorescence of a conventional Texas red-containing molecular beacon probe during PCR thermal cycling.

Fig. 4 is a graph of fluorescence of a Texas red-containing wavelength-shifting molecular beacon probe according to this invention during PCR thermal cycling.

Fig. 5 is a schematic representation of the interaction of a preferred probe of this invention with its target.

Fig. 6 is a graph of fluorescence of Probe 1, a probe according to this invention, and a fluorescein-labeled conventional molecular beacon probe.

Fig. 7 is a graph of fluorescence of Probe 2, a probe according to this invention.

Fig. 8 is a graph of fluorescence of Probe 3, a probe according to this invention.

Fig. 9 is a graph of fluorescence of Probe 4, a probe according to this invention.

Fig. 10 is a graph of fluorescence of probe 5, a probe according to this invention.

Fig. 11 is a graph of fluorescence of Probe 6, a probe according to this invention.

5 Fig. 12 is a graph of fluorescence of Probe 7, a probe according to this invention.

Fig. 13 is a graph of fluorescence of Probe 8, a probe according to this invention.

Fig. 14 is a graph of fluorescence of Probe 9, a probe according to this invention.

Fig. 15 is a graph of fluorescence of Probe 14, a conventional molecular beacon probe, and Probe 13, a wavelength-shifting probe according to this invention during a PCR amplification in which no target for either probe was present.

10 Fig. 16 is a graph of fluorescence of Probe 14 and Probe 13 during a PCR amplification in which target for Probe 14 was amplified.

Fig. 17 is a graph of fluorescence of Probe 14 and Probe 13 during a PCR amplification in which target for Probe 13 was amplified.

15 Fig. 18 is a graph of fluorescence of Probe 14 and Probe 13 during a PCR amplification in which targets for both Probe 14 and Probe 13 were amplified.

Fig. 19 is a graph of fluorescence of Probe 1, and the fluorescence of three other probes that are identical to Probe 1 except that they possess different emitter fluorophores.

Fig. 20 is a graph of the fluorescence of Probe 1 substituted with a Texas-red emitter, measured at 610 nm and plotted as a function of excitation wavelength.

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Detailed Description

25 **[0018]** Hairpin-forming oligonucleotide probes, including molecular beacon probes, that may be modified according to this invention are interactively labeled, hairpin-forming oligonucleotides comprising a stem-and-loop structure. The loop contains a probe sequence complementary to the probe's target. Nucleotide sequences ("arms") flank the probe sequence, and a sequence in one arm is complementary to a sequence in the other arm. When the probe is not hybridized to target, the arms hybridize with one another to form a stem hybrid, which is sometimes referred to as the "stem duplex". This is the closed conformation. When the probe hybridizes to its target, the longer and stronger probe-target hybrid overcomes the stem hybrid and separates the arm sequences. This is the open conformation. In the open conformation an arm may also hybridize to the target. Molecular beacon probes may be free in solution, or they may be tethered to a solid surface. For some molecular beacon probes, which we refer to as "allele-discriminating," only perfectly complementary strands are targets that cause this change under assay conditions; for other embodiments the probe will open despite the presence of one or a few internal mismatches with the target. Molecular beacon probes have a fluorophore attached to one arm and a quencher attached to the other arm. When the arms form a stem, the quencher is very close to the fluorophore and effectively quenches or suppresses its fluorescence, rendering the probe dark. Such probes are described in copending United States patent applications Serial No. 08/439,619 and Serial No. 08/990,176. The fluorophore and quencher, for example, tetramethylrhodamine and DABCYL, need not be a FRET pair.

30 **[0019]** The oligonucleotide sequences of molecular beacon probes modified according to this invention may be DNA, RNA, peptide nucleic acid (PNA) or combinations thereof. Modified nucleotides may be included, for example nitropyrole-based nucleotides or 2'-O-methylribonucleotides. Modified linkages also may be included, for example phosphorothioates. Modified nucleotides and modified linkages may also be incorporated in wavelength-shifting primers according to this invention, subject, as will be recognized, to the requirement that one arm be able to serve a primer for a nucleic acid polymerase.

35 **[0020]** For probes according to this invention, the length of the loop sequence that is target complementary, the length of the stem hybrid and the relation of the two is designed according to the assay conditions for which the probe is to be utilized. Lengths of target-complement sequence and stem hybrid for particular assay conditions can be estimated by known means, tried and adjusted, if necessary. Typical probe sequences for use in PCR assays are in the range of 16 to 25 nucleotides. Typical stem lengths are in the range of 3 to 8, more commonly 4 to 7 nucleotides. The strength of the stem hybrid is adjusted by routine experimentation to achieve proper functioning. In addition to length, the strength of the stem hybrid can be adjusted by altering the G-C content and insertion of destabilizing mismatches, as will be appreciated. One arm can be designed to be partially or completely complementary to the target. If the 3' arm is complementary to the target the probe can serve as a primer for a DNA polymerase. Also, wavelength-shifting molecular beacon probes can be immobilized to solid surfaces, as by tethering, as well as being free-floating.

40 **[0021]** A wide range of fluorophores may be used in probes and primers according to this invention. Available fluorophores include coumarin, fluorescein, tetrachlorofluorescein, hexachlorofluorescein, Lucifer yellow, rhodamine, BOD-IPY, tetramethylrhodamine, Cy3, Cy5, Cy7, eosine, Texas red and ROX. Combination fluorophores such as fluorescein-rhodamine dimers, described, for example, by Lee et al. (1997), *Nucleic Acids Research* 25:2816, are also suitable. Fluorophores may be chosen to absorb and emit in the visible spectrum or outside the visible spectrum, such as in the

ultraviolet or infrared ranges.

[0022] A quencher is a moiety that, when placed very close to an excited fluorophore, causes there to be little or no fluorescence. Suitable quenchers described in the art include particularly DABCYL and variants thereof, such as DABSYL, DABMI and Methyl Red. Fluorophores can also be used as quenchers, because they tend to quench fluorescence when touching certain other fluorophores. Our preferred quenchers are either chromophores such as DABCYL or malachite green, or fluorophores that do not fluoresce in the detection range when the probe is in the open conformation.

[0023] Hairpin-forming probes according to this invention may be utilized in detection assays. They may also be used as detectors in amplification assays, and may be added prior to amplification, in which case quantitative results as to the initial concentration of target may be obtained. Amplification reactions include the polymerase chain reaction (PCR), strand displacement amplification (SDA), nucleic acid sequence based amplification (NASBA), transcription mediated amplification (TMA), the ligase chain reaction (LCR), rolling circle amplification, and RNA-directed RNA amplification catalyzed by an enzyme such as Q-beta replicase. Multiple probes for multiple targets may be used in a single reaction tube or other container for multiplex assays.

[0024] Hairpin-forming primers are used in those of the amplification reactions identified above that include one or more primers. They may be modified according to the present invention have an arm sequence that binds to a nucleic acid target, such that the hairpin-containing primer can be extended by incubation with a nucleic acid polymerase. The loop portion may, but need not be, complementary to the original target strand. Hairpin-containing primers have a stem labeled with a fluorophore on one arm and a quencher on the other arm, similarly to molecular beacon detection probes. Embodiments of the instant invention will be described primarily in connection with molecular beacon detection probes. Workers in the art will understand that the concepts and teachings apply to hairpin primers as well, and will understand how to apply the concepts and particular teachings to hairpin-containing primers.

[0025] Fig. 1 shows the excitation and emission spectra of two fluorophores, fluorescein and tetramethylrhodamine (TMR). Fluorescein has an absorption spectrum 1 with a maximum at 490 nm and an emission spectrum 2 with a maximum at 515 nm. Fluorescein has a Stokes Shift, the difference between the two maxima, of 25 nm. TMR has an excitation spectrum 3 with a maximum at 555 nm and an emission spectrum 4 with a maximum at 575 nm. It has a Stokes shift of 20 nm.

[0026] Fig. 1 shows the relatively small Stokes shift typical of most fluorophores. An instrument producing a stimulation light at a nominal wavelength of, for example, 488 nm, may in fact have an excitation spectrum that includes longer wavelengths, and a detector set for 515 nm may in fact respond to light that includes shorter wavelengths. The fluorescent signal measured by such an instrument would include a background reading from the stimulating light.

[0027] Fig. 1 also shows why there is a serious practical limitation of an instrument having a single stimulating wavelength. If the stimulating wavelength is 490 nm, which is optimum for the excitation of fluorescein, a molecular beacon probe having TMR as the fluorophore would be stimulated only ten percent as well as it would have been by a source at 560 nm. The intensity of its emission would be correspondingly lower. If the excitation source were changed to 560 nm, which is appropriate for TMR, fluorescein would not be excited at all. Thus, the number of different fluorophores that work well is restricted no matter which excitation wavelength is utilized. These effects are illustrated in Fig. 2 and Fig. 3, which present the results of using a conventional molecular beacon probe design as a detector for amplicons in a PCR amplification reaction. The probe design includes a stem-and-loop hairpin structure that is terminally labeled with a fluorophore and the quencher DABCYL oppositely positioned across the stem duplex. Fig. 2 shows what happens when the fluorophore is fluorescein, the excitation source is a blue argon laser (488 nm), and the detector measures fluorescence in the emission range of fluorescein. Fluorescence intensities reported in Fig. 2, and also in Fig. 3 and Fig. 4, were obtained using an Applied Biosystems 7700 PRISM spectrofluorometric thermal cycler (The Perkin-Elmer Corporation), which has sophisticated fluorescence measuring. The fluorescence intensity 21 of a sample containing target molecules increased above the fluorescence intensity 22 of a sample containing no target molecules beginning at about PCR cycle 15 and increased rapidly to a difference of about 4000 units by cycle 30. Fig. 3 shows what happens in a parallel experiment in which the only differences are that the fluorophore of the molecular beacon probe is Texas red instead of fluorescein, and the detector measures fluorescence in the emission range of Texas red. Here again the fluorescence intensity 31 of the sample containing target began to deviate from the fluorescence intensity 32 of the targetless control at about cycle 15, but by cycle 30 the difference was only about 200 units as compared to 4000 seen with the molecular beacon probe containing fluorescein. Texas red is not a useful fluorophore when the stimulating source emits blue light, even with an expensive instrument that has sophisticated fluorescence measurement.

[0028] Fig. 4 shows the results of a third parallel experiment in which the only difference from the second experiment was that the Texas red probe of Fig. 3 was changed to a construction according to the instant invention. Here again the fluorescence intensity 41 of the sample containing target began to deviate from the reading 42 of the targetless control at approximately cycle 15, but by cycle 30 the difference was approximately 3000 units, about seventy-five percent of the magnitude of the fluorescence intensity of the fluorescein-labeled conventional molecular beacon probe utilized in the experiment shown in Fig. 2 and about fifteen times as great as the magnitude of the fluorescence intensity of the conventional molecular beacon probe labeled with Texas red, shown in Fig. 3. This increase was achieved without

sacrificing the property that the probe itself is essentially dark when not hybridized to target as fluorescence intensity 42 shows. Thus, an excitation source at 488 nm is an acceptable source for a Texas red probe according to this invention and, conversely, Texas Red is an acceptable fluorophore for probes and primers according to this invention for use with an excitation source at 488 nm. The emission intensity achieved by the probe according to this invention makes the probe suitable for use not only with sophisticated detection such as the Applied Biosystems PRISM, but also for use with less expensive instruments that have unsophisticated fluorescence measurement.

[0029] The structure and operation of modified molecular beacon probes and hairpin primers according to this invention will be explained by reference to a preferred probe embodiment, shown schematically in Fig. 5. Probe 51 includes a molecular beacon probe hairpin oligonucleotide structure, namely, a loop 52, stem duplex 53, a DABCYL quencher 55 on one end and a fluorescein fluorophore 54 opposite the quencher in a close, quenching relationship across the stem hybrid when the probe is in the closed conformation. The probe includes an extension 57 of several nucleotides past the fluorescein. Extension 57 terminates in a nucleotide that is linked to a Texas red fluorophore 56. In the presence of target strand 58, loop 52 forms hybrid 59 with the target strand, unwinding stem 53 and separating the quencher 55 from the fluorescein 54 and Texas red 56. However, in this open conformation the fluorescein acts as a "harvester" moiety that absorbs energy from the excitation source but transfers a significant portion of the energy, in some constructions the great majority of the energy, to the Texas red fluorophore, which acts as an "emitter" moiety by receiving the transferred energy and emitting it at its characteristic, longer wavelength.

[0030] The function of probe 51 in the open conformation appears to obey the rules of FRET, which require that the two fluorophores be separated by an appropriate distance and that the emission spectrum of the harvester moiety significantly overlaps the absorption spectrum of the emitter moiety. Referring to Fig. 1, the shaded area 5 depicts such a spectral overlap for the two fluorophores whose spectra are presented there. We have discovered that probe 51 is essentially dark in its closed conformation despite the presence of a second fluorophore that makes a FRET pair with fluorescein. Possibly the combination of fluorescein and DABCYL does not make a FRET pair with Texas red. Whatever the mechanism may be, there is little to no fluorescence at 610 nm when the probe is closed. Similarly, there is little to no fluorescence at 515 nm when the probe is closed.

[0031] Probes and primers according to this invention, which we refer to as "wavelength-shifting" molecular beacon probes and "wavelength-shifting" hairpin primers, like their unmodified counterparts, can be made from DNA, RNA, PNA (peptide nucleic acid), or combinations of the foregoing. Modified nucleotides and modified nucleotide linkages may be used in place of naturally occurring nucleotides and linkages. Fluorophores and quenchers can be inserted into the strand in place of a nucleotide. The loop of a wavelength-shifting molecular beacon probe is complementary to the target and has a length of at least seven nucleotides, with a preferred range being from about twelve to about thirty nucleotides, although longer loops can be used. Wavelength-shifting primers may have similar loops; however, their loops need not be complementary to the target, and their loops can be as short as three nucleotides. The arms form a stem duplex of at least three nucleotides with a preferred range being from three to about eight nucleotides, although longer stems are suitable in some applications.

[0032] A first arm of the probe or primer is labeled with a quencher. The probe or primer is labeled with a pair of fluorophores capable of interacting by energy transfer when the quencher is relocated distantly by the opening of the probe or primer. One of the pair is attached to the second arm in a close, quenching relationship with the quencher when the probe is closed. Either the shorter wavelength fluorophore, the "harvester", or the longer wavelength fluorophore, the "emitter", has that relationship to the quencher. The other fluorophore may be placed farther away from the quencher. Alternatively, the quencher may be positioned opposite a point on the other arm that is intermediate to the two fluorophores. When the probe is bound to target or the primer is opened, the harvester and the emitter are separated by a distance appropriate for fluorescence energy transfer. The fluorophore that is not in a close, quenching relationship to the quencher across the stem duplex may be attached to the second arm in the region of the stem or an extension outside the stem, or in some cases it may be attached to the loop. In the closed conformation, as stated above, the quencher and one of the harvester and emitter fluorophores is in a close, quenching relationship, by which we mean that they are sufficiently close to one another that quenching predominates over fluorescence resonance energy transfer. Most preferably the two moieties touch each other. However, separation by a single base pair along the stem duplex is almost always satisfactory. Even greater separations are possible in many instances, namely, 2-4 base pairs or even 5-6 base pairs, particularly if a spacer, described below, is utilized. For these greater separations the helical nature of the stem duplex should be considered for its effect on the distance between the moieties.

[0033] In embodiments in which the emitter fluorophore is in a close, quenching relationship with the quencher moiety when the probe is closed, the harvester and emitter fluorophore must be at an appropriate FRET distance.

[0034] The transfer of energy from the harvester fluorophore to the emitter fluorophore is governed by the rules of fluorescence resonance energy transfer [Stryer (1979) Ann. Rev. Biochem. 47:819], which we use to aid in the design of probes and primers according to this invention. In particular, two rules are considered in order to maximize the efficiency of energy transfer. First, the absorption spectrum of the emitter and the emission spectrum of the harvester must overlap. Second, the two fluorophores must be spaced relative to one another such that they can undergo fluorescence resonance

energy transfer (FRET). The optimal distance between the emitter and harvester fluorophores is a function of two opposing effects. On the one hand, the farther apart the two moieties are, the lower the efficiency of energy transfer; the transfer of energy from the exciter to the emitter is proportional to $1/R^6$, where R is the distance between the two fluorophores. On the other hand, the closer they are, the greater the potential for undesirable quenching of the emitter emission. The distance between the two fluorophores as determined by the number of atoms in the chain separating the two fluorophores can be varied in accordance with the nature of the backbone to which they are attached. They should be sufficiently separated so that there is substantial FRET, and preferably they are sufficiently separated that FRET predominates over quenching. These rules are commonly employed in order to optimize the efficiency of energy transfer from one fluorophore to the other when they are attached to a DNA molecule [Ju et al. (1995) Proc. Natl. Acad. Sci. USA 92:4347; Ju et al. (1995) Anal. Biochem. 231:131; Hung et al. (1998) Anal. Biochem. 252:78; Mathies et al. PCT/US96/13134]. Distances of 20-60 Angstroms are appropriate, which translates to about six to about eighteen nucleotides, with seven nucleotides being a preferred separation and the amount of energy transfer decreasing as separation is increased. For example, with fluorescein as the harvester fluorophore, a DABCYL quencher opposite to and touching the fluorescein, and either JOE, TET, TAMRA, ROX, or Texas red as the emitter fluorophore, a separation of 5 to 8 nucleotides between the two fluorophores provides optimal spacing. We find that with a five-nucleotide separation, FRET is substantial and predominates over quenching. We find that with a four-nucleotide separation there is substantial FRET but also substantially quenching, which is less preferred. The identity of the nucleotides in the spacer has little effect on the efficiency of energy transfer. The sequence of nucleotides in the spacer, however, should not form a hairpin that brings the harvester and emitter fluorophores to a quenching distance. Other kinds of spacers, such as alkyl spacers, can also be used.

[0035] We have discovered that, despite the presence of an emitter capable of receiving energy by transfer from the harvester, probes and primers according to this invention have suppressed emission when closed. We have discovered that suppression occurs when the quencher is in a quenching relationship with the harvester. We have also discovered that suppression occurs when the quencher is in a quenching relationship with the emitter. The mechanism or mechanisms by which suppression occurs are not understood. Nonetheless, as we show in the Examples below, fluorescence of the harvester is suppressed substantially in both cases, and at the same time fluorescence of the emitter is maintained at a low level, so that, when the probe or primer opens, fluorescence of the emitter increases detectably.

[0036] Any fluorophore that has strong absorption in the wavelength range of the available monochromatic light source can be used as the harvester fluorophore. For example, when an argon laser emitting blue light (488 nm) or a blue light emitting diode is used as the excitation source, fluorescein can serve as an excellent harvester fluorophore. Another harvester fluorophore that is efficient in the blue range is 3-(ϵ -carboxy-pentyl)-3'-ethyl-5,5'-dimethylxocarbocyanine (CYA) [Hung et al. (1996) Anal. Biochem. 243:15]. For these harvester fluorophores, the emitter fluorophores can be 2', 7'-dimethoxy-4',5'-dichloro-6-carboxy-fluorescein (JOE), tetrachlorofluorescein (TET), N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), 6-carboxy-X-rhodamine (ROX), Texas red, and a number of cyanine dyes whose absorption spectra share substantial spectral overlap with the emission spectrum of fluorescein and CYA. With sources of different wavelengths, fluorophores may be selected to absorb and emit anywhere along the spectrum from ultraviolet to infrared. Compound fluorophores such as those described in Lee et al. (1997) can be used as a fluorophore.

[0037] Fluorophores and the quencher can be added to the probe by functionalization of the appropriate building blocks (e.g., deoxyribonucleotides) such that the fluorophores will be present on the building blocks prior to the formation of the probe, or they may be conjugated to the probe after formation, as appropriate. Various chemistries known to those of average skill in the art can be used to ensure that the appropriate spacing between the two fluorophores is obtained. In addition fluorophore phosphoramidites, for example a fluorescein phosphoramidite, can be used in place of a nucleoside phosphoramidite. A nucleotide sequence that contains such a substitution is considered to be an "oligonucleotide" as that term is used in this disclosure and in the appended claims, despite the substitution.

[0038] The fluorophores and the quencher can be attached via alkyl spacers to different positions on a nucleotide. The labels can be placed at internal or terminal locations in the oligonucleotide, using commonly available DNA synthesis reagents. The labels can also be placed at internal positions in oligonucleotides by substituting a nucleotide linked to a fluorophore moiety during synthesis. Although, commonly available spacers that employ alkyl chains of several carbons (Glen Research) can be used successfully, the degree of quenching and the extent of energy transfer can be further optimized by varying the length of the spacers.

[0039] Wavelength-shifting molecular beacon probes and hairpin primers according to this invention can be characterized by comparing their fluorescence to corresponding open conventional molecular beacon probes or hairpin primers having only the harvester fluorophore or only the emitter fluorophore. Wavelength-shifting probes and primers, when closed, (a) have harvester emission substantially suppressed as compared to corresponding open, harvester-only probes or primers similarly excited, and (b) have emitter emission substantially lower than emitter-only probes or primers excited at the emitter's excitation maximum. Wavelength-shifting probes and primers, when open, (c) still have harvester emission substantially suppressed as compared to corresponding open, harvester-only probes or primers similarly excited, and (d) have emitter emission both substantially higher than the emitter emission when closed and substantially higher than

corresponding open, emitter-only probes or primers excited at the harvester's excitation maximum.

[0040] Harvester emission suppression is measured against emission from the open conformation of a corresponding conventional molecular beacon probe or hairpin primer labeled only with the harvester and quencher. Alternatively, a wavelength-shifting probe or primer according to this invention may be digested by incubation with DNase I, which separates the label moieties and permits the harvester to fluoresce unhindered. Either of the foregoing can be used to obtain the magnitude, or intensity, of the harvester moiety against which harvester suppression is measured. By "substantially suppressed" we mean that the intensity of fluorescence of the harvester at its emission maximum is at least forty percent, preferably at least fifty percent, and more preferably at least sixty percent lower than either above standard against which it is measured.

[0041] Emitter emission when the probe or primer is in the closed conformation can be measured against emission from the open conformation of a corresponding conventional molecular beacon probe or hairpin primer that is emitter-quencher labeled and excited at the excitation maximum of the emitter. It should be substantially lower. Here again, by "substantially lower" we mean the intensity of fluorescence of the emitter at its emission maximum is at least forty percent, preferably at least fifty percent and more preferably at least sixty percent lower than the standard against which it is measured. This ensures that the emitter is not fluorescing maximally or close to maximally when the probe or primer is closed, permitting a detectable increase when the probe opens. In preferred embodiments that are "dark" when closed, there is little to no fluorescence at the emitter's wavelength, by which we mean that the emission is at least eighty percent lower than the above standard. It is a characteristic of probes and primers according to this invention that, when stimulated at a wavelength appropriate for the harvester, emission at the emitter's emission maximum increases detectably when the probe or primer opens. While an increase of twenty percent is generally a detectable increase, it is preferred that the increase be at least a factor of two, more preferably at least a factor of four. In preferred embodiments that are "dark" when closed, there is an increase of at least a factor of four and most preferably a factor of at least eight. These attributes, and other attributes that may be attractive for particular applications, are described in the Examples that follow.

EXAMPLES

[0042] Several of wavelength-shifting molecular beacon probes of varying constructions were prepared and tested. The following synthesis methods apply to the probes described in these Examples.

[0043] Conventional molecular beacon probes and wavelength-shifting probes according to this invention were synthesized. Labels were attached to the probe sequences either during automated synthesis or by post-synthetic reactions which have been described before [Tyagi and Kramer (1996)]. The quenchers were introduced to the oligonucleotides by any of the following three methods: a controlled-pore glass column was used to introduce a 4-dimethylaminoazobenzene-4'-sulfonyl moiety (DABSYL) at the 3' end of the oligonucleotides during automated synthesis; a succinimidyl ester of 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL) was used when the site of attachment was a primary amino group; and 4-dimethylaminophenylazophenyl-4'-maleimide (DABMI) was used when the site of attachment was a sulphydryl group. Fluorescein was introduced at internal locations in the DNA, either using a fluorescein phosphoramidite that replaces a nucleoside with fluorescein, or by using a fluorescein dT phosphoramidite that introduces a fluorescein moiety at a thymidine ring via a spacer. To link a fluorescein moiety to a terminal location, iodoacetamidofluorescein was coupled to a sulphydryl group. Tetrachlorofluorescein (TET) was introduced during automated synthesis using a 5'-tetrachlorofluorescein phosphoramidite. Other reactive fluorophore derivatives and their respective sites of attachment were: the succinimidyl ester of 5-carboxyrhodamine-6G (RHD) coupled to an amino group; an iodoacetamide of tetramethylrhodamine coupled to a sulphydryl group; an isothiocyanate of tetramethylrhodamine coupled to an amino group; or a sulfonylchloride of Texas red coupled to a sulphydryl group. During the synthesis of these multiply labeled probes the conjugated oligonucleotides were purified by high pressure liquid chromatography after each coupling step.

Example 1

Testing Probe Constructs

[0044] In the series of nine probes described in this example, DABSYL was utilized as the quencher, fluorescein as the harvester and TMR as the emitter. The oligonucleotide sequences of the probes are shown below. The sequences that participate in the formation of a stem duplex are underlined. Internally placed fluorophores are indicated by name only. For example, "fluorescein" refers to a fluorophore moiety that substitutes for a nucleotide in the oligonucleotide. Internally placed fluorophores indicated by T-names, for example, "(T-fluorescein)", refer to a thymidine nucleotide having the fluorophore attached to the thymidine ring via an alkyl spacer.

Probe 1

TMR-5' -TTTTT-fluorescein-CCACGCTTGTGGGTCAACCCCGTGG-3' -
DABSYL

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Probe 2

TMR-5' -TTTTT-fluorescein-CCACGCTTGTGGGTCAACCCCGTGGTTT-3' -
DABSYL

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Probe 3

TMR-5'-CCACGT-fluorescein-TCTTGTGGGTCAACCCCGTGG-3'-DABSYL

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Probe 4

TMR-5' -CCGG (T-fluorescein) CCGCTTGTGGGTCAACCCGACCGG-3' -
DABSYL

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Probe 5

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TMR-5' -TTCC (T-fluorescein) GGCCGCTTGTGGGTCAACCCGCCAGG-3' -
DABSYL

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Probe 6

TMR-5' -TTTT (T-fluorescein) GCGGCCGCTTGTGGGTCAACCCGCCGA-3' -
DABSYL

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Probe 7

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TMR-5' -CACACG (T-fluorescein) CCTGCCGCTTGTGGGTCAACCCGCAGG-3' -
DABSYL

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Probe 8

TMR-5' -CAGCACACG (T-fluorescein) CGCGCGCTTGTGGGTCAACCCGCCGA-3' -
DABSYL

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Probe 9

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5' - (T-fluorescein) CAGCACACG (T-TMR) CGCGCGCTTGTGGGTCAACC
CCGCCGA-3' -DABSYL

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For comparative purposes a conventional fluorescein-DABSYL molecular beacon probe was also synthesized and tested. Its sequence is shown below.

Probe 11

fluorescein-5'-CCACGCTTGTGGGTCAACCCCGTGG-3'-DABSYL

[0045] The probes were tested by subjecting them to an excitation source having a wavelength of 491 nm, in a Photon Technology International QuantaMaster spectrofluorometer, both in the absence of target (closed configuration) and in the presence of excess target (open configuration). Emission spectra were obtained from 500 to 650 nm, that is, across a range that includes the emission of both fluorescein and TMR. Results for probes 1-9 are presented in Figs. 6-14, respectively. Each figure includes fluorescence intensities for open and closed probes. Spectra of samples without target are labeled 61, 71, ..., 141, and spectra of samples with target are labeled 62, 72,, 142.

[0046] Probe 1 is a preferred embodiment of this invention. It has a quencher, in this case DABSYL, terminally attached to one arm, namely, the 3'-terminus of the probe. It has a harvester, in this case fluorescein, in the 5' arm positioned opposite, in this case directly opposite, the quencher when the probe is closed. It has an emitter, in this case TMR, at the terminus of the 5' arm, five nucleotides beyond the fluorescein. Those five nucleotides are an extension of the 5' arm beyond the stem duplex. In this embodiment the harvester and quencher are in the most preferred quenching relationship, that is, they touch.

[0047] Fig. 6 shows emission spectra for Probe 1. In the absence of target, trace 61, there was almost no fluorescence at the fluorescein maximum (515 nm) and very little fluorescence at the TMR maximum (575 nm); that is, the probe was quite dark when closed. In the presence of target, trace 62, there was an 18-fold increase in fluorescence at 575 nm. Fig. 6 also includes trace 63, the emission spectrum of the open, corresponding molecular beacon Probe 11. Because fluorescein emits so strongly as compared to other fluorophores, the vertical scale of Fig. 6 is broken. A comparison of traces 51 and 52 at 515 nm with trace 63 (Probe 11) at that wavelength shows that fluorescein emission was very substantially suppressed in both configurations of Probe 1, even though there was a modest amount of fluorescein emission in the open configuration. In the closed conformation the harvester suppression was greater than ninety-nine percent; in the open conformation, greater than ninety percent. The shape of spectrum 62 remained similar to the shape of spectrum 61. These results demonstrate that Probe 1 shifted emission towards the red as compared to Probe 11.

[0048] Figs. 7-14, unlike Fig. 6, are normalized. The maximum emission when the probe is open is set at 1.0 on the vertical axis. The remaining peaks are fractions of that emission. Figs. 7-14 do not include a trace for a corresponding fluorescein-labeled molecular beacon probe. However, by reference to Fig. 6 it can be appreciated that a fluorescein-labeled molecular beacon probe, when open, has an emission intensity that would be from 3.0 to 6.0 on the vertical scale of Figs. 7-14. Substantial harvester suppression can be discerned from the fact that no fluorescein peak is even twice the intensity of the emitter peak when any of the probes is open.

[0049] Fig. 7 shows the emission spectra of Probe 2. Probe 2 differs from Probe 1 in the position of DABSYL when the Probe is closed. In Probe 2 the 3' end of the probe was extended three nucleotides beyond the stem, moving the quencher to a point intermediate the two fluorophores on the other arm. In the open configuration, trace 72, the spectrum is essentially the same as for Probe 1, as expected. In the closed configuration, trace 71, there was very little fluorescence at either 515 nm or 575 nm, although slightly more than for Probe 1. Probe 2 was quenched quite effectively and was nearly dark when closed. Effective quenching of Probe 2 is not due to FRET.

[0050] Fig. 8 shows the emission spectra of Probe 3. Probe 3 is somewhat reversed from Probe 1 in that a 5' terminal TMR, the emitter, is directly opposite from DABSYL across the stem duplex when the probe is closed. The fluorescein is located inboard from the stem duplex. There is a six-nucleotide separation between the two fluorophores. Spectra 81 and 82 show that Probe 3 exhibited a reversal of color when it opened, which may be useful in particular applications. In the absence of target there was appreciable fluorescence at 515 nm (albeit substantially suppressed), more than double the fluorescence at 575 nm. When Probe 3 opened, however, the opposite was found: fluorescence at 515 nm dropped by more than half, while fluorescence at 575 nm increased by a factor of 4.8. Thus, the color of the probe's fluorescence changed due to the presence of target. Probe 3 was not dark when closed.

[0051] Fig. 9 shows the emission spectra of Probe 4, which is similar to Probe 3 in the placement of the TMR and DABSYL moieties at the end of the stem. Fluorescein is located inboard from TMR, but is located in the stem and attached to a thymidine nucleotide. In the closed configuration Probe 4 was better quenched than Probe 3 at 515 nm and had a lower fluorescence at 575 nm. However, the fluorescence intensity of Probe 4 did not decrease at 515 nm when it opened. It continued to have a modest, suppressed fluorescein emission. Because of its lower fluorescence at 575 nm when closed, as compared to Probe 3, its increase at 575 nm upon opening was three-fold better, namely, a factor of 16.0.

[0052] Figs. 10-13 show the emission spectra of Probes 5-8, respectively. These constructions, when closed, all showed substantial quenching at 515 nm and low signal at 575 nm. When open each had an intensity increase at 575 nm greater than a factor of eight. When open, each had a fluorescein emission that, while appreciable, was substantially

suppressed. All were dark when closed.

[0053] Fig. 14 shows the emission spectra for Probe 9. When Probe 9 was closed, fluorescein emission was very substantially suppressed. When open, fluorescence at 575 nm increased by more than a factor of eight, but suppression of fluorescein was only marginally substantial. While Probe 9 might be expected to have an emission spectrum when open that is very similar to the emission spectrum of Probe 8 when open, a difference was found in the relative intensities of the fluorescein and TMR peaks. Probe 9 had a smaller TMR peak relative to the fluorescein peak. This indicates that the efficiency of FRET between fluorescein and TMR was lower in Probe 9. Why then Probe 9 was better quenched is not clear. Perhaps the nature of the TMR was altered by its association with DABSYL in the closed configuration.

Example 2

Assays

[0054] Two additional probes were prepared, as follows:

Probe 12

Texas red-5'-CCACGCTTGTGGGTCAACCCCGTGG-3'-DABSYL

Probe 13

**Texas red-5' -TTTTT-fluorescein-CCACGCTTGTGGGTCAACCCCGTGG-
3' - DABSYL**

[0055] Probe 12 was a conventional molecular beacon probe terminally labeled with Texas red and DABSYL. It had the same nucleotide sequence as Probe 11, a conventional molecular beacon probe terminally labeled with fluorescein and DABSYL. Probe 13, on the other hand, is a wavelength-shifting molecular beacon probe according to a preferred construction (similar to Probe 1). Either Probe 11, Probe 12 or Probe 13 was added at the beginning of a PCR reaction to a test sample and to a control. The test samples were initiated with target. A reaction initiated with no target served as the control in each case. Each amplification included cycles of PCR in an Applied Biosystems 7700 PRISM spectrofluorometric thermal cycler. The light source in the instrument was an argon laser having a wavelength of 488 nm. Emission of each conventional molecular beacon probe was measured during the annealing phase of each cycle in the emission range of its fluorophore, fluorescein (Probe 11) or Texas red (Probe 12). Emission of the wavelength-shifting molecular beacon probe, Probe 13, was measured in the emission range of the emitter fluorophore, Texas red. The results are presented in Figs. 2-4 and have been discussed earlier in this application.

[0056] Wavelength-shifting probes according to this invention may be used for detection *in situ* and *in vivo*, that is, in living cells. The difference in wavelength between the excitation maximum of the harvester and the emission maximum of the emitter, the Stokes shift of the probes, is larger than conventional probes. This is particularly advantageous detection when a microscope is used to monitor nucleic acids in biological samples. First, microscopes typically utilize a broad wavelength light source rather than a precise, single-wavelength light source. Second, biological samples have natural autofluorescence characterized by a small Stokes shift. Both of the foregoing characteristics contribute to background, which is avoided by wavelength-shifting probes. In certain assays probes may encounter degrading nucleases. Probe 1 illustrates a particular benefit of wavelength-shifting probes in such circumstances. If Probe 1 binds to target, red fluorescence results. However, if Probe 1 is degraded, green fluorescence results. One can see from Fig. 6 that degradation, by separating the harvester from the emitter, will restore fluorescein peak 63, which is stronger than TMR peak 62. Thus, one can see from the fluorescence whether or not degradation is occurring.

[0057] We note that it is of no consequence whether or not most probes according to this invention, Probe 13 for example, are cleaved by the polymerase during amplification, so long as cleavage does not occur between the harvester and the emitter. Where cleavage is found to occur during amplification, one could avoid using an embodiment in which either or both of the harvester and emitter moieties are in the probe-target hybrid, if that is found to lead to significant cleavage between the two of them. One could also use modified nucleotides, such as 2'-O-methylribonucleotides.

Example 3

Multiplex Assay

[0058] A multiplex assay is an assay utilizing at least two probes to test for at least two possible targets in the same

sample. Multiple probes according to this invention for different targets can be used, and they can be used in a mixture with other probes. This example demonstrates the principle and also demonstrates allele discrimination. Wavelength-shifting molecular beacon Probe 13 was utilized as one probe. Another probe, Probe 14, was synthesized for use as a second probe:

Probe 14
 fluorescein-5'-CCACGCTTGTCGGTCAACCCCGTGG-3'-DABSYL

[0059] Probe 14 is a conventional molecular beacon probe terminally labeled with fluorescein and DABSYL. The nucleotide sequence of Probe 14 differed from the nucleotide sequence of Probe 13 by a single nucleotide in the target complement sequence in the loop. A perfectly complementary target was synthesized for each probe. The perfect target for Probe 14 differed from the perfect target for Probe 13 by a single nucleotide.

[0060] Both probes were added to four PCR reaction mixes: a negative control containing no target, a sample containing the target perfectly complementary to Probe 14, a sample containing the target perfectly complementary to Probe 13, and a sample containing both of the targets in equal amounts. In each instance where any target was present, the total amount of target or targets was the same, about 200,000 copies.

[0061] Thermal cycling and fluorescence emission reading was as described in Example 2. The results are presented in Fig. 15 (no target), Fig. 16 (target perfectly complementary to Probe 14), Fig. 17 (target perfectly complementary to Probe 13), and Fig. 18 (both targets). The emissions in the fluorescein range, traces 151, 161, 171 and 181, are presented as dotted lines, and the emissions in the Texas red range, traces 152, 162, 172 and 182, are presented as solid lines.

[0062] Fig. 15 shows that there was no fluorescence in the absence of target: both probes were dark when not hybridized to target. Fig. 16 shows high fluorescence in the fluorescein range caused by perfectly matched target for Probe 14, the fluorescein-labeled conventional molecular beacon probe. Fig. 16 also shows no fluorescence in the Texas red range, demonstrating that Probe 13 was an allele-discriminating probe that was sensitive to a single base-pair mismatch. Fig. 17 shows moderately high fluorescence in the Texas red range caused by perfectly matched target for Probe 13, a wavelength-shifting molecular beacon probe. Fig. 17 also shows no fluorescence in the fluorescein range, demonstrating that Probe 14 was an allele-discriminating probe that was sensitive to a single base-pair mismatch. Fig. 18 shows moderately high fluorescence in both ranges, which should occur because the amount of each target was one-half the total amount. In Fig. 18 the emission level in the Texas red range was 76% of the emission level in the fluorescein range. This is greatly improved compared to what would have occurred with a Texas red-labeled conventional molecular beacon probe stimulated at 488 nm, as Fig. 3 demonstrates, because the signal in the Texas red range would have been only 3% of the emission in the fluorescein range. Whereas a Texas red conventional molecular beacon is not generally suitable for multiplexing with a fluorescein-labeled conventional molecular beacon, a wavelength-shifting molecular beacon with a Texas red emitter is quite satisfactory for such multiplexing.

Example 4

Measuring the Characteristics of Wavelength-Shifting Probes

[0063] Probe 1, which is wavelength-shifting molecular beacon probe according to this invention having TMR as the emitter, was compared to several probes that were identical except for the emitter fluorophore. Three other fluorophores, tetrachlorfluorescein (TET), rhodamine 6G (RHD) and Texas red were utilized. When compared to their conventional molecular beacon counterparts using a 488 nm excitation source, the fluorescence of the wavelength-shifting probes in the open conformation was a much higher percentage of the maximum achievable fluorescence intensity than the corresponding conventional molecular beacon probe would have achieved in the open conformation, if it has been stimulated at its maximum excitation wavelength. Whereas the conventional molecular beacon probes stimulated at 488 nm achieved one-third or less of that maximum, the wavelength-shifting probes achieved two-thirds or more.

[0064] Emission spectra for these probes are shown in Fig. 19, which illustrates the impact of the instant invention as the fluorescence color of the emitter fluorophore departs more and more from the stimulation wavelength of the instrument. The fluorescence intensities are normalized, that is, presented as a fraction of the fluorescence achieved by stimulating the emitter directly at its maximum excitation wavelength. Curves 191 and 192 are the normalized emission spectra for a TET-labeled conventional molecular beacon probe and a corresponding wavelength-shifting molecular beacon probe having TET as the emitter, respectively. Curves 193 and 194 are the normalized emission spectra for an RHD-labeled conventional molecular beacon probe and a corresponding wavelength-shifting molecular beacon probe having RHD as the emitter, respectively. Curves 195 and 196 are analogous curves using TMR, and curves 197 and 198 are analogous curves using Texas red. Fluorescein is the harvester in all cases.

[0065] Curves for the conventional molecular beacon probes, that is, curves 191, 193, 195 and 197, show several things. First, the emission maxima shift to progressively longer wavelengths as the label is changed from TET to RHD

to TMR to Texas red. Second, even for TET, the shortest wavelength fluorophore, excitation at 488 nm produces only about one-third of the emission achievable by excitation at the maximum absorption wavelength for TET, which is 522 nm. Third, the relative emission intensity falls off from one-third as the color of the fluorophore's fluorescence moves toward red, that is to longer wavelengths. For TMR, which has an emission maximum at 575 nm, only ten percent of the maximum achievable fluorescence intensity results. For Texas red, that percentage drops to three percent. Even with an expensive instrument having sophisticated emission detection, such as the Applied Biosystems 7700 PRISM, using TMR with a stimulating source of 488 nm is difficult, and using Texas red is not possible. With a less expensive instrument having unsophisticated detection, using either TMR or Texas red would not be possible. This markedly limits multiplexing possibilities and options, requiring use of fluorophores having closely spaced emission maxima, which are more difficult to resolve.

[0066] Curves for the wavelength-shifting probes according to this invention, that is, curves 192, 194, 196 and 198, also show several things. First, in every case there is increased emission intensity as compared to the corresponding conventional molecular beacon probe. Second, the relative intensities remain very high, about sixty-five percent, as one moves all the way to Texas red. Each of these probes is suitable for use with unsophisticated, less expensive instruments. Third, the amount of improvement over the corresponding conventional molecular beacon probe is greatest for the longest wavelength emitters, TMR and, especially, Texas red. Such marked improvement is significant even for use with an expensive instrument having sophisticated detection. Fourth, fluorescein emission is greatly suppressed. Referring to Fig. 6, one sees that fluorescein is perhaps the brightest fluorophore, roughly five times as bright as Probe 1, a wavelength shifting probe according to this invention. The emission of a corresponding conventional fluorescein molecular beacon probe would be off the scale of Fig. 19. Its intensity would be at least triple the maximum, "1.0", of the vertical axis.

[0067] The changed nature of probes according to this invention is graphically illustrated by considering their excitation spectra. Fig. 20 compares the excitation spectrum of the Texas-red wavelength-shifting molecular beacon described above in this example and the corresponding Texas-red conventional molecular beacon probe. To obtain an excitation spectrum, the spectrofluorometer was set to read emission at 610 nm, the maximum for Texas red, as the excitation wavelength was increased progressively from 400 to 600 nm. Curve 201 for the conventional probe shows strong emission when the excitation wavelength reached 590 nm, the excitation maximum for Texas red. At 488 nm excitation almost no emission occurs. On the other hand, curve 202 for the wavelength-shifting shows not only the peak at 590 nm, but also a second strong peak at 488 nm. The probe has twin excitation ranges, both of which lead to emission at the wavelength of 610 nm.

Example 5

Wavelength-Shifting Primers

[0068] As stated earlier, a wavelength-shifting molecular beacon probe whose 3' arm sequence is Complementary to the target can serve as a primer. However, it is not required that the loop of a hairpin primer be complementary to the target, because specificity can be obtained via an extension of the 3' arm, which creates a priming region. Conventional hairpin primers for PCR amplification can be synthesized and utilized, for example, according to the methods of Nazarenko et al. (1997). They can be modified according to the instant invention. Primer F described by Nazarenko et al. has the following sequence:

5' - 6-fluorescein - CACCTTCACCCTCAGAAGG (T-DABCYL) GACC
AAGTTCAT - 3'

[0069] In Primer F, 6-fluorescein was incorporated by using a 6-fluorescein phosphoramidite during synthesis. The T-DABCYL nucleotide was obtained by synthesizing with AminoModifier C6dT (Glen Research) and attaching DABCYL to the primary amino group. At least the first eighteen nucleotides from the 3' end were complementary to a target, a 172 base-pair segment of human prostate-specific antigen cDNA.

[0070] A primer embodiment according to this invention is made by modifying the sequence of Primer F in several respects. First, a phosphoramidite of fluorescein that can be inserted in the middle of a DNA chain (Glen Research) is utilized to introduce the harvester moiety. Second, six thymidine residues are added past the harvester toward the 5' end. Third, a Texas red moiety is introduced via a sulphhydryl functionality. The primer according to this invention has the following sequence, with portions forming a stem duplex underlined):

Texas red-5'-TTTTT-fluorescein-
CACCTTACCCTAGAAGG (T-DABCYL) GACCAAGTTCAT-3' .

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[0071] In the closed conformation, this primer has DABCYL opposite (with a single nucleotide offset) fluorescein across the stem duplex, with the Texas red on an extension. The construction is analogous to Probe 1 in Example 1. In the open conformation, the DABCYL is displaced from the fluorophores, and the two fluorophores (the harvester fluorescein and the emitter Texas red) are separated by seven nucleotides, a workable FRET separation. A PCR amplification of 30 cycles is carried out using this primer as one of the amplification primers. This construction functions as a wavelength-shifting hairpin primer. Wavelength-shifting hairpin primers of different colors can be multiplexed in amplification reactions. Nazarenko et al. utilized on "exo-minus" DNA polymerase, Pfu^{exo-} DNA polymerase (Stratagene, USA), thereby avoiding possible cleavage during amplification. If cleavage is encountered during a particular amplification reaction using primers according to this invention, cleavage between the harvester and emitter moieties can be avoided as described in Example 2.

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[0072] The Examples are presented for illustration and not as a limitation. Further embodiments will be apparent to workers in the art which fall within the following claims, which are intended as the measure of the invention.

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Claims

1. A fluorescently labeled hairpin-forming oligonucleotide having a closed conformation including a single-stranded loop and a stem duplex and an open conformation not including said stem duplex, wherein hybridization of said loop to a target sequence causes the oligonucleotide to assume its open conformation, said oligonucleotide containing:

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a) a fluorescent emitter moiety having an excitation spectrum and an emission spectrum including a maximum emission wavelength;

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b) a fluorescent harvester moiety having an excitation spectrum including a maximum excitation wavelength, having an emission spectrum that overlaps the excitation spectrum of the emitter moiety and including a maximum emission wavelength, the emission of the harvester moiety at its maximum emission wavelength having a first magnitude when said harvester moiety is unquenched and stimulated at its maximum excitation wavelength; and

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c) a quencher moiety capable of quenching the fluorescence of at least one of the emitter moiety and the harvester moiety, e.g., DABCYL or a variant of DABCYL

wherein when said oligonucleotide is in the closed conformation said quencher moiety is in a quenching relationship to at least one of said harvester and emitter moieties and wherein, when excited at the maximum excitation wavelength of the harvester moiety, emission at the maximum emission wavelength of the harvester moiety is substantially suppressed relative to said first magnitude and emission at the maximum emission wavelength of the emitter moiety has a second magnitude, and when said oligonucleotide is in the open conformation said quencher moiety is not in a quenching relationship with said harvester or said emitter moiety wherein, when excited at the maximum excitation wavelength of the harvester moiety, emission at the maximum emission wavelength of the harvester moiety is substantially suppressed relative to said first magnitude, energy is transferred from the harvester moiety to the emitter moiety, and emission at the maximum emission wavelength of the emitter moiety is detectably greater than said second magnitude.

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2. An oligonucleotide of claim 1 wherein, when said oligonucleotide is in its closed conformation, either:

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a) the emitter moiety is located on said oligonucleotide terminally to said stem duplex, the quencher and harvester moieties are located on said oligonucleotide in said stem duplex, and the harvester and quencher moieties are in a quenching relationship; or

b) said quencher and emitter moieties are located on said oligonucleotide in said stem duplex in a quenching relationship.

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3. An oligonucleotide of claim 1 wherein, when said oligonucleotide is in its open conformation said emission at the maximum emission wavelength of the emitter is at least four times as great as said second magnitude.

4. An oligonucleotide of any one of claims 1 to 3 wherein either:

a) one strand of said stem duplex is complementary to said target strand, whereby said oligonucleotide is capable of serving as a primer for a DNA polymerase; or

b) said oligonucleotide includes a terminal extension capable of serving as a priming region for a DNA polymerase when said oligonucleotide is in its closed conformation; optionally wherein said emitter moiety is located on said terminal extension and said quencher and harvester moieties are located on said oligonucleotide in said stem duplex.

5. An oligonucleotide of any one of claims 1 to 4 wherein at least one of said harvester and emitter moieties forms part of the nucleotide chain of said oligonucleotide, or at least one of said harvester, emitter and quencher moieties is attached to said oligonucleotide through an alkyl linker.

6. An oligonucleotide of any one of claims 1 to 6 wherein said oligonucleotide includes at least one of the group of modified nucleotides and modified linkages, optionally wherein said oligonucleotide comprises peptide nucleic acid (PNA) or includes 2'-O-methylribonucleotides.

7. A reagent kit comprising ingredients for a nucleic acid amplification and detection of amplified product, including an oligonucleotide according to any one of claims 1 to 6, and instructions for carrying out said amplification reaction: optionally said amplification being selected from the group consisting of polymerase chain reaction (PCR), strand displacement amplification (SDA), transcription mediated amplification (TMA), ligase chain reaction (LCR), nucleic acid sequence based amplification (NASBA), rolling circle amplification, and amplification of RNA by an RNA-directed RNA polymerase.

8. A kit according to claim 7 wherein said oligonucleotide is designed to hybridize to a perfectly complementary target sequence but not to hybridize significantly to a sequence differing therefrom by at least one nucleotide.

9. An amplification assay, e.g. an in situ or in vivo assay, comprising adding to a sample that might contain a target strand the reagents to perform amplification reaction and detection including an oligonucleotide according to any one of claims 1 to 6 wherein said amplification reaction is selected from the group consisting of the polymerase chain reaction (PCR), strand displacement amplification (SDA), transcription mediated amplification (TMA), ligase chain reaction (LCR), nucleic acid sequence based amplification (NASBA), rolling circle amplification, and amplification of RNA by an RNA-directed RNA polymerase, and detecting fluorescence emission from said oligonucleotide's emitter moiety;

said assay optionally further comprising adding said oligonucleotide's to a sample that does not contain said target strand and detecting fluorescence emission of said oligonucleotide's emitter moiety.

10. An assay according to claim 9 further comprising detecting fluorescence emission from said oligonucleotide's harvester moiety.

11. An assay according to claim 9 or claim 10 wherein said oligonucleotide is a probe that includes 2'-O-methylribonucleotides.

Patentansprüche

1. Ein fluoreszierend markiertes Haarnadel bildendes Oligonukleotid mit einer geschlossenen Konformation, welche eine einzelsträngige Schleife und einen Stammduplex einschließt, und einer offenen Konformation, welche den Stammduplex nicht einschließt, wobei die Hybridisierung der Schleife mit einer Zielsequenz bewirkt, dass das Oligonukleotid seine offene Konformation annimmt, wobei das Oligonukleotid enthält:

a) einen fluoreszierenden Emitteranteil mit einem Anregungsspektrum und einem Emissionsspektrum, das eine maximale Emissionswellenlänge einschließt;

b) einen fluoreszierenden Harvesteranteil mit einem Anregungsspektrum, das eine maximale Anregungswellenlänge einschließt, mit einem Emissionsspektrum, das mit dem Anregungsspektrum des Emitteranteils überlappt und eine maximale Emissionswellenlänge einschließt, wobei die Emission des Harvesteranteils an seiner maximalen Emissionswellenlänge eine erste Größe aufweist, wenn der Harvesteranteil nicht gequencht wird und an seiner maximalen Anregungswellenlänge angeregt wird; und

c) einen Quencheranteil, welcher in der Lage ist, die Fluoreszenz von wenigstens einem von dem Emitteranteil und dem Harvesteranteil zu quenchen, z.B. DABCYL oder eine Variante von DABCYL,

wobei, wenn das Oligonukleotid in der geschlossenen Konformation vorliegt, sich der Quencheranteil in einer quen-
 chenden Beziehung zu wenigstens einem der Harvester- und Emitteranteile befindet und wobei, wenn bei der
 maximalen Anregungswellenlänge des Harvesteranteils angeregt wird, die Emission bei der maximalen Emissions-
 wellenlänge des Harvesteranteils in Bezug auf die erste Größe im Wesentlichen unterdrückt wird und die Emission
 bei der maximalen Emissionswellenlänge des Emitteranteils eine zweite Größe aufweist, und wenn das Oligonu-
 kleotid in der offenen Konformation vorliegt, sich der Quencheranteil nicht in einer quenchenden Beziehung zu dem
 Harvester- oder dem Emitteranteil befindet, wobei, wenn bei der maximalen Anregungswellenlänge des Harvester-
 anteils angeregt wird, die Emission bei der maximalen Emissionswellenlänge des Harvesteranteils in Bezug auf die
 erste Größe im Wesentlichen unterdrückt wird, Energie von dem Harvesteranteil auf den Emitteranteil übertragen
 wird und die Emission bei der maximalen Emissionswellenlänge des Emitteranteils nachweisbar größer ist als die
 zweite Größe.

2. Ein Oligonukleotid nach Anspruch 1, wobei, wenn das Oligonukleotid in der geschlossenen Konformation vorliegt, entweder:

a) der Emitteranteil auf dem Oligonukleotid terminal zu dem Stammduplex lokalisiert ist, die Quencher- und Harvesteranteile auf dem Oligonukleotid in dem Stammduplex lokalisiert sind und die Harvester- und Quencheranteile sich in einer quenchenden Beziehung befinden; oder

b) die Quencher- und Emitteranteile auf dem Oligonukleotid in dem Stammduplex in einer quenchenden Beziehung lokalisiert sind.

3. Ein Oligonukleotid nach Anspruch 1, wobei, wenn das Oligonukleotid in seiner offenen Konformation vorliegt, die Emission bei der maximalen Emissionswellenlänge des Emitters wenigstens viermal so groß ist wie die zweite Größe.

4. Ein Oligonukleotid nach irgendeinem der Ansprüche 1 bis 3, wobei entweder:

a) ein Strang des Stammduplex zu dem Zielstrang komplementär ist,

wobei das Oligonukleotid in der Lage ist, als ein Primer für eine DNA-Polymerase zu dienen; oder

b) das Oligonukleotid eine terminale Extension einschließt, die in der Lage ist, als eine Primingregion für eine DNA-Polymerase zu dienen, wenn das Oligonukleotid in seiner geschlossenen Konformation vorliegt; wobei gegebenenfalls der Emitteranteil auf der terminalen Extension lokalisiert ist und der Quencher- und Harvesteranteil auf dem Oligonukleotid in dem Stammduplex lokalisiert sind.

5. Ein Oligonukleotid nach irgendeinem der Ansprüche 1 bis 4, wobei wenigstens einer der Harvester- und Emitteranteile einen Teil der Nukleotidkette des Oligonukleotids bildet oder wenigstens einer der Harvester-, Emitter- und Quencheranteile über einen Alkyl-Linker an dem Oligonukleotid befestigt ist.

6. Ein Oligonukleotid nach irgendeinem der Ansprüche 1 bis 5, wobei das Oligonukleotid wenigstens eines/eine aus der Gruppe von modifizierten Nukleotiden und modifizierten Verknüpfungen einschließt, wobei gegebenenfalls das Oligonukleotid Peptidnukleinsäure (PNA) umfasst oder 2'-O-Methylribonukleotide einschließt.

7. Ein Reagenskit, umfassend Zutaten für eine Nukleinsäureamplifikation und einen Nachweis des amplifizierten Produkts, welches ein Oligonukleotid gemäß irgendeinem der Ansprüche 1 bis 6 und Anleitungen zur Durchführung der Amplifikationsreaktion einschließt;

wobei die Amplifikation gegebenenfalls ausgewählt wird aus der Gruppe, bestehend aus der Polymerasekettenreaktion (PCR), der Strangverdrängungsamplifikation (SDA), der transkriptionsvermittelten Amplifikation (TMA), der Ligasekettenreaktion (LCR), der Nukleinsäuresequenz-basierenden Amplifikation (NASBA), der Rolling Circle Amplifikation und der Amplifikation von RNA durch eine RNA-gesteuerte RNA-Polymerase.

8. Ein Kit gemäß Anspruch 7, wobei das Oligonukleotid so gestaltet ist, dass es mit einer perfekt komplementären Zielsequenz hybridisiert, aber nicht wesentlich mit einer Sequenz hybridisiert, welche sich davon durch wenigstens ein Nukleotid unterscheidet.

9. Ein Amplifikationsassay, z.B. ein in situ- oder in vivo-Assay, umfassend das Zugeben zu einer Probe, welche einen Zielstrang enthalten könnte, der Reagenzien, um eine Amplifikationsreaktion und einen Nachweis durchzuführen,

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einschließlich eines Oligonukleotids gemäß irgendeinem der Ansprüche 1 bis 6, wobei die Amplifikationsreaktion ausgewählt wird aus der Gruppe, bestehend aus der Polymerasekettenreaktion (PCR), der Strangverdrängungsamplifikation (SDA), der transkriptionsvermittelten Amplifikation (TMA), der Ligasekettenreaktion (LCR), der Nukleinsäuresequenz-basierenden Amplifikation (NASBA), der Rolling Circle Amplifikation und der Amplifikation von RNA durch eine RNA-gesteuerte RNA-Polymerase, und das Nachweisen von Fluoreszenzemission von dem Emitteranteil des Oligonukleotids;

wobei der Assay gegebenenfalls weiterhin das Zugeben des Oligonukleotids zu einer Probe, welche den Zielstrang nicht enthält, und das Nachweisen einer Fluoreszenzemission von dem Emitteranteil des Oligonukleotids umfasst.

10 **10.** Ein Assay gemäß Anspruch 9, welcher weiterhin das Nachweisen einer Fluoreszenzemission von dem Harvesteranteil des Oligonukleotids umfasst.

15 **11.** Ein Assay gemäß Anspruch 9 oder Anspruch 10, wobei das Oligonukleotid eine Sonde ist, welche 2'-O-Methylribonukleotide einschließt.

Revendications

20 **1.** Oligonucléotide formant une épingle à cheveux à marquage fluorescent ayant une conformation fermée comprenant une boucle simple brin et une tige double brin et une conformation ouverte ne comprenant pas ladite tige double brin, où l'hybridation de ladite boucle à une séquence cible entraîne l'oligonucléotide à adopter sa conformation ouverte, ledit oligonucléotide contenant :

25 a) une fraction émettrice fluorescente ayant un spectre d'excitation et un spectre d'émission comprenant une longueur d'onde d'émission maximum ;

30 b) une fraction récoltrice fluorescente ayant un spectre d'excitation comprenant une longueur d'onde d'excitation maximum, ayant un spectre d'émission qui chevauche le spectre d'excitation de la fraction émettrice et comprenant une longueur d'onde d'émission maximum, l'émission de la fraction récoltrice à sa longueur d'onde d'émission maximum ayant une première amplitude quand ladite fraction récoltrice est non désactivée ' unquenched' et stimulée à sa longueur d'onde d'excitation maximum ; et

c) une fraction désactivatrice capable de désactiver la fluorescence d'au moins un parmi la fraction émettrice et la fraction récoltrice, par exemple le Dabcyl ou un variant du Dabcyl

35 où lorsque ledit oligonucléotide est dans la conformation fermée, ladite fraction désactivatrice est dans une relation de désactivation avec au moins une desdites fractions récoltrice et émettrice et où, quand elle est excitée à la longueur d'onde d'excitation maximum de la fraction récoltrice, l'émission à la longueur d'émission maximum de la fraction récoltrice est sensiblement supprimée par rapport à ladite première amplitude et l'émission à la longueur d'onde d'émission maximum de la fraction émettrice a une deuxième amplitude, et quand ledit oligonucléotide est dans la conformation ouverte ladite fraction désactivatrice n'est pas en relation de désactivation avec ladite fraction

40 récoltrice ou ladite fraction émettrice où, quand elle est excitée à la longueur d'onde d'excitation maximum de la fraction récoltrice, l'émission à la longueur d'onde d'émission maximum de la fraction récoltrice est sensiblement supprimée par rapport à ladite première amplitude, de l'énergie est transférée de la fraction récoltrice à la fraction émettrice, et l'émission à la longueur d'onde d'émission maximum de la fraction émettrice est de manière détectable supérieure à ladite seconde amplitude.

45 **2.** Oligonucléotide selon la revendication 1 où, quand ledit oligonucléotide est dans sa conformation fermée, soit :

50 a) la fraction émettrice est située sur ledit oligonucléotide à l'extrémité de ladite tige double brin, les fractions désactivatrice et récoltrice sont situées sur ledit oligonucléotide dans ladite tige double brin, et les fractions récoltrice et désactivatrice sont dans une relation de désactivation ; soit

b) lesdites fractions désactivatrice et émettrice sont situées sur ledit oligonucléotide dans ladite tige double brin dans une relation de désactivation.

55 **3.** Oligonucléotide selon la revendication 1, où, quand ledit oligonucléotide est dans sa conformation ouverte ladite émission à la longueur d'onde d'émission maximum de l'émetteur est au moins quatre fois aussi élevé que ladite deuxième amplitude.

4. Oligonucléotide selon l'une quelconque des revendications 1 à 3, où soit :

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a) un brin de ladite tige double brin est complémentaire dudit brin cible, moyennant quoi ledit oligonucléotide est capable de servir d'amorce pour une ADN polymérase ; soit

b) ledit oligonucléotide comprend une extension terminale capable de servir de région d'amorçage pour une ADN polymérase quand ledit oligonucléotide est dans sa conformation fermée ; éventuellement où ladite fraction émettrice est située sur ladite extension terminale et lesdites fractions désactivatrice et récoltrice sont situées sur ledit oligonucléotide dans ladite tige double brin.

5
10
5. Oligonucléotide selon l'une quelconque des revendications 1 à 4, où au moins une desdites fractions récoltrice et émettrice fait partie de la chaîne nucléotidique dudit oligonucléotide, ou au moins une desdites fractions récoltrice, émettrice et désactivatrice est attachée audit oligonucléotide par un lieu alkyle.

15
6. Oligonucléotide selon l'une quelconque des revendications 1 à 5, où ledit oligonucléotide comprend au moins un élément du groupe des nucléotides modifiés et des liaisons modifiées, éventuellement où ledit oligonucléotide comprend un acide nucléique peptidique (PNA) ou comprend des 2'-O-méthylribonucléotides.

20
7. Kit de réactifs comprenant les ingrédients pour une amplification d'acide nucléique et la détection du produit amplifié, comprenant un oligonucléotide selon l'une quelconque des revendications 1 à 6, et des instructions pour réaliser ladite réaction d'amplification ;
éventuellement ladite amplification étant choisie dans le groupe constitué par la réaction de polymérisation en chaîne (PCR), l'amplification par déplacement de brin (SDA), l'amplification médiée par la transcription (TMA), la réaction de ligation en chaîne (LCR), l'amplification à base de séquence d'acide nucléique (NASBA), l'amplification par cercle roulant et l'amplification de l'ARN par une ARN polymérase dirigée par l'ARN.

25
8. Kit selon la revendication 7, dans lequel ledit oligonucléotide est conçu pour s'hybrider à une séquence cible parfaitement complémentaire mais pas pour s'hybrider de manière significative à une séquence en différant par au moins un nucléotide.

30
9. Test d'amplification, par exemple un test in situ ou in vivo, comprenant l'ajout à un échantillon qui peut contenir un brin cible des réactifs pour réaliser une réaction d'amplification et la détection comprenant un oligonucléotide selon l'une quelconque des revendications 1 à 6, où ladite réaction d'amplification est choisie dans le groupe constitué par la réaction de polymérisation en chaîne (PCR), l'amplification par déplacement de brin (SDA), l'amplification médiée par la transcription (TMA), la réaction de ligation en chaîne (LCR), l'amplification à base de séquence d'acide nucléique (NASBA), l'amplification par cercle roulant et l'amplification de l'ARN par une ARN polymérase dirigée par l'ARN, et la détection de l'émission de fluorescence de ladite fraction émettrice de l'oligonucléotide ;
35
ledit test comprenant en outre l'ajout dudit oligonucléotide à un échantillon qui ne contient pas ledit brin cible et la détection de l'émission de fluorescence de ladite fraction émettrice de l'oligonucléotide.

40
10. Test selon la revendication 9, comprenant en outre la détection de l'émission de fluorescence de ladite fraction récoltrice de l'oligonucléotide.

45
50
55
11. Test selon la revendication 9 ou la revendication 10, dans lequel ledit oligonucléotide est une sonde qui comprend des 2'-O-méthylribonucléotides.

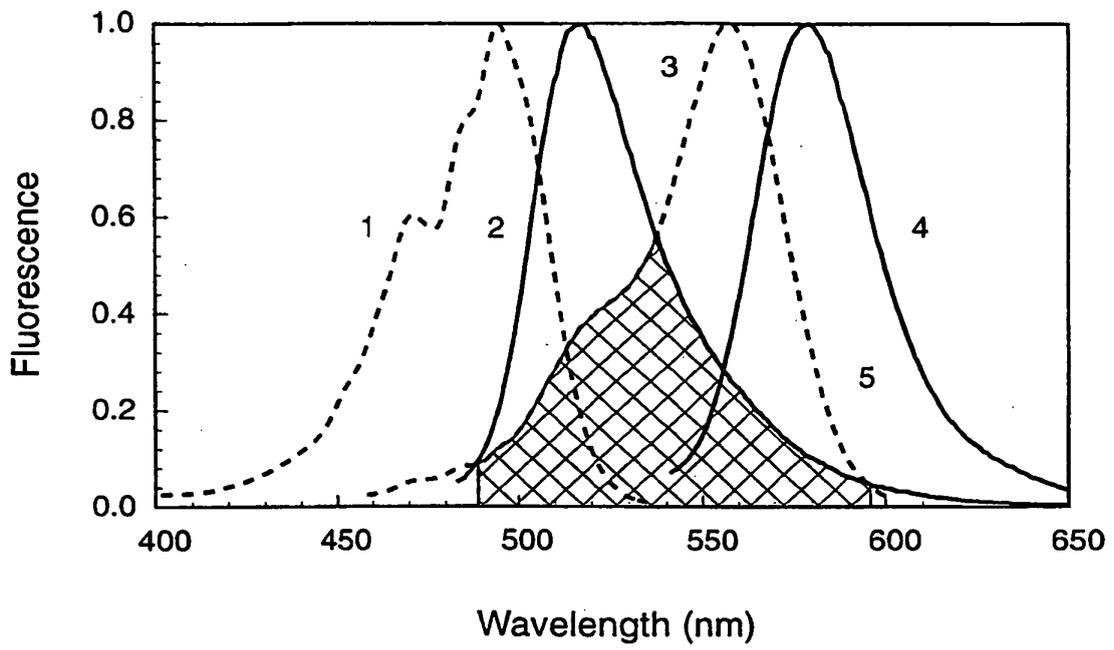


FIG. 1

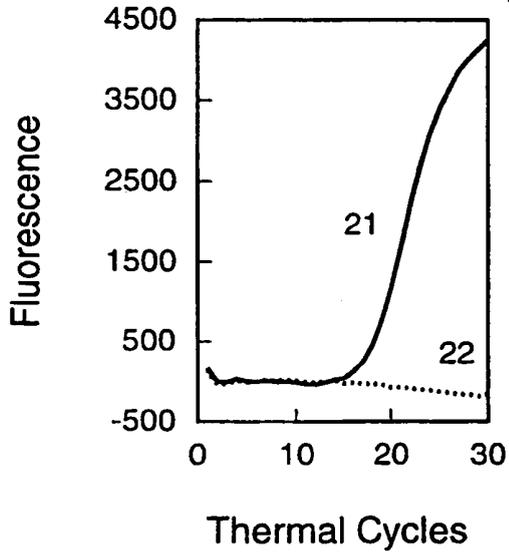


FIG. 2

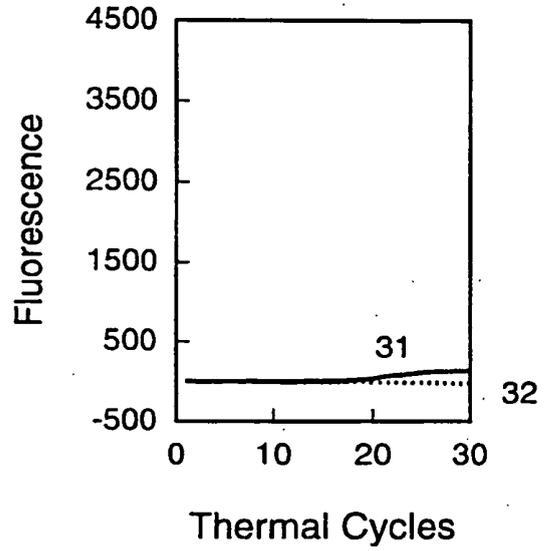


FIG. 3

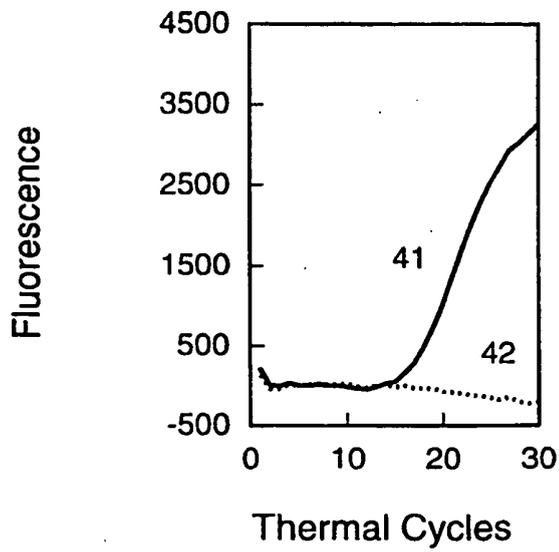


FIG. 4

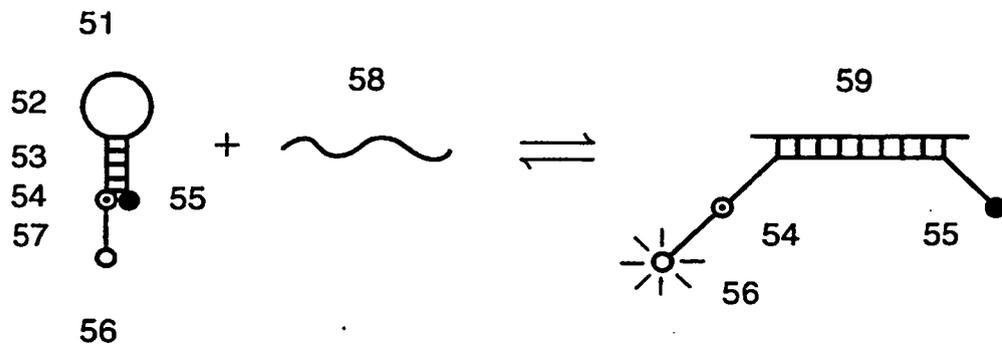


FIG. 5

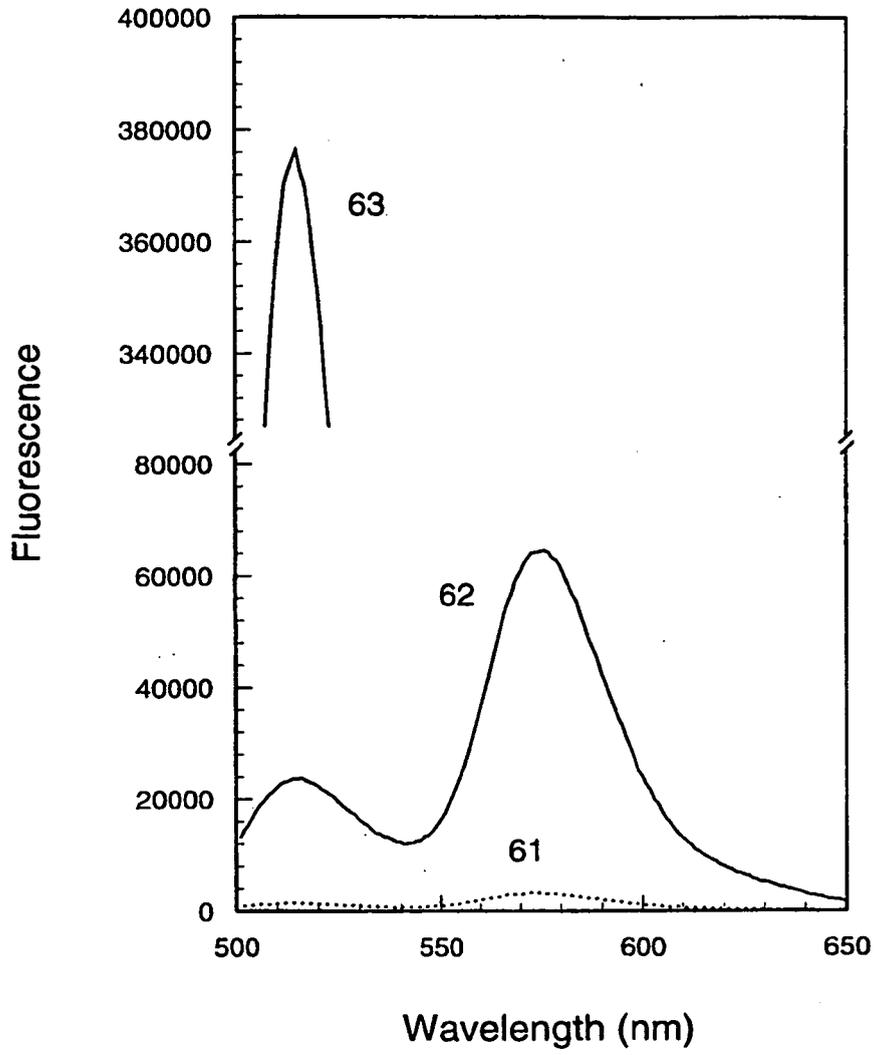


FIG. 6

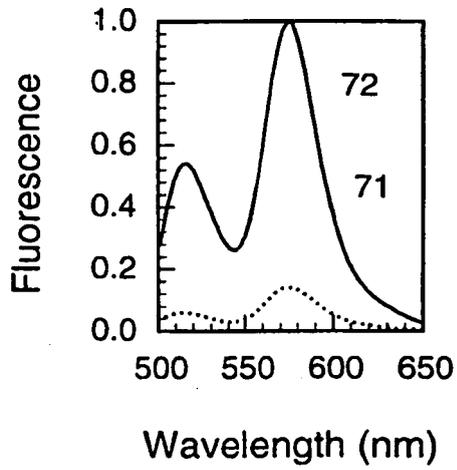


FIG. 7

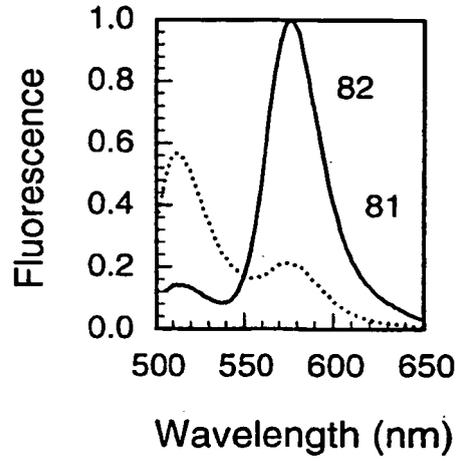


FIG. 8

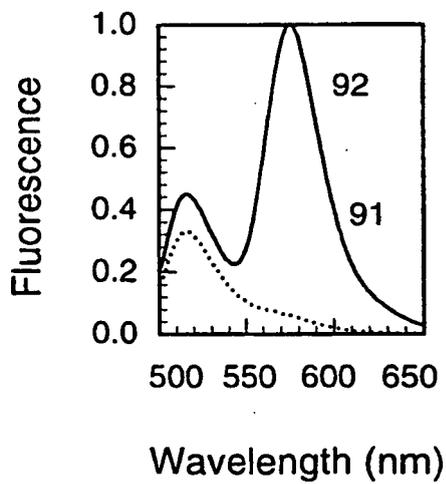


FIG. 9

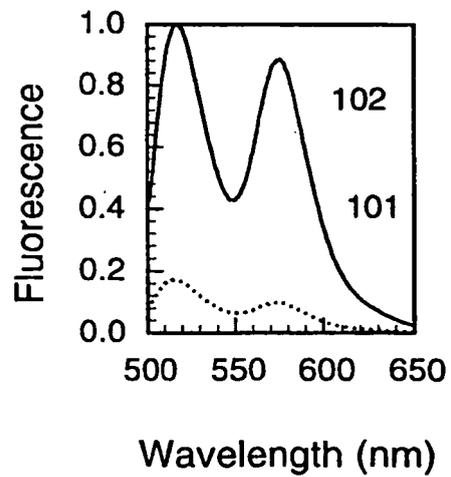


FIG. 10

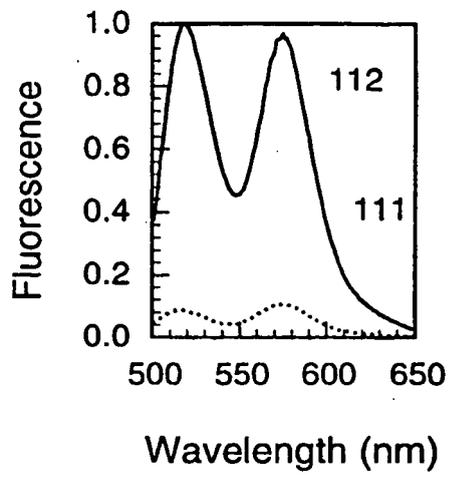


FIG. 11

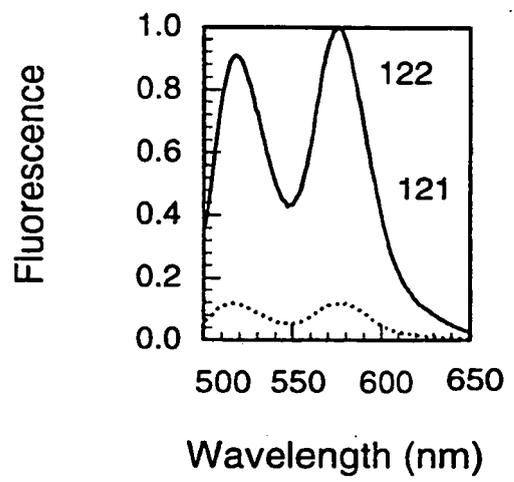


FIG. 12

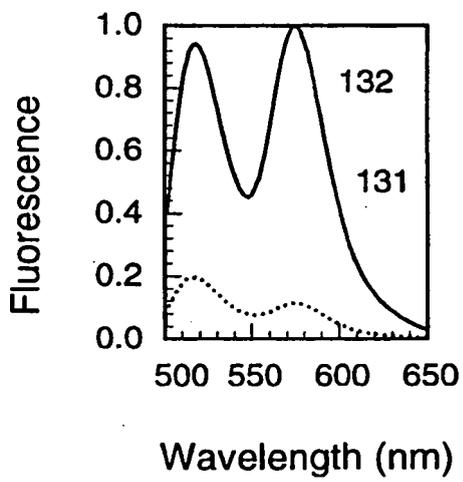


FIG. 13

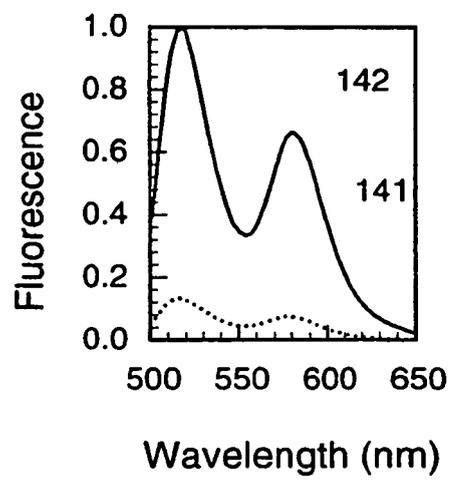


FIG. 14

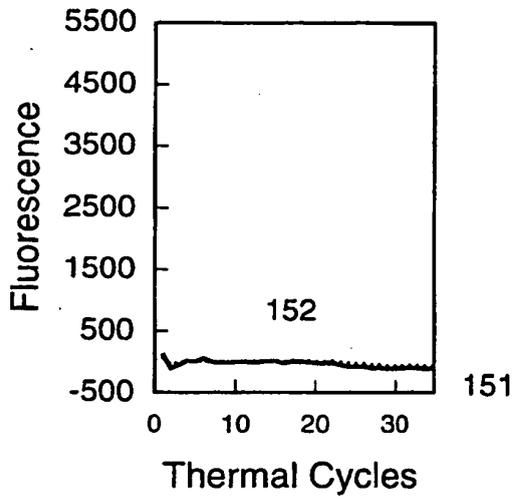


FIG. 15

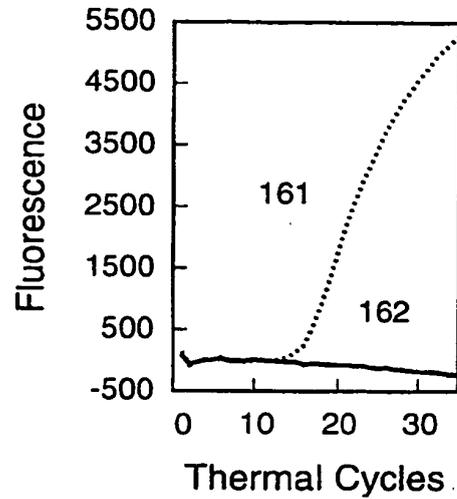


FIG. 16

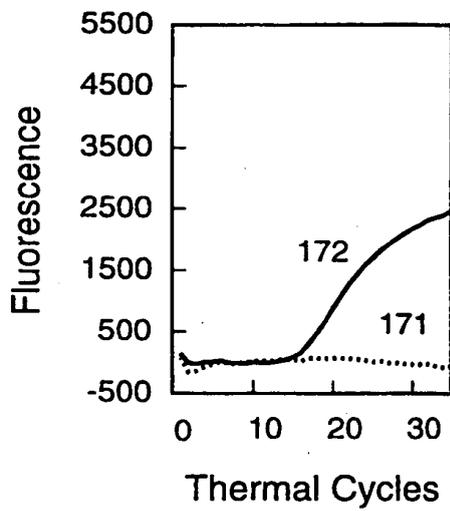


FIG. 17

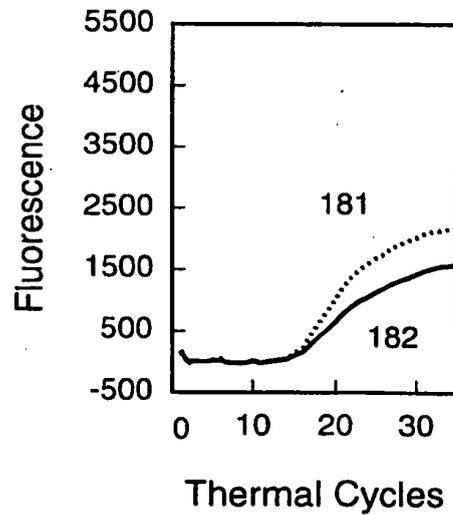


FIG. 18

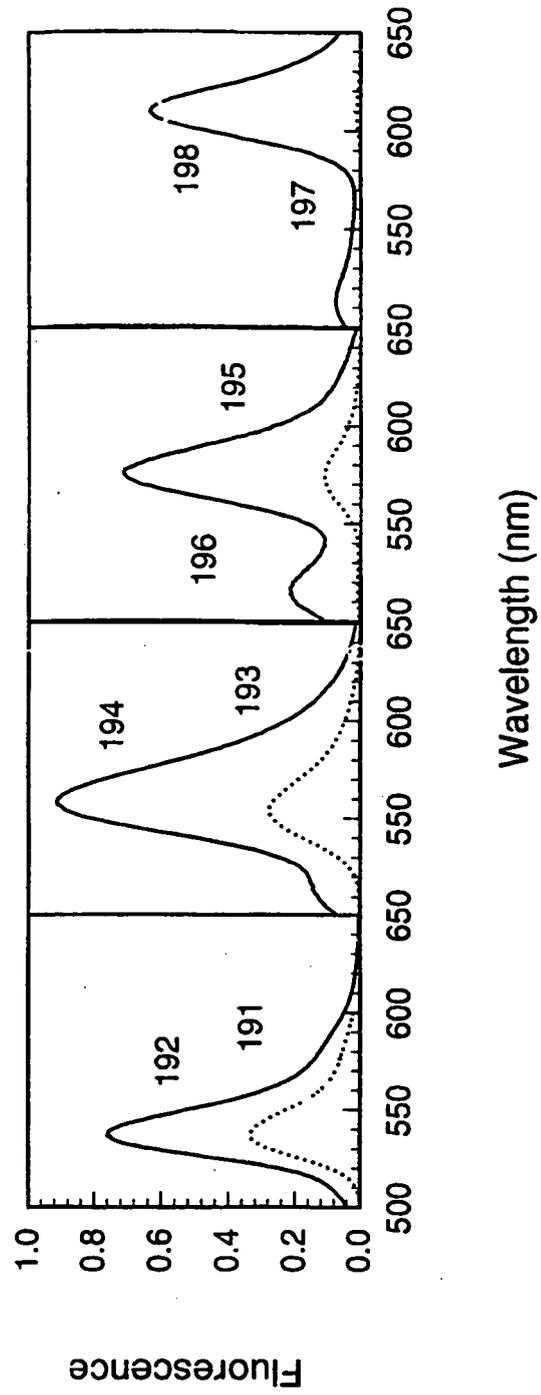


FIG. 19

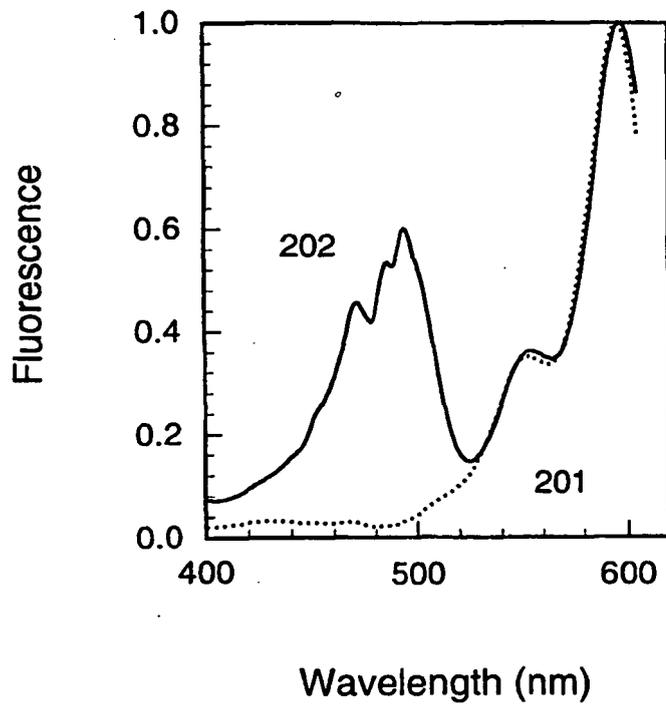


FIG. 20

REFERENCES CITED IN THE DESCRIPTION

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