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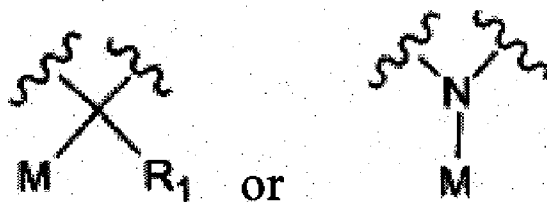
(57) Abstract: A composition, comprising a first oligonucleotide having the structure 5'-Y_1-L_1-X-L_2_2-Y_2-3' Y_1 comprises a se-quence of four or more DNA or RNA nucleotides, including a first nucleotide N_1 having a 3' phosphate covalently linked to L_1. Y_2 comprises a sequence of four or more DNA or RNA nucleotides, including a second nucleotide N_2 having a 5' phosphate covalent-ly linked to L_2. L_1 and L_2 each independently are a direct bond or a $C_1 C_7$ alkyl, alkynyl, alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl, alkylaryl, or alkoxyl group. X is R\ is a hydrogen or a d-Cg alkyl. M is a quencher. The first oligonucleotide is adapted to hybridize to a second oligonucleotide having the structure 3'-Y3-Y4-5'. Y3 comprises a se-quence of four or more DNA or RNA nucleotides, including a third nucleotide N3. Y4 comprises a sequence of four or more DNA or RNA nucleotides, including a fourth nucleotide N4 that is directly attached to nucleotide N_3. If the first oligonucleotide hy-bridizes to the second oligonucleotide, Ni base pairs with N_3 and N2 base pairs with N4.to form a duplex having a T_m that is greater than the T_m of a duplex formed between the second oligonucleotide and a third oligonucleotide having the structure 5'-Y_1-Y_2-3'.

WO 2011/120049 A1

METHODS FOR ENHANCING NUCLEIC ACID HYBRIDIZATION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001]   This patent application claims priority to U.S. Provisional Patent Application No. 61/318,043 filed March 26, 2010, the content of which is incorporated herein by reference in its entirety.

SEQUENCE LISTING

[0002]   The sequence listing is filed with the application in electronic format only and is incorporated by reference herein. The sequence listing text file "**ASFILED_Sequence_WO00**" was created on March 25, 2011, and is 49,479 bytes in size.

FIELD OF THE DISCLOSURE

[0003]   This disclosure pertains to novel oligonucleotide compounds with improved hybridization properties. Methods and reagents are provided which allow internal labeling of oligonucleotides by insertion of labels between adjacent residues without destabilizing the duplex. Because the nucleotide bases are not modified, such labeling groups can be introduced into any sequence. In some embodiments, such modifications increase duplex stability. In some embodiments, the labeling group is a fluorescence quencher. The disclosure further relates to the design of fluorescently labeled oligonucleotide probes with multiple quenching dyes capable of very efficient fluorescence quenching over a broad spectral range.

BACKGROUND

[0004]   Fluorescent energy transfer probes are an important tool in genetic analysis. These probes, also known as dual-labeled probes (DLPs) or self-quenching probes, are generally comprised of a fluorescent donor (a fluorophore) and a quencher linked to an oligonucleotide. This basic design, wherein a signal change is detected once the probe hybridizes to its intended target, is used in a variety of biological applications.

[0005]   One method for detecting hybridization using fluorophores and quenchers is to link fluorescent donors and quenchers to a single oligonucleotide such that there is a detectable difference in fluorescence when the oligonucleotide is unhybridized as compared to when it is hybridized to its complementary sequence. In so-called molecular beacons, a partially self-complementary oligonucleotide is designed to form a hairpin and is labeled with a fluorescent donor at one end of the molecule and a quencher at the other end (U.S. Patent No. 5,925,517). Intramolecular annealing to form the hairpin brings the donor and quencher into close proximity for fluorescent quenching to occur. Intermolecular annealing of such an

oligonucleotide to a target sequence disrupts the hairpin, which increases the distance between the donor and quencher and results in a detectable increase in the fluorescent signal of the donor.

[0006]   Oligonucleotides are not required to form a hairpin structure for this method to work efficiently. The fluorophore and quencher can be placed on an oligonucleotide such that when it is unhybridized and in a random coil conformation, the quencher is able to quench fluorescence from the fluorophore (U.S. Patent No. 5,538,848). Once the oligonucleotide hybridizes to a complementary nucleotide sequence it becomes more extended and the distance between the fluorophore and quencher is increased, resulting in reduced quenching and increased fluorescence.

[0007]   Oligonucleotides labeled in a similar manner can also be used to monitor the kinetics of PCR amplification. In one version of this method, commonly known as a 5'-nuclease cleavage or hydrolysis assay, an oligonucleotide probe is designed to hybridize to the target sequence on the 3' side ("downstream") of one of the amplification primers. During PCR, the 5'-3' exonuclease activity of the DNA polymerase digests the 5' end of the probe thereby separating the fluorophore from the quencher. The fluorescence intensity of the sample increases as an increasing number of probe molecules are digested during the course of amplification (U.S. Pat. No. 5,210,015).

[0008]   DLPs find use in other molecular/cellular biology and diagnostic assays, such as in end-point PCR, in situ hybridizations, in vivo DNA and RNA species detection, single nucleotide polymorphism (SNPs) analysis, enzyme assays, and in vivo and in vitro whole cell assays (see Dirks and Tanke, *Biotechniques* 2006, 40:489-486; Bustin, *Journal of Molecular Endocrinology* 2002, 29:23-39; Mackay, *Clin Microbiol Infect*, 2004, 10:190-212).

[0009]   In one mechanism of fluorescence quenching termed ground state quenching, the fluorophore and the quencher associate to form a ground state complex which is not fluorescent. For ground state quenching to occur there need not be spectral overlap between the fluorophore and the quencher.

[0010]   The most common mechanism of fluorescent quenching is fluorescence resonance energy transfer (FRET). In FRET, energy transfer occurs through space by dipolar coupling between the fluorophore and quencher and requires that there be overlap between the emission spectrum of the fluorescent donor and the absorbance spectrum of the quencher. This requirement complicates the design of probes that utilize FRET because quenchers are limited in their effective wavelength range. For example, the quencher known as BHQ-1, which absorbs light in the wavelength range of about 500-550 nm, quenches fluorescent light emitted by fluorescein, which fluoresces maximally at about 520 nm, but is of limited utility for Texas Red (emission maximum = 615) or Cy5 (emission maximum = 670). In contrast,

the quencher BHQ-3, which absorbs light in the wavelength range of about 650-700 nm is almost completely ineffective at quenching fluorescein but is effective at quenching Cy5. In general, the number of quenchers that are known to be capable of quenching the fluorescence of any given fluorophore is limited.

[0011]   Although fluorescent dyes themselves can be employed to quench fluorescence from other dyes, preferred quenchers will not fluoresce (or minimally fluoresce) so that background fluorescence is minimized. These quenchers are commonly referred to as dark quenchers. Dark quenchers allow for an increased signal to noise ratio in assays that employ DLPs, resulting in increased sensitivity. In addition, the lack of secondary fluorescence facilitates the use of additional fluorophores in multiplexed assay formats which utilize multiple distinct probes each containing a different fluorophore. If a quencher emitted light in a certain region, then additional probes could not bear fluorophores that emit light in that same region.

[0012]   A number of factors are considered in designing a self-quenching probe. These include the ease of synthesis, the compatibility of the fluorophore and quencher, duplex stability, and the specificity of the probe in hybridizing to the intended target.

[0013]   Duplex stability between complementary nucleic acid molecules is frequently expressed as the "melting temperature", T_m, of the duplex. Roughly speaking, the T_m indicates the temperature at which a duplex nucleic acid dissociates into two single strands. Nucleic acid hybridization is generally performed at a temperature slightly below the T_m, so that hybridization between a probe or primer and its target nucleic acid is optimized, while minimizing non-specific hybridization of the probe or primer to other, non-target nucleic acids. Duplex stability and T_m are also important in applications, such as PCR, where thermocycling may be involved. During such thermocycling melting steps, it is important that the sample temperature be raised sufficiently above the T_m so that duplexes of the target nucleic acid and its complement are dissociated. In subsequent steps of reannealing, however, the temperature must be brought sufficiently below the T_m that duplexes of the target nucleic acid and primer are able to form, while still remaining high enough to avoid non-specific hybridization events. For a general discussion, see Rychlik et al., *Nucleic Acids Research* 1990, 18:6409-6412.

[0014]   Shorter oligonucleotides can help increase the specificity of a primer or probe, allowing for the discrimination of even a single mismatch between the probe and a potential complementary target. The shorter the oligonucleotide, the greater the effect of a single-base mismatch on duplex stability. However, the disadvantage of using such short oligonucleotides is that they hybridize weakly, even to a perfectly complementary sequence, and thus must be used at lower temperatures, which are unfavorable for reactions that use

thermal stable enzymes, such as PCR. Certain modified nucleosides such as locked nucleic acids (LNAs) (U.S. Pat. No. 7,060,809) and C5-propynyl pyrimidines (U.S. Pat. No. 5,484,908) can be incorporated into oligonucleotides to increase duplex stability. Many nucleoside analogs, however, especially those having bulkier substituents attached to the base, are destabilizing. For example, fluorescein-dT can destabilize a duplex by up to 4°C (Bioorganic & Medicinal Chemistry Letters, 13:2785-2788 2003).

[0015] Modified nucleosides employed to increase Tm are typically placed internally within an oligonucleotide sequence replacing a natural base. In contrast, non-nucleoside substituents when introduced internally within an oligonucleotide, either as a replacement for a base or as an insertion between bases, generally interfere with hybridization. For example, insertion of an abasic fluorescein group into an oligonucleotide has been observed to destabilize a duplex by 2-4°C (DNA Seq. 4:135-141, 1993).

[0016] There are several classes of compounds that are known to increase binding affinity between complementary nucleic acid strands. One class is major groove binders, which includes proteins or ligands that bind to the major groove (the wider groove around a DNA helix). A second class, minor groove binders (MGBs), include non-covalently bound and covalently bound compounds. Because the minor groove of a helix is narrower in A-T rich regions, some noncovalently bound MBGs recognize the shape of the helix and preferably bind to specific sequence regions. For example, netropsin and distamycin preferably bind to A-T regions (see Bailly and Henichart, ACS, vol.2, 379-393 (1991). Covalently bound MGBs (U.S. Pat. No. 6,084,102) are typically linked to the 5' or 3' end of oligonucleotides (U.S. Pat. App. 2009/0259030) and are known to increase binding affinity and allow for shorter length probes.

[0017] A third class, intercalators, are generally flat polycyclic compounds, examples being acridine or lipticine derivatives (see U.S. Pat. No. 4,835,263). Intercalating compounds stabilize a duplex by fitting in between the bases of the nucleic acid monomers. They can be covalently or noncovalently bound. Some minor groove compounds, such as 4',6-diamidino-2-phenylindole (DAPI), also intercalate.

[0018] Another group of compounds, capping reagents, are terminally attached compounds that favor Watson-Crick duplexes by stacking on the terminal base pair (Dogan, Z. et al., J. Am. Chem. Soc. 2004, 126, 4762-4763). Such groups include stilbene derivatives (Wu, T. et al., J. Am. Chem. Soc. 1995, 117, 8785-8792) and pyrenylmethylpyrrolindol (Narayanan, S. et al., Nucleic Acids Res. 2004, 32, 2901-2911).

[0019] The efficiency of quenching through FRET is extremely sensitive to the distance between the fluorophore and quencher (R_{FQ}), varying with the reciprocal of R_{FQ} to the sixth power. Maximally efficient quenching minimizes background fluorescence and improves the

sensitivity of the 5'-nuclease assay and other hybridization assays in which DLPs are used. Generally, for ease of synthesis and to avoid disruption of hybridization of the probe to the target sequence, the dye and quencher are attached to the ends of the oligonucleotide. For the 5'-nuclease assay, the most common configuration is to attach the dye at the 5'-end of the oligonucleotide and the quencher at the 3'-end. DLPs used in the 5'-nuclease assay are typically 25 to 30 bases in length. Even with the use of T_m enhancing modifications, such as LNA bases or a minor groove binder, probe length is still usually 14 to 18 bases. Any method that permits placement of the fluorophore and quencher in closer proximity within a probe without destabilizing the duplex formed between the probe and its target nucleic acid will improve quencher efficiency and enhance the performance of the probe.
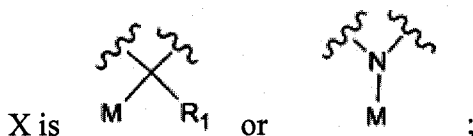
BRIEF SUMMARY

[0020]   This disclosure provides various compositions comprising oligonucleotides having modifying compounds placed internally within the oligonucleotide sequence between nucleotides. Even though these modifying compounds are inserted between adjacent nucleotides (as opposed to being substituted for one of the nucleotides), some of the modified oligonucleotides surprisingly form equally stable, or even more stable, duplexes with their complimentary oligonucleotide sequences as compared to the stability of the duplex formed between the unmodified oligonucleotide and the complimentary oligonucleotide. The modifying groups may include a variety of labels, including but not limited to fluorescence quenchers that enable the design of DLPs with very high quenching efficiency. Because the labeling group is not a modified base, the same modifying compound can be inserted into any position within any oligonucleotide sequence. This disclosure also provides methods for using and making the oligonucleotide compositions.

[0021]   The compositions of the present disclosure each comprise an oligonucleotide having the general structure 5'-Y_1-L_1-X-L_2-Y_2-3', where:

        Y_1 comprises a sequence of one or more DNA and/or RNA nucleotides, including a first nucleotide N_1 having a 3' phosphate covalently linked to L_1;

        Y_2 comprises a sequence of one or more DNA and/or RNA nucleotides, including a second nucleotide N_2 having a 5' phosphate covalently linked to L_2;

        L_1 and L_2 each independently are a direct bond or a C_1-C_7 alkyl, alkynyl, alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl, alkylaryl, or alkoxyl group;

        X is   M R_1 or   M   ;

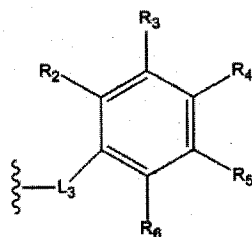R$_1$ is a hydrogen or a C$_1$-C$_8$ alkyl; and

M is a label.

These oligonucleotides may include any desired number of nucleotides, but preferably include 10-50 nucleotides, and even more preferably include 15-35 nucleotides. In some embodiments, L$_1$ and L$_2$ each may be a C$_1$-C$_7$ alkyl, and preferably a C$_2$ alkyl. The 3' phosphate that is covalently linked to L$_1$, and the 5' phosphate that is covalently linked to L$_2$, each independently may be a phosphodiester, a phosphothioate, a phosphodithioate, a methyl phosphonate, a phosphoramidate, a phosphoramidite or a phosphotriester.

[0022]   In some embodiments, Y$_1$ comprises a sequence of four or more DNA and/or RNA nucleotides, Y$_2$ comprises a sequence of four or more DNA and/or RNA nucleotides, M is a first quencher, and the oligonucleotide is adapted to hybridize to a second oligonucleotide having the structure 3'-Y$_3$-Y$_4$-5', where Y$_3$ comprises a sequence of four or more DNA and/or RNA nucleotides, including a third nucleotide N$_3$, Y$_4$ comprises a sequence of four or more DNA and/or RNA nucleotides, including a fourth nucleotide N$_4$ that is directly attached to nucleotide N$_3$. If the first oligonucleotide hybridizes to the second oligonucleotide, then N$_1$ base pairs with N$_3$ and N$_2$ base pairs with N$_4$.to form a duplex having a T_m that is greater than the T_m of a duplex formed between the second oligonuleotide and a third oligonucleotide having the structure 5'-Y$_1$-Y$_2$-3'. In such embodiments, the first oligonucleotide may be labeled with a fluorophore. For example, the fluorophore may be attached to the last nucleotide on the 5' end of the oligonucleotide, and in preferred embodiments, Y$_1$ may comprise a sequence of 8-12 DNA or RNA nucleotides. Compositions comprising the first oligonucleotide also may comprise the second oligonucleotide.

[0023]   In some embodiments, Y$_1$ comprises a sequence of 8-12 DNA and/or RNA nucleotides, such as a sequence of 10 DNA and/or RNA nuceotides, where the nucleotide on the 5' end of Y$_1$ is labeled with a fluorophore, and M is a quencher.

[0024]   In some embodiments, M comprises a fused polycyclic aromatic moiety.
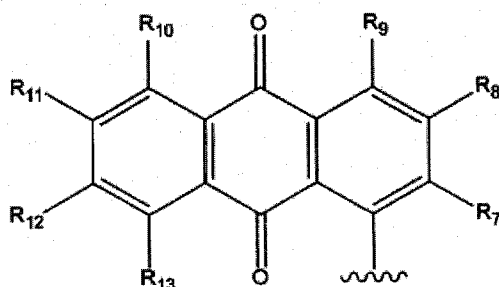
[0025]   In some embodiments, M is:

where L$_3$ is a direct bond or a C$_1$-C$_8$ alkyl, alkenyl, alkenyl, heteroalkyl, substituted alkyl, cycloalkyl, or alkoxyl, where R$_2$-R$_6$ each independently are a hydrogen, an alkyl, an alkenyl, a heteroalkyl, a substituted alkyl, an aryl, a heteroaryl, a substituted aryl, a cycloalkyl, an alkylaryl, an alkoxyl, an electron withdrawing group, or an electron donating group, and

where one of R_2-R_6 is –N=N–P, and where P is a fused polycyclic aromatic moiety. Electron withdrawing groups may be selected from the group consisting of -NO_2, -SO_3^-, -SO_2^-, -CN, -NCS, a ketone, an alkoxyl, an ether, a carboxylic acid and a sulfonyl. Electron donating group is selected from the group consisting of an alkoxyl, a heteroalkoxyl, an alkyl, a cycloalkyl, a heteroalkyl, an amino, an alkylamino, or an arylamino.

[0026]   In some embodiments, M is -L_4-P, where L_4 is an alkyl, an alkynyl, an alkenyl, a heteroalkyl, a substituted alkyl, or an alkoxyl group, and P is a fused polycyclic aromatic moiety. For example, L_4 may be -CH2-O-CH2-CH2-NH- or any other suitable linker.

[0027]   Various of the oligonucleotides described herein include fused polycyclic aromatic moieties P. In some embodiments, P is

where R_7-R_9 each independently are a hydrogen, an alkoxyl, an alkyl, an alkylamino, an arylamino, a cycloalkyl, a heteroalkoxyl, a heteroalkyl, or an amino, and R_{10}-R_{13} each independently are a hydrogen, a nitro, a cyano, a carboxylate, a sulfonyl, a sulfamoyl, an alkenyl, an alkynyl, an amino, an aryl, a heteroaryl, a biaryl, a bialkenyl, a bialkynyl, an alkoxycarbonyl or a carbamoyl. In preferred embodiments, R_9 is
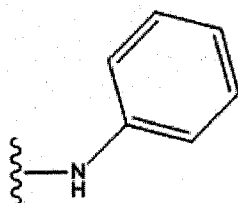
[0028]   In some embodiments, P is
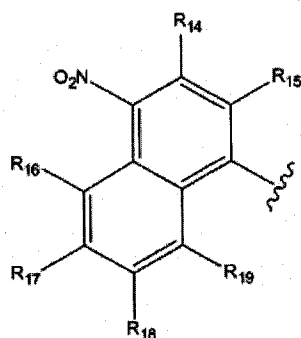
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where R_{14}-R_{19} each independently are a hydrogen, an alkyl, a heteroalkyl, an aryl, a heteroaryl, an electron withdrawing group, or a five or six membered ring structure formed from the R1, R2 pair, the R_3, R_4 pair, the R_4, R_5 pair, or the R_5, R_6 pair.

[0029]   Some of the oligonucleotides disclosed herein include a fluorophore, which may include, but is not limited to, 6-carboxyfluorescein (FAM), 2'7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), tetrachlorofluorescein (TET), 6-carboxyrhodamine (R6G), N,N,N;N'-tetramethyl-6-carboxyrhodamine (TAMRA), 6-carboxy-X-rhodamine (ROX); 1-dimethylaminonaphthyl-5-sulfonate, 1-anilino-8-naphthalene sulfonate, 2-p-toluidinyl-6-naphthalene sulfonate, 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS), a coumarin dye, an acridine dye, indodicarbocyanine 3 (Cy3), indodicarbocyanine 5 (Cy5), indodicarbocyanine 5.5 (Cy5.5), 3-1-carboxy-pentyl)-3'-ethyl-5,5'-dimethyloxacarbocyanine (CyA); 1H,5H,11H,15H-Xantheno[2,3,4-ij:5,6,7-i'j']diquinolizin-18-ium, 9-[2(or 4)-[[[6-[2,5-dioxo-1-pyrrolidinyl)oxy]-6-oxohexyl]amino]sulfonyl]-4(or 2)-sulfophenyl]-2,3,6,7,12,13,16,17-octahydro-inner salt (TR or Texas Red), a BODIPY™ dye, benzoxaazole, stilbene and pyrene. In some embodiments, the fluorophore may be attached to the 5' end, such as to the phosphate at the 5' end of the oligonucleotide.

[0030]   Some of the oligonucleotides disclosed herein include more than one quencher, such as a first internal quencher (described above) and a second quencher. The second quencher may include, but is not limited to dabcyl, Eclipse® quencher, BHQ1, BHQ2 and BHQ3, Iowa Black® FQ, Iowa Black® RQ-n1 or Iowa Black® RQ-n2.

[0031]   This disclosure also provides methods for using and making the oligonucleotide compositions. Methods for use may include methods for detecting target nucleic acids within a sample. For example, such methods may include contacting the sample with an oligonucleotide adapted to hybridize to the target nucleic acid, where the oligonucleotide includes an internal quencher and a fluorophore, and where the fluorescence of the fluorophore is reduced by fluorescence resonance energy transfer to the quencher or by ground state quenching by the quencher when the oligonucleotide is not hybridized to the second oligonucleotide, and detecting an increase in fluorescence indicating the presence of the second oligonucleotide in the sample.

BRIEF DESCRIPTION OF THE DRAWINGS

[0032]   FIGS. 1A, 1B and 1C are structures of modified oligonucleotides tested for their effects on the stability of the duplex formed between the modified oligonucleotide and its complimentary oligonucleotide. The modified oligonucleotides contain various modification

compounds inserted between, and attached to the 3' and 5' phosphates of adjacent nucleotides.

[0033] FIG. 2A is an amplification plot that illustrates the relative fluorescence intensity (R_n) of an HPRT q-PCR assay of the substitution data set for the FQ quencher located 6, 8, 10 and 12 positions from the 5'-fluorophore. FIG. 2B is an amplification plot that illustrates the baseline adjusted fluorescence of results in Figure 2A (ΔR_n).

[0034] FIG. 3A is an amplification plot that illustrates the relative fluorescence intensity (R_n) of the HPRT q-PCR assay of the insertion data set for FQ quencher located 6, 8, 10 and 12 positions from the 5'-fluorophore. FIG. 3B is an amplification plot that illustrates baseline adjusted fluorescence of the data in Figure 3A (ΔR_n).

[0035] FIG. 4A is an amplification plot that shows the results of the baseline adjusted substitution analogs s10FQ and s6FQ and FIG. 4B is an amplification plot that shows the results for the baseline adjusted insertion analogs s10FQ and s6FQ where various plasmid copy target amounts (2×10^2, 2×10^3, 2×10^4, 2×10^5, 2×10^6 and 2×10^7) were tested.

[0036] FIGS. 5A and 5B are amplification plots (baseline comparison (R_n) and baseline adjusted (ΔR_n) comparison respectively) that demonstrate the improved performance of internal FQ (10) by itself or as a dual quenched probe compared to an internal BHQ1 (10) dual quenched probe.

[0037] FIGS. 6A and 6B are amplification plots (baseline comparison (R_n)and baseline adjusted (ΔR_n) respectively) that demonstrate the increased performance of internal FQ (iFQ) by itself or as a dual quenched containing probes compared to internal BHQ1 (iBHQ1) dual quenched probe using a different probe sequence. FIGS. 7A and 7B are amplification plots (baseline comparison (R_n) and baseline adjusted comparison (ΔR_n) respectively) using an AB 7900HT instrument comparing a 5' FAM labeled dual quencher probes iFQ-3'FQ andiFQ-3'RQ-n1, illustrating the enhanced performance of an internally quenched probe in conjunction with a different 3' end quencher.

[0038] FIGS. 7A and 7B are amplification plots for the fluorescein (emission 520 nm) reporter dye probes, wherein the baseline plots are shown in FIG. 7A and baseline normalized plots are shown in FIG. 7B.

[0039] FIGS. 8A and 8B are amplification plots (baseline comparison (R_n) and baseline adjusted comparison (ΔR_n) respectively) using an AB 7900HT instrument comparing a 5' MAX labeled dual quencher probes iFQ-3'FQ andiFQ-3'RQ-n1, illustrating the enhanced performance of an internally quenched probe in conjunction with a different 3' end quencher.

[0040] FIGS. 9A and 9B are amplification plots (baseline comparison (R_n) and baseline adjusted comparison (ΔR_n) respectively) using an iQ5 BioRad instrument comparing a CY3

labeled dual quencher probes iFQ-3'FQ andiFQ-3'RQ-n1, illustrating the enhanced performance of an internally quenched probe in conjunction with a different 3' end quencher.

[0041]   FIGS. 10A and 10B are amplification plots (baseline comparison (R_n) and baseline adjusted comparison (ΔR_n) respectively) using a LC480 Roche instrument comparing a 5'TEX615 labeled dual quencher probes iFQ-3'FQ andiFQ-3'RQ-n1, illustrating the enhanced performance of an internally quenched probe in conjunction with a different 3' end quencher.

[0042]   FIGS. 11 A and 11B are amplification plots (baseline comparison (R_n) and baseline adjusted comparison (ΔR_n) respectively) using a LC480 Roche instrument comparing a 5' CY5 labeled dual quencher probes iFQ-3'FQ andiFQ-3'RQ-n1, illustrating the enhanced performance of an internally quenched probe in conjunction with a different 3' end quencher.

[0043]   FIGS. 12 is a side by side baseline adjusted amplification plot (ΔR_n) where various plasmid copy target amounts (2×10^2, 2×10^3, 2×10^4, 2×10^5, 2×10^6, 2×10^7) were tested for CY5 labeled dual quenched probes with the internal placement of the FQ quencher is between bases 9 and 10, versus 11-12, illustrating the ability to vary the positioning with the internal quencher.

[0044]   FIGS. 13A, 13B, 13C, 13D and 13E are bar charts showing the ΔT_m caused by modifying an oligonucleotide by inserting a modification compound between various nucleotides.

[0045]   FIGS. 14A and 14B are plots showing the dependence of ΔT_m on the position of the insertion within an oligonucleotide for various modification compounds.

DETAILED DESCRIPTION

[0046]   This disclosure provides various compositions comprising oligonucleotides having modifying compounds placed internally within the oligonucleotide sequence between nucleotides. Some of the he modified oligonucleotides surprisingly form equally stable, or even more stable, duplexes with their complimentary oligonucleotide sequences as compared to the stability of the duplex formed between the unmodified oligonucleotide and the complimentary oligonucleotide. The mechanism by which some modifying compounds confer stability to a duplex is unknown. It is particularly surprising and unexpected that modifications of adjacent residues within an oligonucleotide with these compounds should increase duplex stability given the close proximity of the two phosphate groups on either side of reagent when in the double helix. The modifying groups may include a variety of labels, including but not limited to fluorescence quenchers that enable the design of DLPs with very high quenching efficiency. Because the labeling group is not a modified base, the same
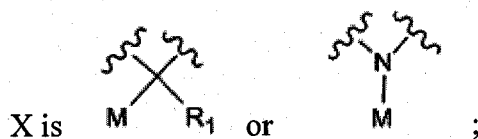
modifying compound can be inserted into any position within any oligonucleotide sequence. This disclosure also provides methods for using and making the oligonucleotide compositions.

[0047]   The compositions of the present disclosure each comprise an oligonucleotide having the general structure 5'-Y_1-L_1-X-L_2-Y_2-3', where:

Y_1 comprises a sequence of one or more DNA and/or RNA nucleotides, including a first nucleotide N_1 having a 3' phosphate covalently linked to L_1;

Y_2 comprises a sequence of one or more DNA and/or RNA nucleotides, including a second nucleotide N_2 having a 5' phosphate covalently linked to L_2;

L_1 and L_2 each independently are a direct bond or a C_1-C_7 alkyl, alkynyl, alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl, alkylaryl, or alkoxyl group;

X is or ;

R_1 is a hydrogen or a C_1-C_8 alkyl; and

M is a label.

FIG. 1 provides a non-exclusive exemplary list of modified oligonucleotides having a modifying compound inserted between adjacent nucleotides. These modified oligonucleotides may include any desired number of nucleotides, but preferably include 10-50 nucleotides, and even more preferably include 15-35 nucleotides. Moreover, depending on the application, the labeled oligonucleotide can be DNA, RNA or a chimeric oligonucleotide containing both DNA and RNA residues. Modified nucleosides such as LNA bases, 2'-O-methyl RNA and purine and pyrimidines analogs also may be included within the sequence. For use as a probe or primer, the length of the oligonucleotide is typically between 15 and 35 residues. Because the label is inserted between adjacent residues (as opposed to being a label attached to a particular nucleotide) the same modifying compound may be used to label essentially any sequence.

[0048]   In some embodiments, L_1 and L_2 each may be a C_1-C_7 alkyl, and preferably a C_2 alkyl. An increase in the stability of the duplex formed upon hybridization of the modified oligonucleotides to its target sequence can be achieved (see Example 1).

[0049]   The 3' phosphate that is covalently linked to L_1, and the 5' phosphate that is covalently linked to L_2, each independently may be a phosphodiester, a phosphothioate, a phosphodithioate, a methyl phosphonate, a phosphoramidate, a phosphoramidite or a phosphotriester. A modified, neutrally charged phosphorous group could be used that would confer even grater stability.

[0050]   In some embodiments, Y_1 comprises a sequence of four or more DNA and/or RNA nucleotides, Y_2 comprises a sequence of four or more DNA and/or RNA nucleotides, M is a first quencher, and the oligonucleotide is adapted to hybridize to a second oligonucleotide having the structure 3'-Y_3-Y_4-5', where Y_3 comprises a sequence of four or more DNA and/or RNA nucleotides, including a third nucleotide N_3, Y_4 comprises a sequence of four or more DNA and/or RNA nucleotides, including a fourth nucleotide N_4 that is directly attached to nucleotide N_3. If the first oligonucleotide hybridizes to the second oligonucleotide, then N_1 base pairs with N_3 and N_2 base pairs with N_4.to form a duplex having a T_m that is greater than the T_m of a duplex formed between the second oligonuleotide and a third oligonucleotide having the structure 5'-Y_1-Y_2-3'. In such embodiments, the first oligonucleotide may be labeled with a fluorophore. For example, the fluorophore may be attached to the last nucleotide on the 5' end of the oligonucleotide, and in preferred embodiments, Y_1 may comprise a sequence of 8-12 DNA or RNA nucleotides for reasons discussed below. Compositions comprising the first oligonucleotide also may comprise the second oligonucleotide.

[0051]   This disclosure also provides optimized positioning of a quencher relative to a fluorophore in a DLP. In some embodiments, the fluorophore may be attached to the nucleotide at the 5'-end of the oligonucleotide (e.g., to the 5' phosphate) and the quencher may be placed internally within the sequence between about 8 and 12 bases from the fluorophore, such as about 10 bases from the fluorophore (see Example 3). As such, in some embodiments, Y_1 comprises a sequence of 8-12 DNA and/or RNA nucleotides, such as a sequence of 10 DNA and/or RNA nuceotides, where the nucleotide on the 5' end of Y_1 is labeled with a fluorophore, and M is a quencher.

[0052]   In some embodiments, M comprises a fused polycyclic aromatic moiety.

[0053]   In some embodiments, M is:
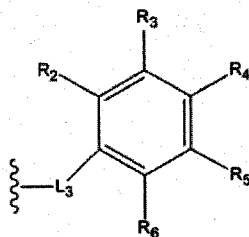
where L_3 may be a direct bond or a C_1-C_8 alkyl, alkenyl, alkenyl, heteroalkyl, substituted alkyl, cycloalkyl, or alkoxyl, where R_2-R_6 each independently may be a hydrogen, an alkyl, an alkenyl, a heteroalkyl, a substituted alkyl, an aryl, a heteroaryl, a substituted aryl, a cycloalkyl, an alkylaryl, an alkoxyl, a ligand (e.g., amino acids, peptides, antibodies, fluorophores, biotin, enzyme conjugates, vitamins, steroids and other lipids, carbohydrates,

13

digoxigenin and other haptens, etc.), an electron withdrawing group, or an electron donating group, and where one of R_2-R_6 is –N=N–P, where P is a fused polycyclic aromatic moiety. Electron withdrawing groups may be selected from the group consisting of -NO_2, -SO_3^-, -SO_2^-, -CN, -NCS, a ketone, an alkoxyl, an ether, a carboxylic acid and a sulfonyl. Electron donating groups may be selected from the group consisting of an alkoxyl, a heteroalkoxyl, an alkyl, a cycloalkyl, a heteroalkyl, an amino, an alkylamino, or an arylamino.

[0054]   In some embodiments, M is -L_4-P, where L_4 may be an alkyl, an alkynyl, an alkenyl, a heteroalkyl, a substituted alkyl, or an alkoxyl group, and P is a fused polycyclic aromatic moiety. For example, L_4 may be -CH2-O-CH2-CH2-NH- or any other suitable linker.

[0055]   Various of the oligonucleotides described herein include fused polycyclic aromatic moieties P. In some embodiments, P may be an anthraquinone quencher having the following formula
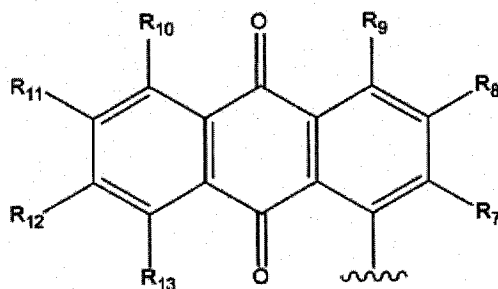
where R_7-R_9 each independently are a hydrogen, an alkoxyl, an alkyl, an alkylamino, an arylamino, a cycloalkyl, a heteroalkoxyl, a heteroalkyl, or an amino, and R_{10}-R_{13} each independently are a hydrogen, a nitro, a cyano, a carboxylate, a sulfonyl, a sulfamoyl, an alkenyl, an alkynyl, an amino, an aryl, a heteroaryl, a biaryl, a bialkenyl, a bialkynyl, an alkoxycarbonyl or a carbamoyl. In preferred embodiments, R_9 is
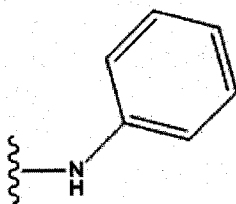
[0056]   In some embodiments, P may be an azo quencher having the following formula
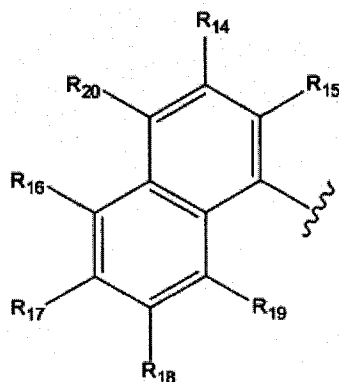
14

where R_{14}-R_{19} each independently may be a hydrogen, an alkyl, a heteroalkyl, an aryl, a heteroaryl, an electron withdrawing group, an electron donating group, or a five or six membered ring structure formed from the R1, R2 pair, the R_3, R_4 pair, the R_4, R_5 pair, or the R_5, R_6 pair, and where R_{20} preferably is an electron withdrawing group, and most preferably – NO_2.

[0057]   Some of the oligonucleotides disclosed herein include a fluorophore, which may include, but is not limited to, 6-carboxyfluorescein (FAM), 2'7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), tetrachlorofluorescein (TET), 6-carboxyrhodamine (R6G), N,N,N;N'-tetramethyl-6-carboxyrhodamine (TAMRA), 6-carboxy-X-rhodamine (ROX); 1-dimethylaminonaphthyl-5-sulfonate, 1-anilino-8-naphthalene sulfonate, 2-p-toluidinyl-6-naphthalene sulfonate, 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS), a coumarin dye, an acridine dye, indodicarbocyanine 3 (Cy3), indodicarbocyanine 5 (Cy5), indodicarbocyanine 5.5 (Cy5.5), 3-1-carboxy-pentyl)-3'-ethyl-5,5'-dimethyloxacarbocyanine (CyA); 1H,5H,11H,15H-Xantheno[2,3,4-ij:5,6,7-i'j']diquinolizin-18-ium, 9-[2(or 4)-[[[6-[2,5-dioxo-1-pyrrolidinyl)oxy]-6-oxohexyl]amino]sulfonyl]-4(or 2)-sulfophenyl]-2,3,6,7,12,13,16,17-octahydro-inner salt (TR or Texas Red), a BODIPY™ dye, benzoxaazole, stilbene and pyrene. In some embodiments, the fluorophore may be attached to the 5' end, such as to the phosphate at the 5' end of the oligonucleotide.

[0058]   Some of the oligonucleotides disclosed herein may include more than one quencher, such as a first internal quencher (described above) and a second quencher. The second quencher may be placed internally or placed at a terminal end of the oligonucleotide. The second quencher may include, but is not limited to, an azo quencher and an anthraquinone quencher, although any quencher may be used. Examples of azo quenchers include, but are not limited to, the azo quencher shown above, dabcyl, Eclipse® quencher, BHQ1, BHQ2 and BHQ3. Examples of anthraquinone quenchers include, but are not limited to, the anthraquinone quencher shown above , Iowa Black® FQ, Iowa Black® RQ-n1 or Iowa Black® RQ-n2 (see, e.g., Laikhter et al., U.S. Patent App. 2004/0110308). Attachment of

multiple quenchers to a probe not only can enhance quenching efficiency but also can provide effective quenching of various fluorophores that fluoresce over a broad spectral range (see Example 7).

[0059]   The compositions of the present disclosure may be used in various assays for detecting target nucleic acids within a sample. Such methods may include contacting the sample with an oligonucleotide adapted to hybridize to the target nucleic acid, where the oligonucleotide includes an internal quencher and a fluorophore, and where the fluorescence of the fluorophore is reduced by fluorescence resonance energy transfer to the quencher and/or by ground state quenching by the quencher when the oligonucleotide is not hybridized to the second oligonucleotide, and detecting an increase in fluorescence indicating the presence of the second oligonucleotide in the sample. In some assays, such as the 5'-nuclease hydrolysis assay, the increase in fluorescence arises from cleavage of the labeled oligonucleotide. In some assays, the oligonucleotide forms a random-coil conformation when the oligonucleotide is unhybridized, such that the fluorescence of the fluorophore is reduced. In some assays, the oligonucleotide comprises a self-complimentary sequence, and the quencher and fluorophore are attached to the oligonucleotide such that the fluorescence of the fluorophore is quenched by the quencher when the nucleic acid polymer undergoes intramolecular base pairing. These assays have many applications, including, but not limited to, monitoring PCR reactions, where synthesis of the PCR product results in an increase in fluorescence.

[0060]   Function of dual-labeled probes in the 5'-nuclease hydrolysis assay requires that the fluorophore be effectively quenched by the quencher and also requires that the chemical modifiers employed (dye and quencher) do not interfere with nuclease cleavage. If cleavage is prevented or rendered inefficient by the presence of the chemical modifications, then fluorophore and quencher remain linked during PCR cycles and no detectable signal is generated. The chemical compositions disclosed herein function to efficiently quench the fluorophore and are compatible with probe hydrolysis using 5'-nuclease positive DNA polymerases, like *Taq* DNA polymerase. The best results are obtained when the internal quencher is positioned as close to the fluorophore as possible (to maximize quenching) yet still permits efficient cleavage of the nucleic acid bases between the dye and quencher (maximize the resulting fluorescent signal). Many fluorescent quenching groups can be placed internally and achieve efficient quenching, however many if not most of these chemical groups interfere with probe cleavage, especially when the distance between fluorophore and quencher is less than 12 nucleotides. This principle is demonstrated in Example 3, where certain quenchers, such as BHQ-1, can achieve very efficient quenching of a 5'-fluorophore, such as Fluorescein (6-FAM), yet the actual magnitude of the fluorescent

16

signal generated during real-time PCR is small, compromising the actual performance and sensitivity of the assay. In contrast, similar internal placement of the quenchers provided herein, are fully compatible with probe hydrolysis and final functional signal generation is large.

[0061]   Traditional DLPs having a 5'-fluorophore and 3'-quencher perform poorly when probe length nears or exceeds 30 nucleotides (nt), because quenching efficiency drops to the point that the probe remains relatively bright even in the quenched state. The present disclosure provides probes of 30 nt length, 35 nt length, or longer as needed for the precise application, having internal quenchers substantially closer to the fluorophore. Quenching in the compositions of this disclosure remains highly efficient as the internal quencher can be inserted the same distance from the fluorophore regardless of probe length. Thus high quality, efficiently quenched probes of an expanded potential length range are possible, which may be of particular importance when working with nucleic acids which are very AT rich. Sequences that are AT-rich have lower melting temperatures and longer probes must be utilized to function in the temperature ranges typically needed for PCR.

[0062]   The compounds of this disclosure can also be utilized in molecular beacon assays. Molecular beacon assays contain probes that contain terminal 3' and 5' ends that self-hybridize to form a stem-loop structure. One end typically contains a terminal fluorophore group and the other end contains a terminal quencher group. When the probe hybridizes to the target the quencher is no longer near the fluorophore and the signal increases. Typically the hybridizing portions of the probes are 4-7 base pairs long. Beacon probes that contain insertions as described herein, preferably within the hybridized portion of the beacon, and more preferably within 1-2 bases from the terminal end, the stability is increased. Therefore shorter hybridizing regions can be used to generate the same performance as a conventional beacon.

[0063]   A wide variety of reactive fluorescent reporter dyes are known in the literature and can be used in the compositions of this disclosure, so long as they are quenched by the precise quencher group or combination of quencher groups employed. The precise fluorophore/quencher pair employed in a dual-labeled probe is usually carefully chosen from a relatively small set of pairings that work well together based upon the emission wavelength of the fluorophore and the absorption wavelength of the quencher. Placement of the quencher in an internal position, as provided here, closer to the fluorophore than is achievable with an end-labeled probe permits efficient quenching even of fluorophore/quencher pairs that usually do not work well together, expanding the utility of the quencher by enabling its use with a wider range of fluorophores. Attachment of a second quencher to the probe can even further expand the useful spectral range.

[0064]   The oligonucleotide probes provided herein may incorporate one or more fluorophores. The fluorophores can be attached internally or at the 5'- or 3'-end. Typically, the fluorophore is an aromatic or heteroaromatic compound and can be a pyrene, anthracene, naphthalene, acridine, stilbene, indole, benzindole, oxazole, thiazole, benzothiazole, cyanine, carbocyanine, salicylate, anthranilate, coumarin, fluoroscein, rhodamine or other like compound. Suitable fluorescent reporters include xanthene dyes, such as fluorescein or rhodamine dyes, including 6-carboxyfluorescein (FAM), 2'7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), tetrachlorofluorescein (TET), 6-carboxyrhodamine (R6G), N,N,N;N'-tetramethyl-6-carboxyrhodamine (TAMRA), 6-carboxy-X-rhodamine (ROX). Suitable fluorescent reporters also include the naphthylamine dyes that have an amino group in the alpha or beta position. For example, naphthylamino compounds include 1-dimethylaminonaphthyl-5-sulfonate, 1-anilino-8-naphthalene sulfonate and 2-p-toluidinyl-6-naphthalene sulfonate, 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS). Other fluorescent reporter dyes include coumarins, such as 3-phenyl-7-isocyanatocoumarin; acridines, such as 9-isothiocyanatoacridine and acridine orange; N-(p-(2-benzoxazolyl)phenyl)maleimide; cyanines, such as indodicarbocyanine 3 (Cy3), indodicarbocyanine 5 (Cy5), indodicarbocyanine 5.5 (Cy5.5), 3-1-carboxy-pentyl)-3'-ethyl-5,5'-dimethyloxacarbocyanine (CyA); 1H,5H,11H,15H-Xantheno[2,3,4-ij:5,6,7-i'j']diquinolizin-18-ium, 9-[2(or 4)-[[[6-[2,5-dioxo-1-pyrrolidinyl)oxy]-6-oxohexyl]amino]sulfonyl]-4(or 2)-sulfophenyl]-2,3,6,7,12,13,16,17-octahydro-inner salt (TR or Texas Red); BODIPY^{TM} dyes; benzoxaazoles; stilbenes; pyrenes; and the like. See Haugland, "Molecular Probes Handbook of Fluorescent Probes and Research Chemicals" for further fluorophore examples.

[0065]   Reagents for incorporation the modification compounds of the present disclosure into oligonucleotides may have the following general structure:

$$
\begin{array}{c}
R_{21} \\
L_1 \!\!-\!\! O \\
M \!\!-\!\! X \\
L_2 \!\!-\!\! O \\
R_{22}
\end{array}
$$
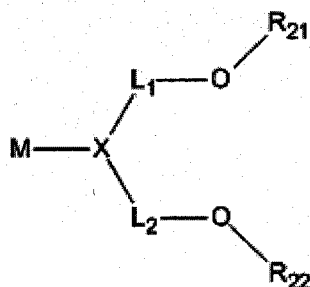
where R$_{13}$ is a protecting group on the oxygen atom, most commonly a trityl group, and preferably a dimethoxytrityl group, and R$_{22}$ is a phosphoramidite, a phosphate group, or a hydrogen phosphate used to couple the reagent to the growing oligonucleotide chain during

synthesis. A phosphoramidite is preferred. A N,N-diisopropyl-β-cyanoethyl phosphoramidite is the most preferred reactive group.

[0066] As used herein, the terms "nucleic acid" and "oligonucleotide," as used herein, refer to polydeoxyribonucleotides (containing 2-deoxy-D-ribose), polyribonucleotides (containing D-ribose), and to any other type of polynucleotide which is an N glycoside of a purine or pyrimidine base. There is no intended distinction in length between the terms "nucleic acid", "oligonucleotide", "oligomer" or "oligo", and these terms will be used interchangeably. These terms refer only to the primary structure of the molecule. Thus, these terms include double- and single-stranded DNA, as well as double- and single-stranded RNA. An oligonucleotide also can comprise nucleotide analogs in which the base, sugar, or phosphate backbone is modified as well as non-purine or non-pyrimidine nucleotide analogs oligonucleotides, which may comprise naturally occurring nucleosides or chemically modified nucleosides. In some embodiments, the compounds comprise modified sugar moieties, modified internucleoside linkages, or modified nucleobase moieties.

[0067] The term "base" as used herein includes purines, pyrimidines and non-natural bases and modifications well-known in the art. Purines include adenine, guanine and xanthine and modified purines such as 8-oxo-N^6-methyladenine and 7-deazaxanthine. Pyrimidines include thymine, uracil and cytosine and their analogs such as 5-methylcytosine and 4,4-ethanocytosine. Non-natural bases include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N^6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N^6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N^6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methyl ester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, nitroindole, and 2,6-diaminopurine.

[0068] The term "base" is sometimes used interchangeably with "monomer", and in this context it refers to a single nucleic acid or oligomer unit in a nucleic acid chain.

[0069] The term "probe" as used herein refers to nucleic acid oligonucleotides that produce a detectable response upon interaction with a target. The probes include at least one detectable moiety, a pair of moieties that form an energy transfer pair detectable upon some

change of state of the probe in response to its interaction with a binding partner, or more than two moieties such as a fluorophore and more than one quencher.

[0070]   The term "primer," as used herein, refers to an oligonucleotide capable of acting as a point of initiation of DNA synthesis under suitable conditions. Such conditions include those in which synthesis of a primer extension product complementary to a nucleic acid strand is induced in the presence of four different nucleoside triphosphates and an agent for extension (e.g., a DNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. A primer is preferably a single-stranded DNA. The appropriate length of a primer depends on the intended use of the primer but typically ranges from 6 to 50 nucleotides, preferably from 15-35 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template nucleic acid, but must be sufficiently complementary to hybridize with the template. The design of suitable primers for the amplification of a given target sequence is well known in the art and described in the literature cited herein. Primers can incorporate additional features which allow for the detection or immobilization of the primer but do not alter the basic property of the primer, that of acting as a point of initiation of DNA synthesis. For example, primers may contain an additional nucleic acid sequence at the 5' end which does not hybridize to the target nucleic acid, but which facilitates cloning or detection of the amplified product. The region of the primer which is sufficiently complementary to the template to hybridize is referred to herein as the hybridizing region.

[0071]   The term "hybridization," as used herein, refers to the formation of a duplex structure by two single-stranded nucleic acids due to complementary base pairing. Hybridization can occur between fully complementary nucleic acid strands or between "substantially complementary" nucleic acid strands that contain minor regions of mismatch. Conditions under which hybridization of fully complementary nucleic acid strands is strongly preferred are referred to as "stringent hybridization conditions" or "sequence-specific hybridization conditions". Stable duplexes of substantially complementary sequences can be achieved under less stringent hybridization conditions; the degree of mismatch tolerated can be controlled by suitable adjustment of the hybridization conditions. Those skilled in the art of nucleic acid technology can determine duplex stability empirically considering a number of variables including, for example, the length and base pair composition of the oligonucleotides, ionic strength, and incidence of mismatched base pairs, following the guidance provided by the art (see, e.g., Sambrook et al., 1989, Molecular Cloning–A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York;

Wetmur, 1991, Critical Review in Biochem. and Mol. Biol. 26(3/4):227-259; and Owczarzy et al., 2008, *Biochemistry*, 47: 5336-5353, which are incorporated herein by reference).

[0072]   The term "amplification reaction" refers to any chemical reaction, including an enzymatic reaction, which results in increased copies of a template nucleic acid sequence or results in transcription of a template nucleic acid. Amplification reactions include reverse transcription, the polymerase chain reaction (PCR), including Real Time PCR (see U.S. Pat. Nos. 4,683,195 and 4,683,202; PCR Protocols: A Guide to Methods and Applications (Innis et al., eds, 1990)), and the ligase chain reaction (LCR) (see Barany et al., U.S. Pat. No. 5,494,810). Exemplary "amplification reactions conditions" or "amplification conditions" typically comprise either two or three step cycles. Two step cycles have a high temperature denaturation step followed by a hybridization/elongation (or ligation) step. Three step cycles comprise a denaturation step followed by a hybridization step followed by a separate elongation or ligation step.

[0073]   The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope.

EXAMPLE 1

[0074]   This example demonstrates the improved stability of probes containing modifications of the present disclosure compared to a selection of other compounds.

[0075]   *Oligonucleotide synthesis and purification.* DNA oligonucleotides were synthesized using solid phase phosphoramidite chemistry, deprotected and desalted on NAP-5 columns (Amersham Pharmacia Biotech, Piscataway, NJ) according to routine techniques (Caruthers *et al., Methods Enzymol* 1992, 211:3-20). The oligomers were purified using reversed-phase high performance liquid chromatography (RP-HPLC). The purity of each oligomer was determined by capillary electrophoresis (CE) carried out on a Beckman P/ACE MDQ system (Beckman Coulter, Inc., Fullerton, CA). All single strand oligomers were at least 90% pure. Electrospray-ionization liquid chromatography mass spectroscopy (ESI-LCMS) of the oligonucleotides was conducted using an Oligo HTCS system (Novatia, Princeton, NJ), which consisted of ThermoFinnigan TSQ7000, Xcalibur data system, ProMass data processing software and Paradigm MS4™ HPLC (Michrom BioResources, Auburn, CA). Protocols recommended by manufacturers were followed. Experimental molar masses for all single strand oligomers were within 1.5 g/mol of expected molar mass. These results confirm identity of the oligomers.

[0076]   *Preparation of DNA samples.* Melting experiments were carried out in buffer containing 3.87 mM NaH_2PO_4, 6.13 mM Na_2HPO_4, 1 mM Na_2EDTA and 1000 mM NaCl. 1 M NaOH was used to titrate each solution to pH 7.0. Total sodium concentrations were

estimated to be 1020 mM. The DNA samples were thoroughly dialyzed against melting buffer in a 28-Well Microdialysis System (Life Technologies, Carlsbad, CA) following the manufacturer's recommended protocol. Concentrations of DNA oligomers were estimated from the samples' UV absorbance at 260 nm in a spectrophotometer (Beckman Coulter, Inc., Fullerton, CA), using extinction coefficients for each oligonucleotide that were estimated using the nearest neighbor model for calculating extinction coefficients. (See, Warshaw *et al.*, *J. Mol. Biol.* 1966, 20:29-38).

[0077]   *Internal modifications studied.* FIGS. 1A-1C show the structures of modified portions of the various modified oligonucleotides studied in this example and in Example 2. The FQ (Integrated DNA Technologies, Inc., sometimes referred to as "iFQ" in this application), was introduced into oligonucleotides using phosphoramidite reagents at the time of synthesis. See Example 10 for synthesis of the phosphoramidite. In the first series of duplexes, the iFQ group was placed as an insertion between bases in the duplex so that a 10 base top strand annealed to a 10 base bottom strand and the iFQ group was not aligned to a base. Additionally, 10-mer oligonucleotides with C3 spacer insertions were also synthesized and studied. The C3 spacer represents the control wherein a linear insertion of a phosphate group plus propanediol is placed between bases, which is similar to the iFQ insertions without having the nathylene-azo ring structures present. Extinction coefficients at 260 nm of iFQ was estimated to be 13340; the C3 spacer does not contribute to UV absorbance. Two 20 base and two 25 base duplexes of similar design were also studied. A second set of 10 base duplexes was studied where the iFQ group was placed as a substitution such that a 10 base top strand (5 bases – iFQ – 5 bases) was annealed to an 11 base bottom strand so that the iFQ group functioned as a substitution or replacement for a base. Four duplexes of this design were tested, one comprising each of the 4 bases (AGCT) to pair with the iFQ group.

[0078]   *Measurement of melting curves.* Oligomer concentrations were measured at least twice for each sample. If the estimated concentrations for any sample differed more than 4%, the results were discarded and new absorbance measurements were performed. To prepare oligonucleotide duplexes, complementary DNA oligomers were mixed in 1:1 molar ratio, heated to 367 K (*i.e.*, 94 °C) and slowly cooled to an ambient temperature. Each solution of duplex DNA was diluted with melting buffer to a total DNA concentration (C_T) of 2 µM.

[0079]   Melting experiments were conducted on a single beam Beckman DU 650 spectrophotometer (Beckman-Coulter) with a Micro T_m Analysis accessory, a Beckman High Performance Peltier Controller (to regulate the temperature), and 1 cm path-length cuvettes. Melt data were recorded using a PC interfaced to the spectrophotometer. UV-absorbance values at 268 nm wavelength were measured at 0.1 degree increments in the temperature range from 383 to 368 K (*i.e.*, 10 - 95 °C). Both heating (*i.e.*, denaturation) and cooling (*i.e.*,

renaturation) transition curves were recorded in each sample at a controlled rate of temperature change (24.9 ± 0.3°C per hour). Sample temperatures were collected from the internal probe located inside the Peltier holder, and recorded with each sample's UV-absorbance data. Melting profiles were also recorded for samples of buffer alone (no oligonucleotide), and these blank profiles were digitally subtracted from melting curves of the DNA samples. To minimize systematic errors, at least two melting curves were collected for each sample in different cuvettes and in different positions within the Peltier holder.

[0080] *Determination of melting temperatures.* To determine each sample's melting temperature, the melting profiles were analyzed using methods that have been previously described (see, Doktycz *et al., Biopolymers* 1992, 32:849-864; Owczarzy *et al., Biopolymers* 1997, 44:217-239; Owczarzy R., *Biophys. Chem.* 2005, 117: 207-215.). Briefly, the experimental data for each sample was smoothed, using a digital filter, to obtain a plot of the sample's UV-absorbance as a function of its temperature. The fraction of single-stranded oligonucleotide molecules, θ, was then calculated from that plot. The melting temperature or T_m of a sample was defined as the temperature where $\theta = 0.5$.

[0081] Table 1 lists the sequences tested, the internal quenchers used, and the resulting melting temperatures.

Table 1: Melting Temperatures for nucleic acids containing internal quencher moieties, where iFQ = internal FQ azo quencher, and iSpC3 = internal C3 spacer.

SEQ ID NO.	Duplex Sequence	N	T_m (C)	ΔT_m (C)
1	5'-ATCGTTGCTA-3'	10	43.85	0.0
2	3'-TAGCAACGAT-5'	10		
3	5'-ATC/iFQ/GTTGCTA-3'	10	48.05	4.2
2	3'-TAGCAACGAT-5'	10		
4	5'-ATCG/iFQ/TTGCTA-3'	10	48.55	4.7
2	3'-TAGCAACGAT-5'	10		
5	5'-ATCGT/iFQ/TGCTA-3'	10	46.35	2.5
2	3'-TAGCAACGAT-5'	10		
6	5'-CTTGGATCGTTGCTAGTAGG-3'	20	69.55	0.0
7	3'-GAACCTAGCAACGATCATCC-5'	20		
8	5'-CTTGGATCGT/iFQ/TGCTAGTAGG-3'	20	71.35	1.8
7	3'-GAACCTAGCAACGATCATCC-5'	20		
9	5'-CACTTGGATCGTTGCTAGTAGGGTC-3'	25	76.15	0.0
10	3'-GTGAACCTAGCAACGATCATCCCAG-5'	25		

SEQ ID NO.	Duplex Sequence	N	T_m (C)	ΔT_m (C)
11	5'-CACTTGGATC/iFQ/GTTGCTAGTAGGGTC-3'	25	77.05	0.9
10	3'-GTGAACCTAGCAACGATCATCCCAG-5'	25		
12	5'-ATC/iSpC3/GTTGCTA-3'	10	36.35	-7.5
2	3'-TAGCAACGAT-5'	10		
13	5'-ATCG/iSpC3/TTGCTA-3'	10	36.55	-7.3
2	3'-TAGCAACGAT-5'	10		
14	5'-ATCGT/iSpC3/TGCTA-3'	10	32.55	-11.3
2	3'-TAGCAACGAT-5'	10		
5	5'-ATCGT/iFQ/TGCTA-3'	10	47.35	3.5
15	3'-TAGCA/iSpC3/ACGAT-5'	10		
5	5'-ATCGT/iFQ/TGCTA-3'	10	42.24	-1.6
16	3'-TAGCAAACGAT-5'	11		
5	5'-ATCGT/iFQ/TGCTA-3'	10	45.27	1.4
17	3'-TAGCACACGAT-5'	11		
5	5'-ATCGT/iFQ/TGCTA-3'	10	40.44	-3.4
18	3'-TAGCAGACGAT-5'	11		
5	5'-ATCGT/iFQ/TGCTA-3'	10	45.27	1.4
19	3'-TAGCATACGAT-5'	11		

[0082]   Three different insertion placement sites were studied using a 10-mer oligonucleotide scaffold. Use of the shorter sequences most clearly demonstrates the potential effects on T_m and testing different placement sites illustrate that the T_m effects can be sequence context dependent. The relative ΔT_m shifts for the modified vs. unmodified 10mer sequences were averaged and are summarized in Table 2 below.

Table 2. Average ΔT_m shifts for three 10mer sequences with internal modifiers

Modifier	iFQ (insertion)	iFQ (substitution)	iSpC3
ΔT_m	+3.8°C	-0.6°C	-8.7°C

[0083]   As Tables 1 and 2 illustrate, disrupting a DNA sequence with a modification that is small and offers no steric hindrance (or stabilization) like a propanediol group (C3 spacer) has a significant negative impact on the T_m of a duplex (ΔT_m of -8.7°C). In contrast, the napthylene-azo-class quencher studied (iFQ) significantly stabilized the duplex compared with the iC3 control. A greater degree of stabilization was seen when the iFQ was placed as

24

an insertion (ΔT_m of +12.5°C relative to the iSpC3) than when the iFQ was placed as a base substitution (ΔT_m of +8.1°C relative to the iSpC3). Unexpectedly, use of the iFQ group as an insertion between bases stabilized the duplex compared to the unmodified parent duplex (ΔT_m of +3.8°C relative to the unmodified duplex), while base substitution resulted in slight destabilization (ΔT_m of -0.6°C relative to the unmodified duplex).

[0084]   Therefore internal incorporation of the napthylene-azo group within a DNA duplex stabilizes the duplex when placed as an insertion between bases.

[0085]   Certain anthraquinone groups can stabilize a duplex when placed on the ends (J. Am. Chem. Soc., 131:12671-12681, 2009); however this effect has not been described for internal placement or using napthylene-azo compounds. Therefore the use of internal napthylene-azo-class quenchers would be preferred to maintain duplex stability.

EXAMPLE 2

[0086]   The following example compares 10 base pair sets of duplexes with varying modification insertions placed at varying location along the 10-mer oligonucleotide. The structures of the modified portions of the various modified oligonucleotides are illustrated in FIGS. 1A-1C. As noted earlier, the synthesis of FQ phosphoramidite is described in Example 10. The structure "IB 1.1" is synthesized in the same manner as FQ except an aminoanthraquinone reagent is used instead of 4-nitro-1-napthylamine. The IB RQ quenchers are anthraquinone-based compounds (U.S. Pat. Application 2004/0110308) which are commonly used with red wavelength fluorescent dyes.

[0087]   The preparation of the DNA samples, the measurement of melting curves and determination of melting temperatures were performed as in Example 1. Table 3 lists the resulting Tm data for each duplex studied, as well as the ΔT_m relative to the duplex formed with the unmodified oligonucleotide.

Table 3:

SEQ ID No.	Sequence		T_m	ΔT_m
1	5' ATCGTTGCTA		43.9	-
2	3' TAGCAACGAT			
20	5' ATC/GTTGCTA	N-MDA "/"	31.0	-12.9
2	3' TAG CAACGAT			
21	5' ATC/GTTGCTA	C2 "/"	37.8	-6.1
2	3' TAG CAACGAT			
22	5' ATC/GTTGCTA	2,2 DMP "/"	34.9	-9.0

SEQ ID No.	Sequence		T_m	ΔT_m
2	3' TAG CAACGAT			
12	5' ATC/GTTGCTA	iSpC3 "/"	36.3	-7.6
2	3' TAG CAACGAT			
23	5' ATC/GTTGCTA	C4 "/"	30.1	-13.8
2	3' TAG CAACGAT			
24	5' ATC/GTTGCTA	C5 "/"	27.3	-16.6
2	3' TAG CAACGAT			
25	5' ATC/GTTGCTA	C6 "/"	26.1	-17.8
2	3' TAG CAACGAT			
26	5' ATC/GTTGCTA	C7 "/"	24.7	-19.1
2	3' TAG CAACGAT			
27	5' ATC/GTTGCTA	iSpS9 "/"	28.5	-15.4
2	3' TAG CAACGAT			
28	5' ATC/GTTGCTA	idSp "/"	33.5	-10.4
2	3' TAG CAACGAT			
3	5' ATC/GTTGCTA	iFQ "/"	48.0	+4.1
2	3' TAG CAACGAT			
29	5' ATC/GTTGCTA	iBHQ2 "/"	45.0	+1.1
2	3' TAG CAACGAT			
30	5' ATC/GTTGCTA	iRQ-n1 "/"	38.7	-5.2
2	3' TAG CAACGAT			
31	5' ATC/GTTGCTA	iRQ-n2 "/"	46.0	+2.1
2	3' TAG CAACGAT			
32	5' ATC/GTTGCTA	iEc "/"	40.1	-3.8
2	3' TAG CAACGAT			
33	5' ATC/GTTGCTA	IB 1.1 "/"	47.6	+3.7
2	3' TAG CAACGAT			
34	5' ATC/GTTGCTA	NPDA "/"	29.3	-14.6
2	3' TAG CAACGAT			
35	5' ATCG/TTGCTA	N-MDA "/"	34.1	-9.8
2	3' TAGC AACGAT			
36	5' ATCG/TTGCTA	C2 "/"	38.6	-5.3
2	3' TAGC AACGAT			
37	5' ATCG/TTGCTA	2,2 DMP "/"	35.5	-8.4

SEQ ID No.	Sequence		T_m	ΔT_m
2	3′ TAGC AACGAT			
13	5′ ATCG/TTGCTA	iSpC3 "/"	36.6	-7.3
2	3′ TAGC AACGAT			
38	5′ ATCG/TTGCTA	C4 "/"	32.0	-11.9
2	3′ TAGC AACGAT			
39	5′ ATCG/TTGCTA	C5 "/"	28.1	-15.8
2	3′ TAGC AACGAT			
40	5′ ATCG/TTGCTA	C6 "/"	26.5	-17.4
2	3′ TAGC AACGAT			
41	5′ ATCG/TTGCTA	C7 "/"	24.7	-19.2
2	3′ TAGC AACGAT			
42	5′ ATCG/TTGCTA	iSpS9 "/"	29.1	-14.8
2	3′ TAGC AACGAT			
43	5′ ATCG/TTGCTA	idSp "/"	34.1	-9.8
2	3′ TAGC AACGAT			
4	5′ ATCG/TTGCTA	iFQ "/"	48.6	+4.7
2	3′ TAGC AACGAT			
44	5′ ATCG/TTGCTA	iBHQ2 "/"	43.3	-0.6
2	3′ TAGC AACGAT			
45	5′ ATCG/TTGCTA	iRQ-n1 "/"	34.7	-9.2
2	3′ TAGC AACGAT			
46	5′ ATCG/TTGCTA	iRQ-n2 "/"	44.7	+0.8
2	3′ TAGC AACGAT			
47	5′ ATCG/TTGCTA	iEc "/"	35.7	-8.2
2	3′ TAGC AACGAT			
48	5′ ATCG/TTGCTA	IB 1.1 "/"	45.9	+2.1
2	3′ TAGC AACGAT			
49	5′ ATCG/TTGCTA	NPDA "/"	30.0	-13.9
2	3′ TAGC AACGAT			
50	5′ ATCGT/TGCTA	C2 "/"	34.0	-9.9
2	3′ TAGCA ACGAT			
51	5′ ATCGT/TGCTA	2,2 DMP "/"	33.1	-10.8
2	3′ TAGCA ACGAT			
14	5′ ATCGT/TGCTA	iSpC3 "/"	32.6	-11.3

SEQ ID No.	Sequence		T_m	ΔT_m
2	3' TAGCA ACGAT			
52	5' ATCGT/TGCTA	C4 "/"	26.4	-17.5
2	3' TAGCA ACGAT			
53	5' ATCGT/TGCTA	C5 "/"	23.3	-20.6
2	3' TAGCA ACGAT			
54	5' ATCGT/TGCTA	C6 "/"	21.9	-22.0
2	3' TAGCA ACGAT			
55	5' ATCGT/TGCTA	C7 "/"	20.4	-23.5
2	3' TAGCA ACGAT			
56	5' ATCGT/TGCTA	iSpS9 "/"	24.4	-19.5
2	3' TAGCA ACGAT			
57	5' ATCGT/TGCTA	idSp "/"	31.8	-12.1
2	3' TAGCA ACGAT			
5	5' ATCGT/TGCTA	iFQ "/"	46.3	+2.4
2	3' TAGCS ACGAT			
58	5' ATCGT/TGCTA	iBHQ2 "/"	41.5	-2.4
2	3' TAGCA ACGAT			
59	5' ATCGT/TGCTA	iRQ-n1 "/"	32.1	-11.8
2	3' TAGCA ACGAT			
60	5' ATCGT/TGCTA	iRQ-n2 "/"	42.6	-1.3
2	3' TAGCA ACGAT			
61	5' ATCGT/TGCTA	iEc "/"	33.1	-10.8
2	3' TAGCA ACGAT			
62	5' ATCGT/TGCTA	IB 1.1 "/"	44.7	+0.8
2	3' TAGCA ACGAT			
63	5' ATCGT/TGCTA	NPDA "/"	26.8	-17.1
2	3' TAGCA ACGAT			
64	5' A/TCGTTGCTA	N-MDA "/"	40.9	-3.0
2	3' T AGCAACGAT			
65	5' A/TCGTTGCTA	C2 "/"	43.5	-0.4
2	3' T AGCAACGAT			
66	5' A/TCGTTGCTA	2,2 DMP "/"	43.4	-0.4
2	3' T AGCAACGAT			
67	5' A/TCGTTGCTA	iSpC3 "/"	44.6	+0.7

SEQ ID No.	Sequence		T_m	ΔT_m
2	3' T AGCAACGAT			
68	5' A/TCGTTGCTA	C4 "/"	43.3	-0.6
2	3' T AGCAACGAT			
69	5' A/TCGTTGCTA	C5 "/"	41.5	-2.4
2	3' T AGCAACGAT			
70	5' A/TCGTTGCTA	C6 "/"	41.6	-2.3
2	3' T AGCAACGAT			
71	5' A/TCGTTGCTA	C7 "/"	43.2	-0.7
2	3' T AGCAACGAT			
72	5' A/TCGTTGCTA	iSpS9 "/"	41.5	-2.4
2	3' T AGCAACGAT			
73	5' A/TCGTTGCTA	idSp "/"	43.0	-0.9
2	3' T AGCAACGAT			
74	5' A/TCGTTGCTA	iFQ "/"	51.8	+7.9
2	3' T AGCAACGAT			
75	5' A/TCGTTGCTA	iBHQ2 "/"	48.9	+5.0
2	3' T AGCAACGAT			
76	5' A/TCGTTGCTA	iRQ-n1 "/"	48.2	+4.3
2	3' T AGCAACGAT			
77	5' A/TCGTTGCTA	iRQ-n2 "/"	49.1	+5.2
2	3' T AGCAACGAT			
78	5' A/TCGTTGCTA	iEc "/"	44.5	+0.6
2	3' T AGCAACGAT			
79	5' A/TCGTTGCTA	IB 1.1 "/"	51.2	+7.3
2	3' T AGCAACGAT			
80	5' A/TCGTTGCTA	NPDA "/"	41.0	-2.9
2	3' T AGCAACGAT			
81	5' ATCGTTGCT/A	C2 "/"	44.2	+0.3
2	3' TAGCAACGA T			
82	5' ATCGTTGCT/A	2,2 DMP "/"	43.8	-0.1
2	3' TAGCAACGA T			
83	5' ATCGTTGCT/A	iSpC3 "/"	44.5	+0.6
2	3' TAGCAACGA T			
84	5' ATCGTTGCT/A	C4 "/"	43.4	-0.5

SEQ ID No.	Sequence		T_m	ΔT_m
2	3' TAGCAACGA T			
85	5' ATCGTTGCT/A	C5 "/"	42.8	-1.0
2	3' TAGCAACGA T			
86	5' ATCGTTGCT/A	C6 "/"	43.3	-0.6
2	3' TAGCAACGA T			
87	5' ATCGTTGCT/A	C7 "/"	40.9	-3.0
2	3' TAGCAACGA T			
88	5' ATCGTTGCT/A	iSpS9 "/"	44.2	+0.3
2	3' TAGCAACGA T			
89	5' ATCGTTGCT/A	idSp "/"	44.7	+0.8
2	3' TAGCAACGA T			
90	5' ATCGTTGCT/A	iFQ "/"	50.3	+6.4
2	3' TAGCAACGA T			
91	5' ATCGTTGCT/A	iBHQ2 "/"	50.7	+6.8
2	3' TAGCAACGA T			
92	5' ATCGTTGCT/A	iRQ-n1 "/"	48.6	+4.7
2	3' TAGCAACGA T			
93	5' ATCGTTGCT/A	iRQ-n2 "/"	47.5	+3.6
2	3' TAGCAACGA T			
94	5' ATCGTTGCT/A	iEc "/"	45.8	+1.9
2	3' TAGCAACGA T			
95	5' ATCGTTGCT/A	IB 1.1 "/"	50.9	+7.0
2	3' TAGCAACGA T			
96	5' ATCGTTGCT/A	NPDA "/"	43.9	+0.0
2	3' TAGCAACGA T			

[0088]   FIGS. 13A, 13B, 13C, 13D and 13E are bar charts each showing the ΔT_m caused by each of the modifications at a particular location within the nucleotide. FIGS. 14A and 14B are plots showing the dependence of ΔT_m on the position of the insertion within an oligonucleotide for each of the various modification compounds. In general, the insertion of a modification nearer the end of an oligonucleotide is less destabilizing than when it is inserted towards the middle of the oligonucleotide. The spacer modifications were all destabilizing, with the degree of destabilization increasing as the size of the spacer increased.

[0089] The FQ modification had a positive effect on stability whether it was inserted near an end or in the middle of the duplex. The IB1.1 had nearly the same stability profile as FQ. The iRQ-n2 modification was also positive or negligible in its effect on duplex stability.

EXAMPLE 3

[0090] This example details the predictive modeling for oligonucleotides containing an internal quencher.

[0091] The thermodynamic impact of internal FQ azo quencher (iFQ) modification was determined from the difference between modified and native duplex DNAs. Melting experiments were conducted at 2μM DNA concentration (C_t) and in 1M Na$^+$ buffer. Transition enthalpies (ΔH°) and entropies (ΔS°) where obtain from fits to individual melting profiles (Petersheim, M.& Turner, D.H. (1983) *Biochemistry*, 22, 256-263). The equilibrium constant K_a was calculated from the fraction of broken base pairs (θ) at each temperature,

$$K_a = \frac{2(1-\theta)}{\theta^2 C_t} \qquad (1)$$

[0092] The graphs of -lnK_a vs. 1/T were least-square fit to straight lines and thermodynamic parameters were calculated from the slope and the intercept,

$$-\ln K_a = \frac{\Delta H^\circ}{RT} - \frac{\Delta S^\circ}{R} \qquad (2)$$

[0093] The fits were limited to the range of θ from 0.15 to 0.85, where θ and K_a are the most accurate. The symbol R is the ideal gas constant (1.9865 cal/(mol*K)). Reported values of ΔH°, and ΔS° are averages from at least four heating and cooling melting profiles. This thermodynamic analysis assumes that the transition enthalpies and entropies are temperature-independent and melting transitions proceed in two-state manner. The results are summarized in Table 4. Average thermodynamic effect of inserted FQ azo quencher could be estimated from the following relationships,

ΔH°(modified oligo) = ΔH° (native oligo) -1 272 cal/mol

ΔS°(modified oligo) = ΔS° (native oligo) -1.44 cal/(mol.K)

When these equations were employed to predict melting temperatures of 20 and 25 base pair duplexes (Table 5), the average error of predictions was 0.7°C.

31

Table 4: Thermodynamic effects of internal FQ modifications

SEQ ID No.	DNA sequence (5' to 3')[a]	ΔH^{o} cal/mol	$\Delta\Delta H^{o}$ cal/mol	ΔS^{o} cal/(mol.K)	$\Delta\Delta S^{o}$ cal/(mol.K)
1	ATCGTTGCTA	-68012		-185.62	
3	ATC/iFQ/GTTGCTA	-71827	-3815	-194.73	-9.12
4	ATCG/iFQ/TTGCTA	-69783	-1771	-188.00	-2.38
5	ATCGT/iFQ/TGCTA	-66241	1771	-178.45	7.17
	Average effect		-1272		-1.44

[a]Complementary DNA strand was 5'-TAGCAACGAT-3'. Calculations were done using non-rounded values.

Table 5: Accuracy of T_m predictions for two duplex DNAs containing internal FQ quencher that were not used to derive equations (1) and (2).

SEQ ID No.	DNA sequence (5' to 3')[a]	N_{bp}	Exper. T_m (°C)	Predicted T_m (°C)	Error of T_m prediction (°C)
8	CTTGGATCGT/iFQ/TGCTAGTAGG	20	71.3	71.6	0.3
11	CACTTGGATC/iFQ/GTTGCTAGTAGGGTC	25	77.1	78.2	1.1

[a]Melting temperatures were calculated using the nearest-neighbor model (SantaLucia, J., Jr. (1998) *Proc. Natl. Acad. Sci. USA*, 95, 1460–1465) and equations (1) and (2).

[0094]   The low error of prediction demonstrates the consistency and predictability of the effect of the addition of an internal quencher into an oligonucleotide.

EXAMPLE 4

[0095]   This example demonstrates the functional performance through quantitative real time PCR (q-PCR) using probes containing the novel internal quenchers disclosed herein.

[0096]   Validated qPCR assays were used to assess the performance of the different designs of fluorescence quenched probes. The primer sequences employed were specific for the human HPRT gene (NM_00194); probe and primer sequences and are listed below in Table 6.

Table 6: Sequences used in q-PCR assays

SEQ ID No.	Sequence Name	Sequence
97	HPRT Forward	GACTTTGCTTTCCTTGGTCAG

SEQ ID No.	Sequence Name	Sequence
98	HPRT Reverse	GGCTTATATCCAACACTTCGTG
99	HPRT Probe	ATGGTCAAGGTCGCAAGCTTGCTGGT

[0097] All oligonucleotides were synthesized by IDT (Integrated DNA Technologies, Coralville, IA). Probe oligonucleotides were HPLC purified. Mass identity of all oligonucleotides was verified by mass spectrometry. All target amplicons were cloned and sequence verified. The target plasmids were linearized by restriction endonuclease digestion and 10 fold serial dilutions were performed to create standard curves.

[0098] HPRT q-PCR reactions were comprised of 0.4 U Immolase DNA Polymerase (Bioline, Taunton, MA), 0.8 mM dNTP mix, 3 mM $MgCl_2$, and primer/probe concentrations at 200 nM each. Q-PCR reactions were performed on a Roche Lightcycler® 480 platform. Plasmid copy number standards were run in triplicate starting with $2x10^7$ down to $2x10^2$ (10 fold increments). The thermocycling profile used was $95^{10:00}$-$(95^{0:15}$-$60^{1:00})$ x 40. Data analysis was performed using software supplied by the manufacturer.

[0099] **Effects of placement of internal FQ (iFQ).** A series of oligonucleotide probes having a 5'-FAM reporter dye and an internal iFQ quencher were synthesized using the HPRT probe sequence varying the relative placement of the iFQ group. In one set the iFQ group was placed as a base substitution (replacing a base within the sequence). In another set the iFQ group was placed as a base insertion between residues. The distance of the iFQ from the 5'-dye was varied including 6, 8, 10, or 12 positions from the 5'-end. Henceforth, a modification placed as an insertion at position 6 is indicated as an "i6" and as a substitution at position 8 is indicated as a "s8", etc. The probes were used in qPCR as outlined above using the HPRT primers (SEQ ID Nos. 97 and 98) and $2x10^6$ copies of a cloned HPRT amplicon plasmid target. Amplification plots for the substitution series probes (SEQ ID Nos. 100-103) are shown in FIG. 2A and baseline normalized plots are shown in FIG. 2B. Amplification plots for the insertion series (SEQ ID Nos. 104-107) are shown in FIG. 3A and baseline normalized plots are shown in FIG. 3B. It is clear that precise placement of the iFQ group within the probe affected probe characteristics, with changes seen in both the baseline fluorescence, magnitude of signal generation, and quantification cycle number (Cq, the cycle number where amplification signal is first detected).

[00100] Relative metrics for assessing probe quality from the amplification plots are reported in Table 7, including the baseline fluorescence measured in the qPCR device (as a measure of quenching efficiency) and the ΔR_n, the difference in the fluorescence intensity between the start and end of the qPCR run (as a measure of the magnitude of signal

generated). Having low baseline fluorescence coupled with high relative ΔR_n signal generation leads to improved probe performance.

Table 7: Internal placement of iFQ in FAM labeled probes (HPRT assay)

SEQ ID No.	Sequence (Substitution)		Baseline fluorescence	ΔR_n
100	FAM-ATGGTCAAGGT/iFQ/GCAAGCTTGCTGGT-SpC3	(s12)	3.2	8.0
101	FAM-ATGGTCAAG/iFQ/TCGCAAGCTTGCTGGT-SpC3	(s10)	2.2	7.8
102	FAM-ATGGTCA/iFQ/GGTCGCAAGCTTGCTGGT-SpC3	(s8)	1.7	7.0
103	FAM-ATGGT/iFQ/AAGGTCGCAAGCTTGCTGGT-SpC3	(s6)	1.1	3.3
	Sequence (Insertion)			
104	FAM-ATGGTCAAGGT/iFQ/CGCAAGCTTGCTGGT-SpC3	(i12)	6.0	11.6
105	FAM-ATGGTCAAG/iFQ/GTCGCAAGCTTGCTGGT-SpC3	(i10)	4.0	13.7
106	FAM-ATGGTCA/iFQ/AGGTCGCAAGCTTGCTGGT-SpC3	(i8)	2.0	12.5
107	FAM-ATGGT/iFQ/CAAGGTCGCAAGCTTGCTGGT-SpC3	(i6)	1.7	6.8

[00101] All probe designs functioned in the above qPCR assay, thus the iFQ group can be used in the methods of this disclosure as either a base substitution or as an insertion between bases. Placing the iFQ group as a base substitution resulted in slightly better quenching than when used as an insertion between bases, however as a group the substitution probes showed lower signal generation. Further, iFQ placed as a base substitution is slightly destabilizing (lowers T_m) whereas iFQ placed as a base insertion is stabilizing (increases T_m) (see Example 1 above). Given the better signal generation and improved duplex stabilization properties, placement of the iFQ as an insertion is considered to be a more preferred embodiment and all subsequent examples will be done using only iFQ insertion probes.

[00102] The relative distance of the iFQ group from the 5'-FAM reporter dye had a significant impact on baseline fluorescence and on signal generation. In FIG. 3A, background fluorescence levels were i6 < i8 < i10 < i12. Placing the quencher group closer to the reporter dye reduced background fluorescence. This effect was expected as FRET based quenching improves with the proximity of the reporter and quencher. Unexpectedly, the relative placement of the iFQ group also affected the magnitude of signal generation during an amplification run and final functional fluorescence (positive signal) was i10 > i8 > i12 >> i6 (FIG. 3B). Interestingly, the i6 probes showed poor fluorescence signal for both the insertion and substitution series. This reduced signal compromised assay quality and delayed the Cq point for these probes, indicating lower assay sensitivity. We hypothesize that placing the iFQ group too close to the fluorophore results in probes that are not fully cleaved by the

DNA polymerase during amplification (5'-nuclease assay format), resulting in decreased release of reporter dye from quencher. Peak probe performance is realized at a point which is a compromise between quenching, which improves as quencher and fluorophore are placed more closely, and cleavage, which improves as quencher and fluorophore are separated by a greater number of nucleic acid bases. The precise range where this relationship is optimal is non-obvious and may be different for different reporter dye / quencher combinations. For this reporter dye / quencher combination, optimum performance of the assay was seen with i8 and i10 placement. For these probes, background fluorescence was low and signal generation was high.

EXAMPLE 5

[00103]   The following example demonstrates the efficacy of the internal quencher probe design at varying target concentrations.

[00104]   Example 4 examined the performance of 8 different probe designs using a single concentration of target nucleic acid. In the present example, six concentrations of target were tested in HPRT qPCR assays comparing performance of four of the probes from Example 4. HPRT specific probes using internal FQ (iFQ) quencher with FAM reporter were employed including substitution design s6 and s10 (SEQ ID Nos. 103 and 101) and insertion design i6 and i10 (SEQ ID Nos. 107 and 105). Amplification reactions were run as outlined in Example 3 using input target plasmid copy numbers of 2×10^2, 2×10^3, 2×10^4, 2×10^5, 2×10^6 and 2×10^7. Each reaction was run in triplicate.

[00105]   FIG. 4A shows the results of the baseline adjusted substitution set and FIG. 4B shows the results for the baseline adjusted insertion set. There is a clear progression of the amplification plot curves corresponding to the expected difference of ~3.3 cycles for every 10-fold change of input target nucleic acid, wherein the curves align from left to right the highest concentration of template to the lowest concentration. The insertion probe set outperformed the substitution probe set at all concentrations tested as evidenced by the improved magnitude of signal generated for all comparable data points. Further, the quencher i10 placement outperformed the quencher i6 placement series. Subsequent examples will therefore focus on use of the substitution i10 probe design.

EXAMPLE 6

[00106]   The following example demonstrates the use of an inserted internal quencher coupled with a second quencher positioned at the 3'-end of the probe.

[00107]   A new series of probes were synthesized targeting the HPRT gene all having a 5'-fluorescein reporter dye (6-FAM) and having quenchers located at varying positions, including compositions having two quencher groups in the same probe molecule. Internal

quenchers were added as insertions between bases. Single quencher probes (FQ quencher on the 3'-terminus, FQ quencher at the i10 position) were compared with dual quencher version of the same sequences. Dual quencher probes were made using the FQ chemical group or the Black Hole Quencher™-1 (BHQ1, shown below), a different commercially available dark quencher (Biosearch Technologies, Novato, CA). Table 8 lists the probe sequences tested. HPRT qPCR assays were performed as described in Example 4, using 2 x 10^6 copies of an HPRT amplicon-containing plasmid as target.
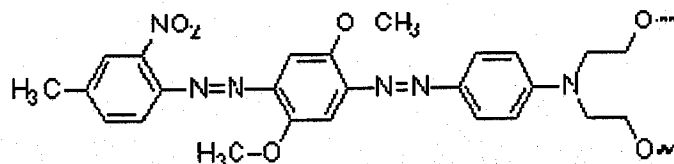
BHQ1

Table 8: Single and Dual Quenched Probes

SEQ ID No.	Probe Name	Sequence	Baseline fluorescence	ΔR_n
105	i10FQ	FAM-ATGGTCAAG/iFQ/GTCGCAAGCTTGCTGGT-SpC3	4.0	12.7
108	3'FQ	FAM-ATGGTCAAGGTCGCAAGCTTGCTGGT-FQ	10.5	11.0
109	i10FQ +3'FQ	FAM-ATGGTCAAG/iFQ/GTCGCAAGCTTGCTGGT-FQ	2.6	14.0
110	i10BHQ +3'BHQ	FAM-ATGGTCAAG/iBHQ1/GTCGCAAGCTTGCTGGT-BHQ1	2.5	7.5

[00108] These four probes were used in qPCR as outlined in Example 4 above using the HPRT primers (SEQ ID Nos. 97 and 98) and 2×10^6 copies of a cloned HPRT amplicon plasmid target. Amplification plots are shown in FIG. 5A and baseline normalized plots are shown in FIG. 5B. Relative metrics for assessing probe quality from the amplification plots are also reported in Table 8, including the baseline fluorescence measured in the qPCR device (as a measure of quenching efficiency) and the ΔR_n seen between the start and end of the qPCR run (as a measure of the magnitude of signal generated). Having low baseline fluorescence coupled with high relative ΔR_n signal generation leads to improved probe performance.

[00109] The traditional probe design with a 5'-reporter dye and 3'-quencher (SEQ ID No. 108) showed the highest baseline fluorescence (i.e., worst quenching). The single quencher

internal i10 placement probe (SEQ ID No. 105) showed significantly lower baseline fluorescence and the dual-quencher probes (SEQ ID Nos. 109, 110) showed the lowest baseline fluorescence (i.e., best quenching). Thus the dual quencher probes in FIGS. 5A and 5B showed slightly better performance than the i10FQ quencher alone, demonstrating that the presence of two quenchers not only does not negatively affect their quenching properties but rather improves the overall quenching properties.

[00110]   The relative fluorescence signal generated using the different probe designs also varied with quencher type and placement. The three FQ probes generated a ΔR_n of 11-14 with the highest relative fluorescent signal produced by the dual-quencher i10FQ-3'-FQ probe (SEQ ID No. 109). Interestingly, the same design using the alternative commercial dark quencher BHQ-1 (SEQ ID No. 110) performed significantly worse, showing a ΔR_n of only 7.5. This probe also showed a delayed Cq value (the cycle number where amplification signal is first detected), indicating worse functional sensitivity. This clearly demonstrates that all dark quencher chemical compositions are not functionally interchangeable and that the napthylene-azo quencher (FQ) performs in a superior fashion using the methods of this disclosure.

EXAMPLE 7

[00111]   Examples 4, 5, and 6 were performed using a single probe sequence specific for the human HPRT gene. The following example illustrates that the functional qPCR results detailed in Examples 4-6 are consistent when run using a different probe sequence, for a different target gene, using a different thermal cycler platform and different reagents.

[00112]   A new qPCR assay was employed specific for a strain of the H1N1 Influenza virus (SW H1, also known as the "swine flu"). Primer and probe sequences and are listed below in Table 9.

Table 9: Sequences used in an Influenza qPCR assay

SEQ ID No.	Sequence Name	Sequence
111	SW H1 Forward	GTGCTATAAACACCAGCCTYCCA
112	SW H1 Reverse	CGGGATATTCCTTAATCCTGTRGC
113	SW H1 Probe	CAGAATATACATCCAGTCACAATTGGAAAA

[00113]   A set of Influenza virus H1N1 specific (SW H1) probes were synthesized and qPCR assays were performed using the same methods as described in Example 4 except that the assays were run on an Applied Biosystems 7900HT Sequence Detection System (Applied

Biosystems, Foster City, CA) according to manufacturer's instructions, using 1X TaqMan Gene Expression Master Mix (Life Technologies, Carlsbad, CA), 250 nM probe and 1000 nM primers per the Center for Disease Control (CDC) document (CEC REF# I-007-005 Protocol for Detection and Characterization of Swine Influenza, 2009) recommendations. Assays run using different probe sequences and targets in the present example demonstrate that the performance of the probes of this disclosure are not sequence dependent, instrument dependent or polymerase formulation dependent.

[00114] The assays were run in duplicate with 2×10^6 copies of plasmid target used. Table 10 lists the probe sequences tested. All internal quencher groups were placed as insertions. Relative metrics for assessing probe quality from the amplification plots are also reported in Table 10, including the baseline fluorescence measured in the qPCR device (as a measure of quenching efficiency) and the ΔR_n seen between the start and end of the qPCR run (as a measure of the magnitude of signal generated). Note that the numbers reported are "relative fluorescence units" and that this example was performed on a different machine than the plots shown in Examples 4-6 above. The absolute numbers are different between machines; however the relative performance of the various probe designs is directly comparable.

Table 10: Single and dual quenched influenza SW H1 assays

SEQ ID No.	Name	Sequence	Baseline	ΔR_n
114	3'FQ	FAM-CAGAATATACATCCAGTCACAATTGGAAAA-FQ	2.6	1.5
115	i10FQ	FAM-CAGAATATA/iFQ/CATCCAGTCACAATTGGAAAA-SpC3	0.7	2.4
116	iF10Q +3'FQ	FAM-CAGAATATA/iFQ/CATCCAGTCACAATTGGAAAA-FQ	0.6	2.9
117	i10BHQ1 +3'BHQ1	FAM-CAGAATATA/iBHQ1/CATCCAGTCACAATTGGAAAA-BHQ1	0.45	0.8

[00115] Amplification plots for the influenza qPCR assays are shown in FIG. 6A and baseline normalized amplification plots are shown in FIG. 6B. Similar to the results described in Example 6 for the HPRT assays, the probe containing the 3'FQ quencher alone did not perform as well as any of the internal FQ containing probes. In particular, the internal-quencher probes showed markedly lower baseline (background) fluorescence, with the dual-quencher probes having the lowest background. As before, the dual quencher probes outperformed the single quencher probes, with the iFQ+3'FQ combination working the best. The SWH1 probe is 30 bases long and represents a relatively long probe sequence for use as

a dual-quenched probe in hydrolysis assays, which contributes to the greater difference seen between 3'-quencher probes and internal quencher probes in this example compared with the HPRT probes in Example 6 (the HPRT probe is 26 bases long). This illustrates another benefit of the methods of this disclosure. Long probes (as are frequently required in AT-rich target sequences) perform poorly using 3'-quencher design; however probe length does not affect performance of internally quenched probes.

[00116]   Similar to the results observed in Example 6 above, the dual-quencher i10FQ+3'FQ probe performed the best of the set tested, showing both low baseline fluorescence and high positive signal strength. Of note, again in the influenza probe sequence context the dual-quencher i10BHQ1+3'BHQ1 probe showed low baseline fluorescence with very low signal strength and functioned poorly in the assay, showing a delayed Cq value relative to the other probes.

EXAMPLE 8

[00117]   The following example demonstrates the efficacy of the internal quencher probes of the present disclosure when used with various fluorophores.

[00118]   In previous examples, all probes contained a 5'-fluorescein reporter dye (6-FAM). Typically, quencher molecules perform well with a limited subset of reporter dyes that are matched such that the fluorophore fluorescence emission wavelength overlaps well with the absorbance wavelengths of the quencher. A dye with emission in the red region, such as Cy5, typically requires use of a different quencher than one that is useful with a dye that has a shorter wavelength emission spectra, such as fluorescein. The probes in this example comprise different fluorophores having a wide range of emission wavelengths (Table 10) and demonstrate that the use of internal quencher probes in the dual-quencher format function well across a wide spectral range. All probes in the present example place the iFQ quencher at position i10 as an insertion. The dual-quencher probes of the present example are made using the i10FQ combined with either a 3'-FQ or with a 3'-IB RQ-n1 (also referred to as "RQ"). In traditional single 3'-quencher probe format, the FQ quencher (which has a peak absorbance around 534 nm) is typically employed with reporter dyes having emission in the 500-580 nm wavelength range. The RQ quencher (which has a bimodal peak absorbance around 610 and 640 nm) is typically employed with reporter dyes having emission in the 550-700 nm wavelength range.

Table 10. Fluorescent reporter dyes and their excitation and emission wavelengths.

Dye	Excitation Max (nm)	Emission Max (nm)
6-FAM	495	520

Dye	Excitation Max (nm)	Emission Max (nm)
MAX	524	557
Cy3	550	564
TEX-615	596	615
Cy5	648	668

[00119] A series of dual quencher probes specific for the human HPRT gene were synthesized having an insertion of the FQ quencher at the i10 position and either a 3'-FQ or a 3'-RQ quencher. Probe sequences are shown in Table 11 below. The probes were used in qPCR as described in Example 4 above using the HPRT primers (SEQ ID Nos. 97 and 98) and 2×10^6 copies of a cloned HPRT amplicon plasmid target. The FAM and MAX probes were run using the Applied Biosystems AB7900HT Sequence Detection platform. The Cy3 probes were run using the BIO-RAD iQ5 platform. The TEX615 and Cy5 probes were run using the Roche LightCycler 480 platform.

[00120] Examples 6 and 7 demonstrated that the dual-quencher iFQ-3'FQ combination performed slightly better for FAM reporter dye probes than iFQ alone, although the single quencher iFQ probes also performed well. Amplification plots for the fluorescein (emission 520 nm) reporter dye probes (SEQ ID Nos. 109 and 118) are shown in FIG. 7A and baseline normalized plots are shown in FIG. 7B. In this comparison, performance is nearly identical for the i10FQ-3'FQ vs. i10FQ-3'RQ probes, even though the RQ quencher is best suited for red wavelength dyes. The i10FQ-3'FQ probe did, however, perform slightly better.

[00121] Amplification plots for the MAX (emission 557 nm) reporter dye probes (SEQ ID Nos. 119 and 120) are shown in FIG. 8A and baseline normalized plots are shown in FIG. 8B. Baseline quenching was nearly identical for the i10FQ-3'FQ vs. i10FQ-3'RQ probes, however peak signal intensity was superior with the i10FQ-3'FQ probe. Both designs worked well but the i10FQ-3'FQ design is preferred.

[00122] Amplification plots for the Cy3 (emission 564 nm) reporter dye probes (SEQ ID Nos. 121 and 122) are shown in FIG. 9A and baseline normalized plots are shown in FIG. 9B. Baseline quenching was slightly lower for the i10FQ-3'FQ vs. i10FQ-3'RQ probes, and peak signal intensity was also slightly superior with the i10FQ-3'FQ probe. Both designs worked well but the i10FQ-3'FQ design is preferred.

[00123] Amplification plots for the TEX615 (emission 615 nm) reporter dye probes (SEQ ID Nos. 123 and 124) are shown in FIG. 10A and baseline normalized plots are shown in FIG. 10B. Baseline quenching was slightly lower for the i10FQ-3'RQ vs. i10FQ-3'FQ probes, however peak signal intensity was superior with the i10FQ-3'FQ probe. Both designs worked well but the i10FQ-3'FQ design is preferred.

40

[00124]   Amplification plots for the Cy5 (emission 668 nm) reporter dye probes (SEQ ID Nos. 125 and 126) are shown in FIG. 11A and baseline normalized plots are shown in FIG. 11B. Baseline quenching was lower for the i10FQ-3'RQ vs. i10FQ-3'FQ probes; however peak signal intensity was identical for both designs. Both designs worked well but the i10FQ-3'RQ design showed lower baseline fluorescence.

[00125]   The relative metrics for assessing probe quality from the amplification plots are also reported in Table 12, including the baseline fluorescence measured in the qPCR device (as a measure of quenching efficiency) and the ΔR_n seen between the start and end of the qPCR run (as a measure of the magnitude of signal generated). Having low baseline fluorescence coupled with high relative ΔR_n signal generation leads to improved probe performance. Note that different real-time PCR machines were employed for different probe pairs (see above) and that the arbitrary fluorescence units reporting fluorescence intensity vary between platforms.

Table 12: Dual-quencher probes with various reporter dyes and an internal i10FQ with either 3'-FQ or 3'-RQ

SEQ ID No.	Name	Sequence	Baseline fluorescence	ΔR_n
109	FAM i10FQ-3'FQ	FAM-ATGGTCAAG/iFQ/GTCGCAAGCTTGCTGGT-FQ	2.9	14
118	FAM i10FQ-3'RQ	FAM-ATGGTCAAG/iFQ/GTCGCAAGCTTGCTGGT-RQ	3.1	13.8
119	MAX i10FQ-3'FQ	MAX-ATGGTCAAG/iFQ/GTCGCAAGCTTGCTGGT-FQ	0.2	2.1
120	MAX i10FQ-3'RQ	MAX-ATGGTCAAG/iFQ/GTCGCAAGCTTGCTGGT-RQ	0.2	1.9
121	Cy3 i10FQ-3'FQ	Cy3-ATGGTCAAG/iFQ/GTCGCAAGCTTGCTGGT-FQ	350	1500
122	Cy3 i10FQ-3'RQ	Cy3-ATGGTCAAG/iFQ/GTCGCAAGCTTGCTGGT-RQ	250	1300
123	TEX i10FQ-3'FQ	TEX-ATGGTCAAG/iFQ/GTCGCAAGCTTGCTGGT-FQ	0.8	3.4
124	TEX i10FQ-3'RQ	TEX-ATGGTCAAG/iFQ/GTCGCAAGCTTGCTGGT-RQ	0.7	2.4
125	Cy5 i10FQ-3'FQ	Cy5-ATGGTCAAG/iFQ/GTCGCAAGCTTGCTGGT-FQ	0.4	1.5

SEQ ID No.	Name	Sequence	Baseline fluorescence	ΔR_n
126	Cy5 i10FQ-3'RQ	Cy5-ATGGTCAAG/iFQ/GTCGCAAGCTTGCTGGT-RQ	0.2	1.5

[00126] The use of an internal quencher paired with a 3'-quencher in the above dual-quencher probes enables use of a single quencher compound (FQ) in probes with reporter dyes ranging from FAM to Cy5. This novel format thus permits use of a quencher with dyes that it is not suitable to work with when used in the traditional 3'-quencher alone probe design format.

EXAMPLE 9

[00127] The following example demonstrates that placement of the internal iFQ insertion can vary with the Cy5 reporter dye.

[00128] The probes studied in Example 8 all employed the FQ quencher placed as an insertion at position i10. This example compares function of a dual-quenched Cy5 probes having i10FQ vs. i12FQ.

[00129] Probe sequences are shown in Table 13 below. The probes were used in qPCR as described in Example 4 above using the HPRT primers (SEQ ID Nos. 97 and 98) and 2×10^7 to 2×10^2 copies of a cloned HPRT amplicon plasmid target. The Cy5 probes were run using the Roche LightCycler 480 platform.

Table 13: Dual-quencher probes with Cy5 reporter dye comparing i10FQ vs. i12FQ

SEQ ID No.	Name	Sequence
125	Cy5 i10FQ-3'FQ	Cy5-ATGGTCAAG/iFQ/GTCGCAAGCTTGCTGGT-FQ
127	Cy5 i12FQ-3'FQ	Cy5-ATGGTCAAGGT/iFQ/CGCAAGCTTGCTGGT-RQ

The baseline adjusted amplification plot showing superimposed traces of all 6 dilution curves for both probes is shown in FIG. 12. Peak signal intensity was slightly superior for the i10FQ-3'FQ probe than for the i12FQ-3'FQ probe, however both probes performed equally well in quantitative detection of the target standard curve dilution set. Precise Cq values measured for each probe / target dilution are shown in Table 14 below, where it can be seen that the Cq values are nearly identical between the two probes.

Table 14. Cq values comparing sensitivity of i10FQ-3'FQ vs. i12FQ-3-FQ for an HPRT assay using Cy5 reporter dye

Copy Number	CY5 i10FQ-3'FQ	CY5 i12FQ-3'FQ
2E7	14.2	14.4
2E6	17.5	17.4
2E5	20.9	21.0
2E4	24.8	24.6
2E3	28.0	27.9
2E2	31.6	32.4

[00130]   Although there may be range in location for placement of the iFQ insertion for different probes, the preferred location may vary with different reporter dyes. More importantly, there exists some flexibility in placement such that iFQ group place anywhere in the i8-i12 range should work well.

EXAMPLE 10

[00131]   This example demonstrates the synthesis of a phosphoramidite useful for oligonucleotide synthesis that is derivatized with a quencher of the present disclosure. The synthetic method is shown below in Schemes 1 and 2.

[00132]   **Mono-DMT-phenyl diethanolamine (2):** A solution of 10 g of phenyl diethanolamine in 100 mL of pyridine was mixed for 3-4 h at room temperature with a solution of 6 g dimethoxytrityl-chloride (DMT-Cl) in 150 mL of a 98:2 dichloromethane/pyridine solution. The reaction mixture was concentrated to dryness under vacuum. The residue was dissolved in 200 mL of ethyl acetate, washed with two portions of 100 mL of deionized water, and the organic layer dried over Na_2SO_4. The organic solution was concentrated and purified by column chromatography using a 300 g of silica gel column developed with 30/65/5 ethyl acetate/hexanes/triethylamine to yield 5.25g (20% yield) of mono-DMT-phenyl diethanolamine. TLC: R_f 0.55 (EtAc/hexanes/Et$_3$N – 40/55/5). ^{1}H NMR (CDCl$_3$) δ 7.38 (d, J=8 Hz, 2H), 7.27 (d, J=8 Hz, 4H), 7.38 (d, J=8 Hz, 2H), 7.24-7.12 (m, 6H), 6.76 (d, J=8 Hz, 4H), 6.66 (d, J=8 Hz, 2H), 3.74 (s, 6H), 3.74 (t, J=7.5 Hz, 2H), 3.54 (t, J=7.5 Hz, 2H), 3.51 (t, J=7.5 Hz, 2H), 3.33 (t, J=7.5 Hz, 2H), 2.23 (br. s, 1H).
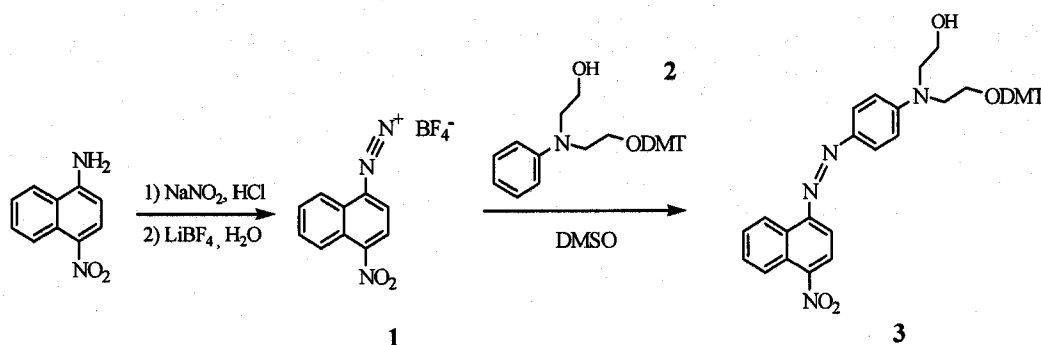
Scheme 1

[00133]   **Mono-DMT-4-(1-nitro-4-naphthylazo)-*N*,*N*-diethanolaniline (3)**:   Cold concentrated HCl (17 mL) was added dropwise at 0 °C over 15 min to a suspension of 4-nitro-1-naphthylamine (2 g) in cold water (6 mL) at 0 °C. Then $NaNO_2$ (1.6 g) in cold water (4 mL) was added dropwise at 0 °C over 15 min and the 4-nitro-1-naphthylamine dissolved upon stirring. $LiBF_4$ (1.38 g) in H_2O (3 mL) was added dropwise at 0°C. The reaction mixture was stirred at 0°C for 30 min. A brownish yellow powder (3.08 g) of naphthyl-1-nitro-4-tetrafluoroborate azonium salt **(1)** was obtained after filtering and rinsing the solution with cold water, methanol, and ether. A solution of 4 g of mono-DMT-phenyl diethanolamine **(2)** in 50 mL of dimethylsulfoxide (DMSO) was added with stirring at 10-15 °C over 10-15 min to a chilled solution of 2.8 g of azonium salt **(1)** in 50 mL of DMSO at 10-15 °C in a water bath. After an additional 15 min of stirring, 3 mL of triethylamine was added to the reaction mixture followed by 100 mL of ethyl acetate. The reaction mixture was washed with 3x30 mL of deionized water and the organic layer was dried over Na_2SO_4. The solvent was removed and product was purified by column chromatography with 300 g of silica gel to provide 1.8 g of mono-DMT-4-(1-nitro-4-naphtylazo)-*NN*-diethanolaniline **(3)**. TLC: R_f 0.65 (DCM/Et$_3$N – 80/20). ^{1}H NMR (CDCl$_3$) δ 9.04 (d, J=8.4 Hz, 1H), 8.68 (d, J=8.4 Hz, 1H), 8.34 (d, J=8.4 Hz, 1H), 7.96 (d, J=9.2 Hz, 2H), 7.81-7.71 (m, 3H), 7.39 (d, J=8 Hz, 2H), 7.27 (d, J=8 Hz, 4H), 7.24-7.19 (m, 3H), 6.78 (d, J=8 Hz, 4H), 6.77 (d, J=8 Hz, 2H), 3.88 (t, J=7.5 Hz, 2H), 3.75 (s, 6H), 3.78-368 (m, 4H), 3.47 (t, J=7.5 Hz, 2H), 1.57 (br. s, 1H).
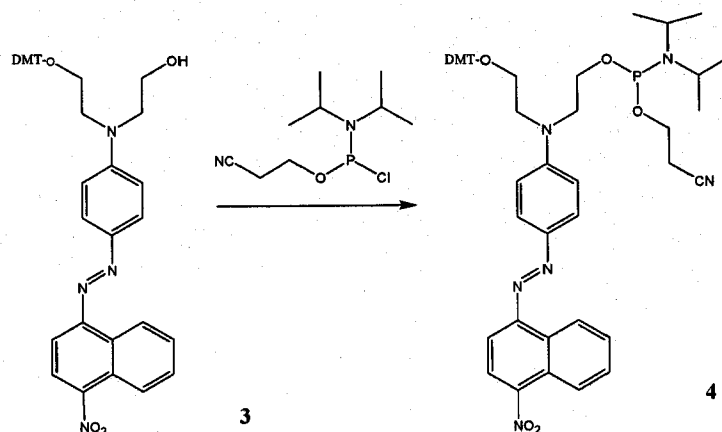
Scheme 2

[00134]   Mono-DMT-4-(1-nitro-4-naphthylazo)-*N,N*-diethanolaniline

phosphoramidite (4): A solution of 0.2 ml of *N,N*-diisopropylamino-cyanoethyl-
phosphoramidolchloride was stirred into a solution of 0.3 g of alcohol **(3)** in 20 mL of
anhydrous THF and 1 mL of triethylamine for 5 min at 0-5 °C. After 15 min of additional
stirring the reaction mixture was warmed to room temperature. The solvent was evaporated
under a vacuum and the residue purified by column chromatography through 50 g of silica
gel (EtOAc/PE/TEA: 10/85/5 – 40/55/5). TLC: R_f 0.65 (DCM/Et$_3$N – 80/20). ^{1}H NMR
(CDCl$_3$) δ 9.05 (d, J=8.4 Hz, 1H), 8.68 (d, J=8.4 Hz, 1H), 8.34 (d, J=8.4 Hz, 1H), 7.96 (d,
J=9.2 Hz, 2H), 7.81-7.71 (m, 3H), 7.39 (d, J=8 Hz, 2H), 7.27 (d, J=8 Hz, 4H), 7.24-7.19 (m,
3H), 6.78 (d, J=8 Hz, 4H), 6.76 (d, J=8 Hz, 2H), 3.85-3.75 (m, J=7.5 Hz, 4H), 3.76 (s, 6H),
3.70 (t, J=7.5 Hz, 2H), 3.41 (t, J=7.5 Hz, 2H), 2.58 (t, J=8.0 Hz, 2H), 1.20 (s, 3H), 1.18 (s,
3H), 1.17 (s, 3H), 1.15 (s, 3H). ^{31}P NMR δ 148.39.

[00135]   The phosphoramidite **(4)** can be added to an oligonucleotide during synthesis
using standard phosphoramidite oligonucleotide synthetic techniques.

EXAMPLE 11

[00136]   The following example demonstrates the utility of the disclosed insertions in a
molecular beacon probe.

[00137]   All Molecular Beacon oligonucleotides were synthesized and purified by
Integrated DNA Technologies, Inc. Molecular beacon oligonucleotides at 200 nM were
incubated in a buffer consisting of 16.0mM (NH$_4$)$_2$SO$_4$, 67.0mM Tris-HCl pH 8.3, 0.01%
Tween-20 in the presence and absence of a 1000 nM complementary oligonucleotide. The
samples were incubated at 30°C in a CFX884 Real Time System (BioRad, Hercules, CA),
and subjected to an increase in temperature from 30-95°C at a ramp rate of 1°C/min, with
fluorescence measurements taken at each degree interval. First derivative analysis of the

fluorescence curves yielded the melting temperature of the beacon alone (stem T_m) and the melting temperature of the probe plus complement (loop T_m). Each sample independently was measured a minimum of three times.

[00138] Table 15 lists the sequences that were synthesized and tested. The "RQ" is IBRQn-1.

Table 15: Molecular beacon sequences

SEQ ID No.	Name	Sequence
128	beacon	CGCGATCAGACAAGGAGTGGGCTTCATGGATCGCG
129	complement	TTACATGAAGCCCACTCCTTGTCTATC
130	3' Dabcyl	/56-FAM/CGCGATCAGACAAGGAGTGGGCTTCATGGATCGCG/3Dabcyl/
131	3' BHQ-1	/56-FAM/CGCGATCAGACAAGGAGTGGGCTTCATGGATCGCG/3BHQ-1/
132	3' FQ	/56-FAM/CGCGATCAGACAAGGAGTGGGCTTCATGGATCGCG/3FQ/
133	FQ0-FQ	/56-FAM/CGCGATCAGACAAGGAGTGGGCTTCATGGATCGCG/FQ/FQ/
134	FQ1-FQ	/56-FAM/CGCGATCAGACAAGGAGTGGGCTTCATGGATCGC/FQ/G/3FQ/
135	FQ2-FQ	/56-FAM/CGCGATCAGACAAGGAGTGGGCTTCATGGATCG/FQ/CG/3FQ/
136	FQ3-FQ	/56-FAM/CGCGATCAGACAAGGAGTGGGCTTCATGGATC/FQ/GCG/3FQ/
137	FQ4-FQ	/56-FAM/CGCGATCAGACAAGGAGTGGGCTTCATGGAT/FQ/CGCG/3FQ/
138	FQ5-FQ	/56-FAM/CGCGATCAGACAAGGAGTGGGCTTCATGGA/FQ/TCGCG/3FQ/
139	FQ6-FQ	/56-FAM/CGCGATCAGACAAGGAGTGGGCTTCATGG/FQ/ATCGCG/3FQ/
140	3' RQSp	/56-FAM/CGCGATCAGACAAGGAGTGGGCTTCATGGATCGCG/3RQSp/
141	FQ0-RQ	/56-FAM/CGCGATCAGACAAGGAGTGGGCTTCATGGATCGCG/FQ//3RQSp/
142	FQ1-RQ	/56-FAM/CGCGATCAGACAAGGAGTGGGCTTCATGGATCGC/FQ/G/3RQSp/
143	FQ2-RQ	/56-FAM/CGCGATCAGACAAGGAGTGGGCTTCATGGATCG/FQ/CG/3RQSp/
144	FQ3-RQ	/56-FAM/CGCGATCAGACAAGGAGTGGGCTTCATGGATC/FQ/GCG/3RQSp/
145	FQ4-RQ	/56-FAM/CGCGATCAGACAAGGAGTGGGCTTCATGGAT/FQ/CGCG/3RQSp/
146	FQ5-RQ	/56-FAM/CGCGATCAGACAAGGAGTGGGCTTCATGGA/FQ/TCGCG/3RQSp/
147	FQ6-RQ	/56-FAM/CGCGATCAGACAAGGAGTGGGCTTCATGG/FQ/ATCGCG/3RQSp/

46

[00139]   Table 16 lists the results of the assay for each beacon.

Table 16

Quencher	Stem T_m	Stem ΔT_m	Loop T_m	Loop ΔT_m
3' Dabcyl	66		63	
3' BHQ-1	70		62.7	
3' FQ	70	-	63	-
FQ0-FQ	72	2	62	-1
FQ1-FQ	68	-2	63	0
FQ2-FQ	70	0	62	-1
FQ3-FQ	71	1	62	-1
FQ4-FQ	72	2	62	-1
FQ5-FQ	74	4	63	0
FQ6-FQ	75.3	5.3	64	1
3' RQSp	74	-	61	-
FQ0-RQ	74	0	63	2
FQ1-RQ	70	-4	62	1
FQ2-RQ	71	-3	61	0
FQ3-RQ	71	-3	61	0
FQ4-RQ	73	-1	62	1
FQ5-RQ	76	2	62	1
FQ6-RQ	77	3	61.3	0.3

[00140]   The stability is improved when the FQ quencher is placed internally close to the 3' quencher, particularly with a 3' RQ quencher.

[00141]   All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[00142]   The use of the terms "a" and "an" and "the" and similar referents in the context of this disclosure (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a

shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the various inventions disclosed herein and does not pose a limitation on the scope of any inventions unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[00143]   Preferred embodiments of various inventions are described herein, including the best mode known to the inventors for carrying out the various inventions. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the various inventions to be practiced otherwise than as specifically described herein. Accordingly, this disclosure includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the disclosure unless otherwise indicated herein or otherwise clearly contradicted by context.

WHAT IS CLAIMED IS:

1.   A composition, comprising:
        a first oligonucleotide having the structure 5'-Y_1-L_1-X-L_2-Y_2-3', wherein:
                Y_1 comprises a sequence of four or more DNA or RNA nucleotides,
        including a first nucleotide N_1 having a 3' phosphate covalently linked to L_1;
                Y_2 comprises a sequence of four or more DNA or RNA nucleotides,
        including a second nucleotide N_2 having a 5' phosphate covalently linked to L_2;
                L_1 and L_2 each independently are a direct bond or a C_1-C_7 alkyl, alkynyl,
        alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl,
   alkylaryl, or alkoxyl group;

        X is $\overset{M}{\diagup}\overset{R_1}{\diagdown}$ or $\overset{N}{\underset{M}{|}}$;
        R_1 is a hydrogen or a C_1-C_8 alkyl; and
        M is a quencher;
        wherein the first oligonucleotide is adapted to hybridize to a second oligonucleotide
having the structure 3'-Y_3-Y_4-5', wherein:
                Y_3 comprises a sequence of four or more DNA or RNA nucleotides,
        including a third nucleotide N_3; and
                Y_4 comprises a sequence of four or more DNA or RNA nucleotides,
        including a fourth nucleotide N_4 that is directly attached to nucleotide N_3;
        wherein if the first oligonucleotide hybridizes to the second oligonucleotide, N_1 base
pairs with N_3 and N_2 base pairs with N_4.to form a duplex having a T_m that is greater than the
T_m of a duplex formed between the second oligonuleotide and a third oligonucleotide having
the structure 5'-Y_1-Y_2-3'.

2.   The composition of claim 1, wherein the first oligonucleotide comprises 15-35
nucleotides.

3.   The composition of either of claims 1 and 2, wherein L_1 and L_2 each is a C_1-C_7
alkyl.

4.   The composition of claim 3, wherein L_1 and L_2 are C_2 alkyls.

5.   The composition of any of claims 1-4, wherein the 3' phosphate that is covalently linked to L_1, and the 5' phosphate that is covalently linked to L_2, each independently are a phosphodiester, a phosphothioate, a phosphodithioate, a methyl phosphonate, a phosphoramidate, a phosphoramidite or a phosphotriester.

6.   The composition of any of claims 1-5, wherein:
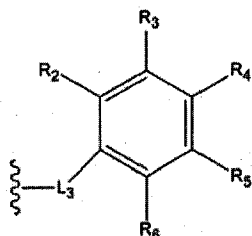
M is

;

L$_3$ is a direct bond or a C_1-C_8 alkyl, alkenyl, alkenyl, heteroalkyl, substituted alkyl, cycloalkyl, or alkoxyl;

R_2-R_6 each independently are a hydrogen, an alkyl, an alkenyl, a heteroalkyl, a substituted alkyl, an aryl, a heteroaryl, a substituted aryl, a cycloalkyl, an alkylaryl, an alkoxyl, an electron withdrawing group, or an electron donating group, and one of

R_2-R_6 is

; and
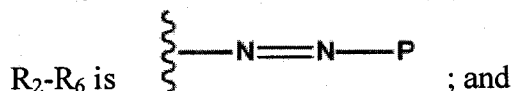
P is a fused polycyclic aromatic moiety.

7.   The composition of 6, wherein the electron withdrawing group is selected from the group consisting of -NO$_2$, -SO$_3$⁻, -SO$_2$⁻, -CN, -NCS, a ketone, an alkoxyl, an ether, a carboxylic acid and a sulfonyl.

8.   The composition of either of claims 6 and 7, wherein the electron donating group is selected from the group consisting of an alkoxyl, a heteroalkoxyl, an alkyl, a cycloalkyl, a heteroalkyl, an amino, an alkylamino, or an arylamino.
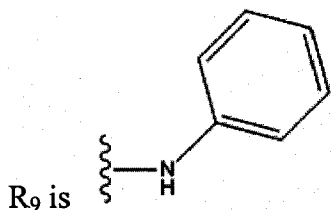
9.   The composition of any of claims 6-8, wherein:
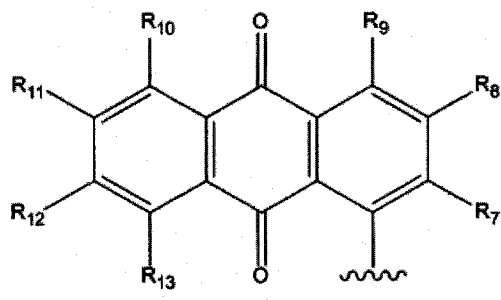
P is

;

R$_7$-R$_9$ each independently are a hydrogen, an alkoxyl, an alkyl, an alkylamino, an arylamino, a cycloalkyl, a heteroalkoxyl, a heteroalkyl, or an amino; and

R$_{10}$-R$_{13}$ each independently are a hydrogen, a nitro, a cyano, a carboxylate, a sulfonyl, a sulfamoyl, an alkenyl, an alkynyl, an amino, an aryl, a heteroaryl, a biaryl, a bialkenyl, a bialkynyl, an alkoxycarbonyl or a carbamoyl.

10.   The composition of claim 9, wherein:

R$_9$ is

.
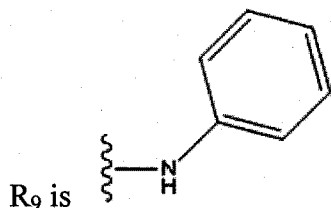
11.   The composition of any of claims 6-8, wherein:
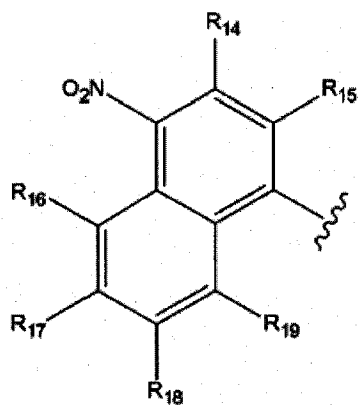
P is

; and

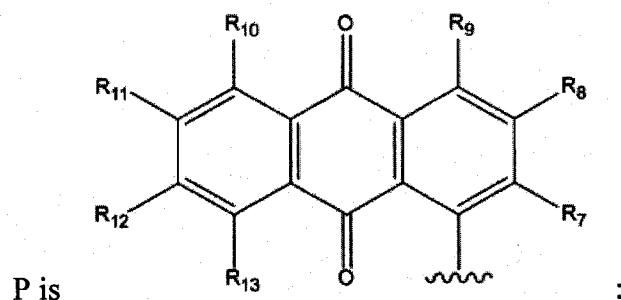R$_{14}$-R$_{19}$ each independently are a hydrogen, an alkyl, a heteroalkyl, an aryl, a heteroaryl, an electron withdrawing group, or a five or six membered ring structure formed from the R1, R2 pair, the R$_3$, R$_4$ pair, the R$_4$, R$_5$ pair, or the R$_5$, R$_6$ pair.

12.   The composition of claim 1, wherein M is -L$_4$-P, wherein L$_4$ is an alkyl, an alkynyl, an alkenyl, a heteroalkyl, a substituted alkyl, or an alkoxyl group, and P is a fused polycyclic aromatic moiety.

13.   The composition of claim 12, wherein L$_4$ is -CH2-O-CH2-CH2-NH-.

14.   The composition of either of claims 12 and 13, wherein:
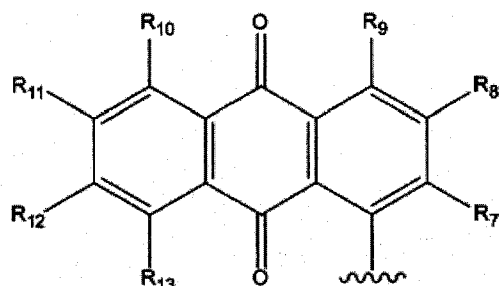
P is

R$_7$-R$_9$ each independently are a hydrogen, an alkoxyl, an alkyl, an alkylamino, an arylamino, a cycloalkyl, a heteroalkoxyl, a heteroalkyl, or an amino; and

R$_{10}$-R$_{13}$ each independently are a hydrogen, a nitro, a cyano, a carboxylate, a sulfonyl, a sulfamoyl, an alkenyl, an alkynyl, an amino, an aryl, a heteroaryl, a biaryl, a bialkenyl, a bialkynyl, an alkoxycarbonyl or a carbamoyl.

15.   The composition of claim 14, wherein:
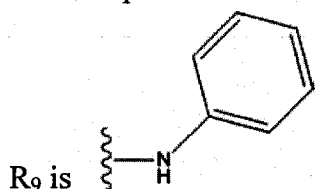
R$_9$ is

16.   The composition of any of claims 1-15, wherein the first oligonucleotide is labeled with a fluorophore.

17.   The composition of claim 16, wherein the fluorophore is 6-carboxyfluorescein (FAM), 2'7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), tetrachlorofluorescein (TET), 6-carboxyrhodamine (R6G), N,N,N;N'-tetramethyl-6-carboxyrhodamine (TAMRA), 6-carboxy-X-rhodamine (ROX); 1-dimethylaminonaphthyl-5-sulfonate, 1-anilino-8-naphthalene sulfonate, 2-p-toluidinyl-6-naphthalene sulfonate, 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS), a coumarin dye, an acridine dye, indodicarbocyanine 3 (Cy3), indodicarbocyanine 5 (Cy5), indodicarbocyanine 5.5 (Cy5.5),

3-1-carboxy-pentyl)-3'-ethyl-5,5'-dimethyloxacarbocyanine (CyA); 1H,5H,11H,15H-Xantheno[2,3,4-ij:5,6,7-i'j']diquinolizin-18-ium, 9-[2(or 4)-[[[6-[2,5-dioxo-1-pyrrolidinyl)oxy]-6-oxohexyl]amino]sulfonyl]-4(or 2)-sulfophenyl]-2,3,6,7,12,13,16,17-octahydro-inner salt (TR or Texas Red), a BODIPY™ dye, benzoxaazole, stilbene or pyrene.
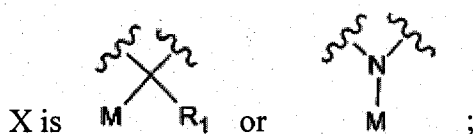
18.   The composition of either of claims 16 and 17, wherein the fluorophore is attached to the 5' end.

19.   The composition of any of claims 16-18, wherein Y_1 comprises a sequence of 8-12 DNA or RNA nucleotides.

20.   The composition of any of claims 16-19, wherein the first oligonucleotide is labeled with a second quencher.

21.   The composition of claim 20, wherein the second quencher is dabcyl, Eclipse® quencher, BHQ1, BHQ2 and BHQ3, Iowa Black® FQ, Iowa Black® RQ-n1 or Iowa Black® RQ-n2.

22.   The composition of claim 21 wherein the second quencher is Iowa Black® FQ, Iowa Black® RQ-n1 or Iowa Black® RQ-n2.

23.   The composition of any of claims 1-22, further comprising the second oligonucleotide hybridized to the first oligonucleotide.

24.   A composition comprising:

an oligonucleotide having the structure 5'-Y_1-L_1-X-L_2-Y_2-3', wherein

Y_1 comprises a sequence of 8-12 DNA or RNA nucleotides, including a first nucleotide N_1 having a 3' phosphate covalently linked to L_1, and where the nucleotide on the 5' end of Y_1 is labeled with a fluorophore;

Y_2 comprises a sequence of one or more DNA or RNA nucleotides, including a second nucleotide N_2 having a 5' phosphate covalently linked to L_2;

L_1 and L_2 each independently are a direct bond or a C_1-C_7 alkyl, alkynyl, alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl, alkylaryl, or alkoxyl group;

X is   M R_1 or   M   ;

R_1 is a hydrogen or a C_1-C_8 alkyl; and

M is a quencher.
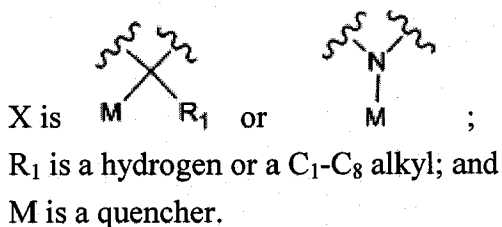
25.   The composition of claim 24, wherein the oligonucleotide comprises 15-35 nucleotides.

26.   The composition of either of claims 24 and 25, wherein L_1 and L_2 each is a C_1-C_7 alkyl.

27.   The composition of claim 26, wherein L_1 and L_2 are C_2 alkyls.

28.   The composition of any of claims 24-27, wherein the 3' phosphate that is covalently linked to L_1, and the 5' phosphate that is covalently linked to L_2, each independently are a phosphodiester, a phosphothioate, a phosphodithioate, a methyl phosphonate, a phosphoramidate, a phosphoramidite or a phosphotriester.

29.   The composition of any of claims 24-28, wherein:
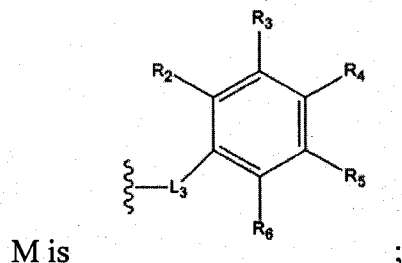
M is   ;

L_3 is a direct bond or a C_1-C_8 alkyl, alkenyl, alkenyl, heteroalkyl, substituted alkyl, cycloalkyl, or alkoxyl;

R_2-R_6 each independently are a hydrogen, an alkyl, an alkenyl, a heteroalkyl, a substituted alkyl, an aryl, a heteroaryl, a substituted aryl, a cycloalkyl, an alkylaryl, an alkoxyl, an electron withdrawing group, or an electron donating group, and one of

R_2-R_6 is   ; and
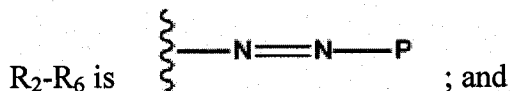
P is a fused polycyclic aromatic moiety.

30.   The composition of claim 29, wherein the electron withdrawing group is selected from the group consisting of -NO_2, -SO_3^-, -SO_2^-, -CN, -NCS, a ketone, an alkoxyl, an ether, a carboxylic acid and a sulfonyl.

31.   The composition of either of claims 29 and 30, wherein the electron donating group is selected from the group consisting of an alkoxyl, a heteroalkoxyl, an alkyl, a cycloalkyl, a heteroalkyl, an amino, an alkylamino, or an arylamino.
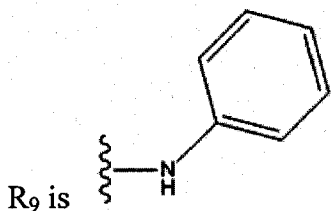
32.   The composition of any of claims 29-31, wherein:
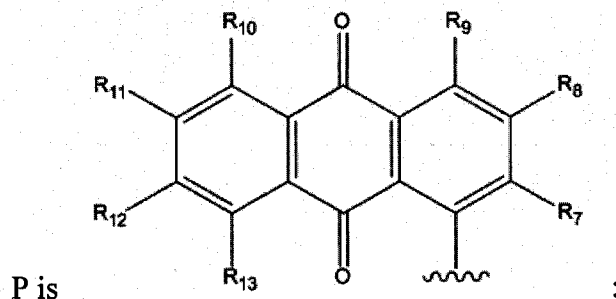
P is   ;

R_7-R_9 each independently are a hydrogen, an alkoxyl, an alkyl, an alkylamino, an arylamino, a cycloalkyl, a heteroalkoxyl, a heteroalkyl, or an amino; and

R_{10}-R_{13} each independently are a hydrogen, a nitro, a cyano, a carboxylate, a sulfonyl, a sulfamoyl, an alkenyl, an alkynyl, an amino, an aryl, a heteroaryl, a biaryl, a bialkenyl, a bialkynyl, an alkoxycarbonyl or a carbamoyl.

33.   The composition of claim 32, wherein:

R$_9$ is   .

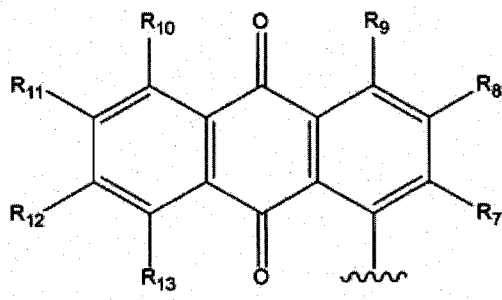34.   The composition of any of claims 29-31, wherein:
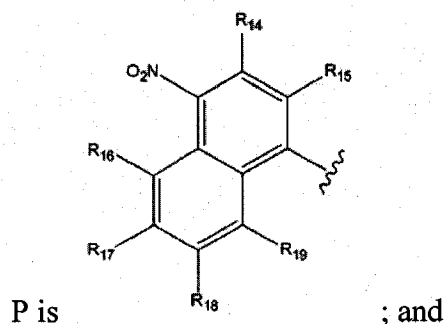
P is   ; and

R$_{14}$-R$_{19}$ each independently are a hydrogen, an alkyl, a heteroalkyl, an aryl, a
heteroaryl, an electron withdrawing group, or a five or six membered ring structure formed
from the R1, R2 pair, the R$_3$, R$_4$ pair, the R$_4$, R$_5$ pair, or the R$_5$, R$_6$ pair.

35.   The composition of claim 24, wherein M is -L$_4$-P, wherein L$_4$ is an alkyl, an alkynyl,
an alkenyl, a heteroalkyl, a substituted alkyl, or an alkoxyl group, and P is a fused
polycyclic aromatic moiety.

36.   The composition of claim 35, wherein L$_4$ is -CH2-O-CH2-CH2-NH-.

37.   The composition of either of claims 35 or 36, wherein:

P is   ;

R$_7$-R$_9$ each independently are a hydrogen, an alkoxyl, an alkyl, an alkylamino, an
arylamino, a cycloalkyl, a heteroalkoxyl, a heteroalkyl, or an amino; and

R$_{10}$-R$_{13}$ each independently are a hydrogen, a nitro, a cyano, a carboxylate, a sulfonyl, a sulfamoyl, an alkenyl, an alkynyl, an amino, an aryl, a heteroaryl, a biaryl, a bialkenyl, a bialkynyl, an alkoxycarbonyl or a carbamoyl.

38.   The composition of claim 37, wherein:

R$_9$ is
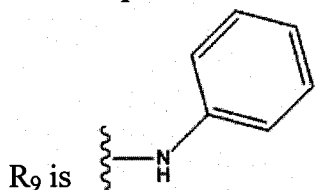
39.   The composition of any of claims 24-38, wherein F is 6-carboxyfluorescein (FAM), 2'7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), tetrachlorofluorescein (TET), 6-carboxyrhodamine (R6G), N,N,N;N'-tetramethyl-6-carboxyrhodamine (TAMRA), 6-carboxy-X-rhodamine (ROX); 1-dimethylaminonaphthyl-5-sulfonate, 1-anilino-8-naphthalene sulfonate, 2-p-toluidinyl-6-naphthalene sulfonate, 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS), a coumarin dye, an acridine dye, indodicarbocyanine 3 (Cy3), indodicarbocyanine 5 (Cy5), indodicarbocyanine 5.5 (Cy5.5), 3-1-carboxy-pentyl)-3'-ethyl-5,5'-dimethyloxacarbocyanine (CyA); 1H,5H,11H,15H-Xantheno[2,3,4-ij:5,6,7-i'j']diquinolizin-18-ium, 9-[2(or 4)-[[[6-[2,5-dioxo-1-pyrrolidinyl)oxy]-6-oxohexyl]amino]sulfonyl]-4(or 2)-sulfophenyl]-2,3,6,7,12,13,16,17-octahydro-inner salt (TR or Texas Red), a BODIPY™ dye, benzoxaazole, stilbene or pyrene.

40.   The composition of any of claims 24-39, wherein F is attached to the 5' phosphate at the 5' end of the oligonucleotide.

41.   The composition of any of claims 24-40, wherein the oligonucleotide is labeled with a second quencher.

42.   The composition of claim 41, wherein the second quencher is dabcyl, Eclipse® quencher, BHQ1, BHQ2 and BHQ3, Iowa Black® FQ, Iowa Black® RQ-n1 or Iowa Black® RQ-n2.

43.   The composition of claim 41 wherein the second quencher is Iowa Black® FQ, Iowa Black® RQ-n1 or Iowa Black® RQ-n2.

57

44.   A composition comprising:

an oligonucleotide having the structure 5'-Y_1-L_1-X-L_2-Y_2-3', wherein

Y_1 comprises a sequence of one or more DNA or RNA nucleotides, including a first nucleotide N_1 having a 3' phosphate covalently linked to L_1;

Y_2 comprises a sequence of one or more DNA or RNA nucleotides, including a second nucleotide N_2 having a 5' phosphate covalently linked to L_2;

L_1 and L_2 each independently are a direct bond or a C_1-C_7 alkyl, alkynyl, alkenyl, heteroalkyl, substituted alkyl, aryl, heteroaryl, substituted aryl, cycloalkyl, alkylaryl, or alkoxyl group;

X is   M R_1 or   M   ;

R_1 is a hydrogen or a C_1-C_8 alkyl; and

M is a label comprising a fused polycyclic aromatic moiety.

45.   The composition of claim 44, wherein the oligonucleotide comprises 15-35 nucleotides.

46.   The composition of either of claims 44 and 45, wherein L_1 and L_2 each is a C_1-C_7 alkyl.

47.   The composition of claims 46, wherein L_1 and L_2 are C_2 alkyls.

48.   The composition of any of claims 44-47, wherein the 3' phosphate that is covalently linked to L_1, and the 5' phosphate that is covalently linked to L_2, each independently are a phosphodiester, a phosphothioate, a phosphodithioate, a methyl phosphonate, a phosphoramidate, a phosphoramidite or a phosphotriester.

49.   The composition of any of claims 44-48, wherein:
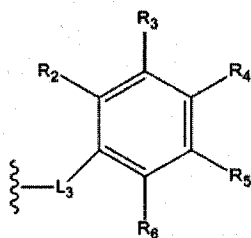
M is

L_3 is a direct bond or a C_1-C_8 alkyl, alkenyl, alkenyl, heteroalkyl, substituted alkyl, cycloalkyl, or alkoxyl;

R_2-R_6 each independently are a hydrogen, an alkyl, an alkenyl, a heteroalkyl, a substituted alkyl, an aryl, a heteroaryl, a substituted aryl, a cycloalkyl, an alkylaryl, an alkoxyl, an electron withdrawing group, or an electron donating group, and one of R_2-R_6 comprises the fused polycyclic aromatic moiety.

50.   The composition of claim 49, wherein the one of R_2-R_6 comprising the fused polycyclic aromatic moiety is

; and
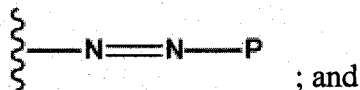
P is the fused polycyclic aromatic moiety.

51.   The composition of either of claims 49 and 50, wherein the electron withdrawing group is selected from the group consisting of -NO$_2$, -SO$_3^-$, -SO$_2^-$, -CN, -NCS, a ketone, an alkoxyl, an ether, a carboxylic acid and a sulfonyl.

52.   The composition of any of claims 49-51, wherein the electron donating group is selected from the group consisting of an alkoxyl, a heteroalkoxyl, an alkyl, a cycloalkyl, a heteroalkyl, an amino, an alkylamino, or an arylamino.

53.   The composition of any of claims 50-52, wherein:
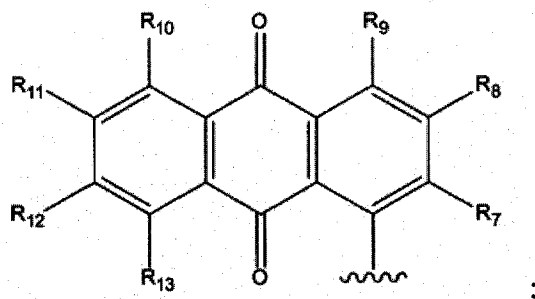
P is

;

R_7-R_9 each independently are a hydrogen, an alkoxyl, an alkyl, an alkylamino, an arylamino, a cycloalkyl, a heteroalkoxyl, a heteroalkyl, or an amino; and

R_{10}-R_{13} each independently are a hydrogen, a nitro, a cyano, a carboxylate, a sulfonyl, a sulfamoyl, an alkenyl, an alkynyl, an amino, an aryl, a heteroaryl, a biaryl, a bialkenyl, a bialkynyl, an alkoxycarbonyl or a carbamoyl.

54.   The composition of claim 53, wherein:

R$_9$ is .

55.   The composition of any of claims 50-52, wherein:
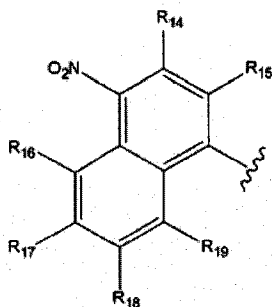
P is ; and

R$_{14}$-R$_{19}$ each independently are a hydrogen, an alkyl, a heteroalkyl, an aryl, a heteroaryl, an electron withdrawing group, or a five or six membered ring structure formed from the R1, R2 pair, the R$_3$, R$_4$ pair, the R$_4$, R$_5$ pair, or the R$_5$, R$_6$ pair.

56.   The composition of claim 44, wherein M is -L$_4$-P, wherein L$_4$ is an alkyl, an alkynyl, an alkenyl, a heteroalkyl, a substituted alkyl, or an alkoxyl group, and P is a fused polycyclic aromatic moiety.

57.   The composition of claim 56, wherein L$_4$ is -CH2-O-CH2-CH2-NH-.

58.   The composition of either of claims 56 and 57, wherein:

P is ;

R$_7$-R$_9$ each independently are a hydrogen, an alkoxyl, an alkyl, an alkylamino, an arylamino, a cycloalkyl, a heteroalkoxyl, a heteroalkyl, or an amino; and

R_{10}-R_{13} each independently are a hydrogen, a nitro, a cyano, a carboxylate, a sulfonyl, a sulfamoyl, an alkenyl, an alkynyl, an amino, an aryl, a heteroaryl, a biaryl, a bialkenyl, a bialkynyl, an alkoxycarbonyl or a carbamoyl.

59.   The composition of claim 58, wherein:

R_9 is
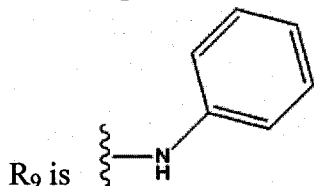
60.   The composition of any of claims 44-59, wherein the oligonucleotide is labeled with a fluorophore.

61.   The composition of claim 60, wherein the fluorophore is 6-carboxyfluorescein (FAM), 2'7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), tetrachlorofluorescein (TET), 6-carboxyrhodamine (R6G), N,N,N;N'-tetramethyl-6-carboxyrhodamine (TAMRA), 6-carboxy-X-rhodamine (ROX); 1-dimethylaminonaphthyl-5-sulfonate, 1-anilino-8-naphthalene sulfonate, 2-p-toluidinyl-6-naphthalene sulfonate, 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS), a coumarin dye, an acridine dye, indodicarbocyanine 3 (Cy3), indodicarbocyanine 5 (Cy5), indodicarbocyanine 5.5 (Cy5.5), 3-1-carboxy-pentyl)-3'-ethyl-5,5'-dimethyloxacarbocyanine (CyA); 1H,5H,11H,15H-Xantheno[2,3,4-ij:5,6,7-i'j']diquinolizin-18-ium, 9-[2(or 4)-[[[6-[2,5-dioxo-1-pyrrolidinyl)oxy]-6-oxohexyl]amino]sulfonyl]-4(or 2)-sulfophenyl]-2,3,6,7,12,13,16,17-octahydro-inner salt (TR or Texas Red), a BODIPY™ dye, benzoxaazole, stilbene or pyrene.

62.   The composition of either of claims 60 or 61, wherein the fluorophore is attached to the 5' phosphate at the end of the oligonucleotide.

63.   The composition of any of claims 60-62, wherein the oligonucleotide is labeled with a second quencher.

64.   The composition of claim 63, wherein the second quencher is dabcyl, Eclipse® quencher, BHQ1, BHQ2 and BHQ3, Iowa Black® FQ, Iowa Black® RQ-n1 or Iowa Black® RQ-n2.

65.   The composition of claim 64 wherein the second quencher is Iowa Black® FQ, Iowa Black® RQ-n1 or Iowa Black® RQ-n2.

66.   A method of detecting a target oligonucleotide in a sample, comprising:
        contacting the sample with the composition of any of claims 1-23, wherein the target oligonucleotide is the second oligonucleotide, the first oligonucleotide is labeled with a fluorophore, and fluorescence of the fluorophore is reduced by fluorescence resonance energy transfer to the quencher or by ground state quenching by the quencher when the oligonucleotide is not hybridized to the second oligonucleotide; and
        detecting an increase in fluorescence indicating the presence of the second oligonucleotide in the sample.

67.   The method of claim 66, wherein fluorescence is reduced by fluorescence resonance energy transfer when the first oligonucleotide is not hybridized to the second oligonucleotide.

68.   The method of claim 66, wherein fluorescence is reduced by ground state quenching when the first oligonucleotide is not hybridized to the second oligonucleotide.

69.   The method of claim 66, wherein fluorescence is reduced by both fluorescence resonance energy transfer and ground state quenching when the first oligonucleotide is not hybridized to the second oligonucleotide.

70.   The method of any of claims 66-69, wherein the increase in fluorescence arises from cleavage of the labeled oligonucleotide.

71.   The method of any of claims 66-70, wherein the oligonucleotide forms a random-coil conformation when the oligonucleotide is unhybridized, such that the fluorescence of the fluorophore is reduced.

72.   The method of any of claims 66-70, wherein the oligonucleotide comprises a self-complimentary sequence and wherein the quencher and the fluorophore are attached to the oligonucleotide such that the fluorescence of the fluorophore is quenched by the quencher when the nucleic acid polymer undergoes intramolecular base pairing.

73.   The method of any of claims 66-72, wherein the method is used in a PCR reaction wherein synthesis of PCR product results in an increase in fluorescence.

74.   A method of detecting a target nucleic acid in a sample, comprising:
       contacting the sample with the composition of any of claims 24-43, wherein the oligonucleotide is adapted to hybridize to the target nucleic acid, and fluorescence of the fluorophore F is reduced by fluorescence resonance energy transfer to the quencher or by ground state quenching by the quencher when the oligonucleotide is not hybridized to the second oligonucleotide; and
       detecting an increase in fluorescence indicating the presence of the second oligonucleotide in the sample.

75.   The method of claim 74, wherein fluorescence is reduced by fluorescence resonance energy transfer when the first oligonucleotide is not hybridized to the second oligonucleotide.

76.   The method of either of claims 74, wherein fluorescence is reduced by ground state quenching when the first oligonucleotide is not hybridized to the second oligonucleotide.

77.   The method of either of claims 74, wherein fluorescence is reduced by both fluorescence resonance energy transfer and ground state quenching when the first oligonucleotide is not hybridized to the second oligonucleotide.

78.   The method of any of claims 74-77, wherein the increase in fluorescence arises from cleavage of the labeled oligonucleotide.

79.   The method of any of claims 74-78, wherein the oligonucleotide forms a random-coil conformation when the oligonucleotide is unhybridized, such that the fluorescence of the fluorophore is reduced.

80.   The method of any of claims 74-78, wherein the oligonucleotide comprises a self-complimentary sequence and wherein the quencher and the fluorophore are attached to the oligonucleotide such that the fluorescence of the fluorophore is quenched by the quencher when the nucleic acid polymer undergoes intramolecular base pairing.

81.   The method of any of claims 74-80, wherein the method is used in a PCR reaction wherein synthesis of PCR product results in an increase in fluorescence.

82.   A method of detecting a target oligonucleotide in a sample, comprising:
       contacting the sample with the composition of any of claims 44-65, wherein the oligonucleotide is adapted to hybridize to the target nucleic acid, the oligonucleotide is labeled with a fluorophore, and fluorescence of the fluorophore is reduced by fluorescence resonance energy transfer to the quencher or by ground state quenching by the quencher when the oligonucleotide is not hybridized to the second oligonucleotide; and
       detecting an increase in fluorescence indicating the presence of the second oligonucleotide in the sample.

83.   The method of claim 82, wherein fluorescence is reduced by fluorescence resonance energy transfer when the first oligonucleotide is not hybridized to the second oligonucleotide.

84.   The method of claim 82, wherein fluorescence is reduced by ground state quenching when the first oligonucleotide is not hybridized to the second oligonucleotide.

85.   The method of claim 82, wherein fluorescence is reduced by both fluorescence resonance energy transfer and ground state quenching when the first oligonucleotide is not hybridized to the second oligonucleotide.

86.   The method of any of claims 82-85, wherein the increase in fluorescence arises from cleavage of the labeled oligonucleotide.

87.   The method of any of claims 82-86, wherein the oligonucleotide forms a random-coil conformation when the oligonucleotide is unhybridized, such that the fluorescence of the fluorophore is reduced.

88.   The method of any of claims 82-86, wherein the oligonucleotide comprises a self-complimentary sequence and wherein the quencher and the fluorophore are attached to the oligonucleotide such that the fluorescence of the fluorophore is quenched by the quencher when the nucleic acid polymer undergoes intramolecular base pairing.

89.   The method of any of claims 82-87, wherein the method is used in a PCR reaction wherein synthesis of PCR product results in an increase in fluorescence.
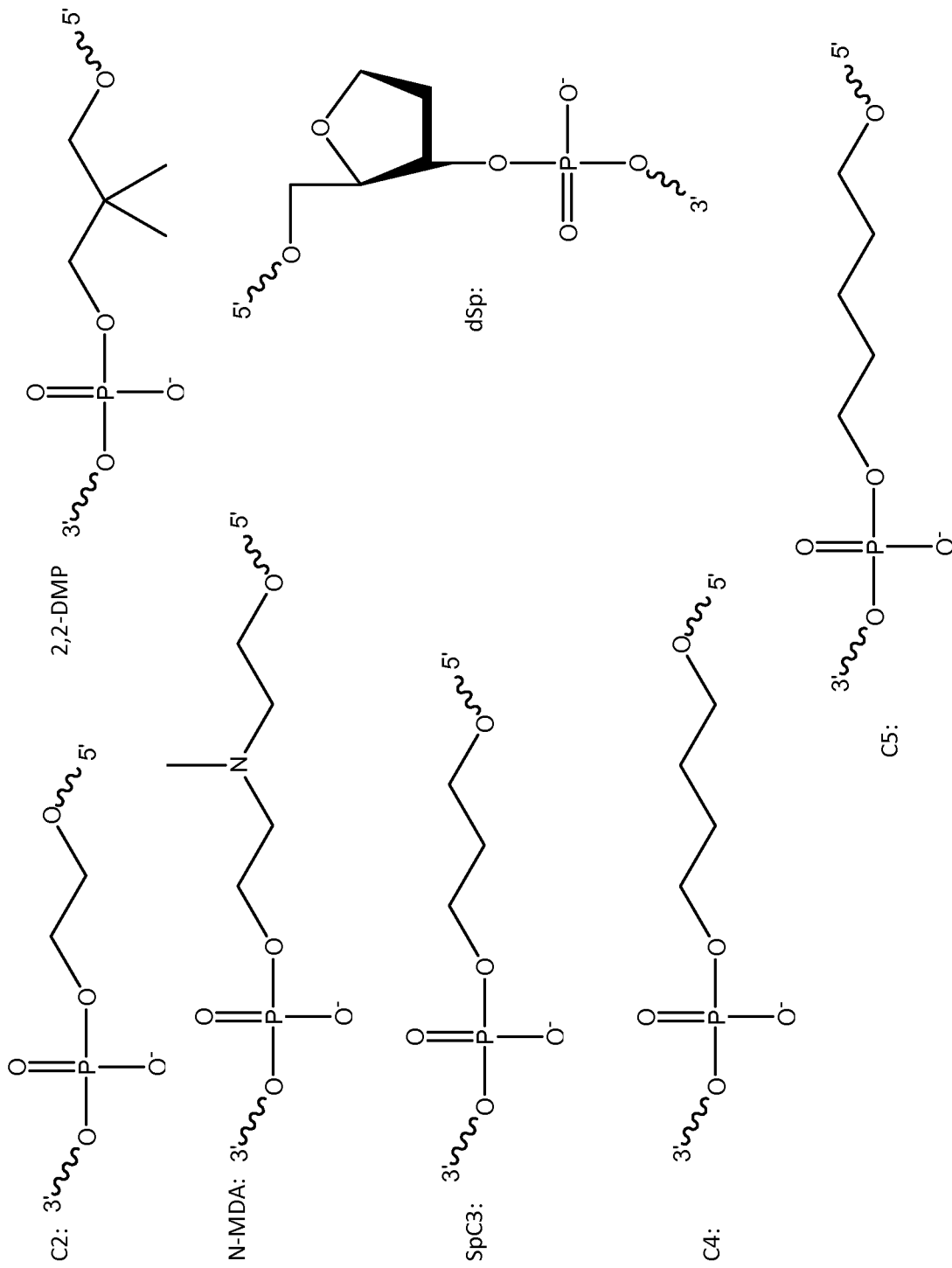
FIG. 1A
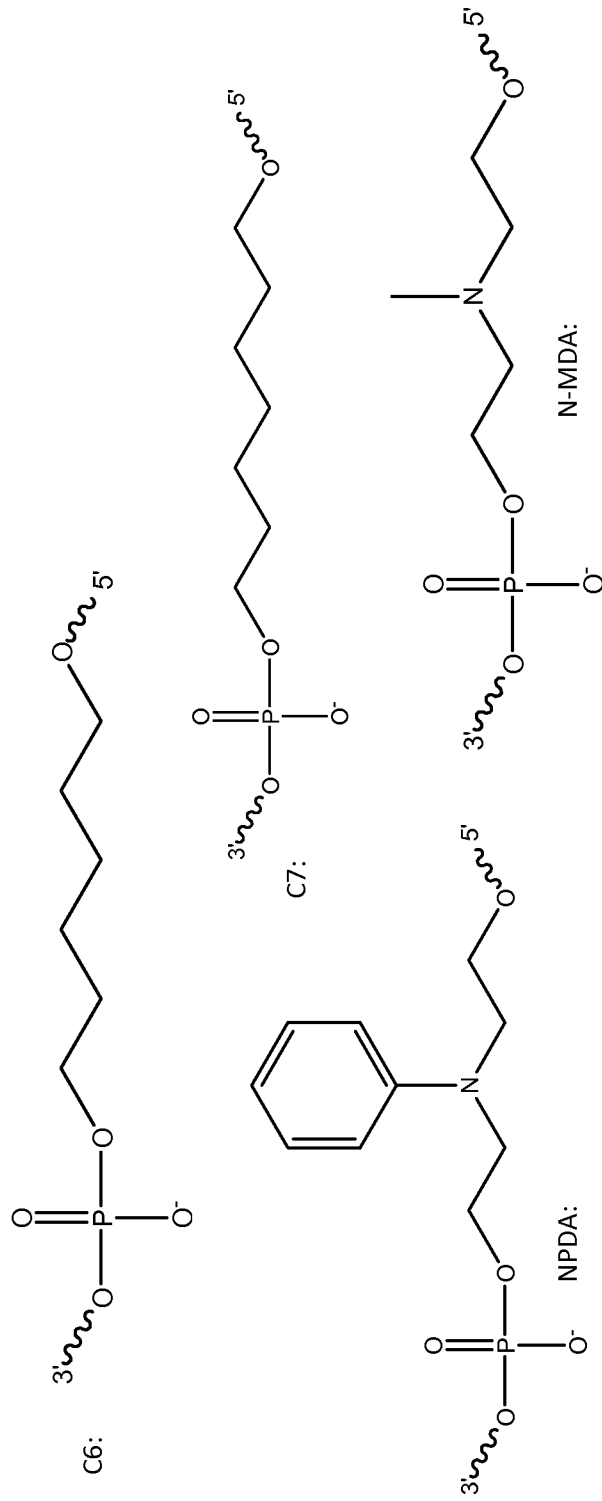
FIG. 1A (cont'd)

IB 1.1:

FQ:

FIG. 1B

IB RQ-n2:

IB RQ-n1:
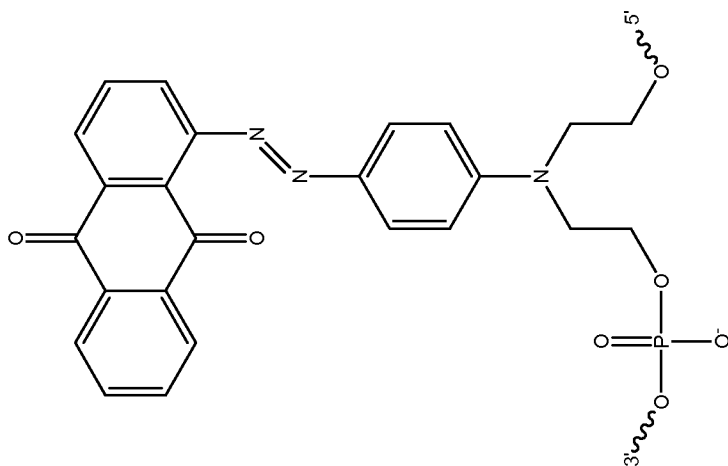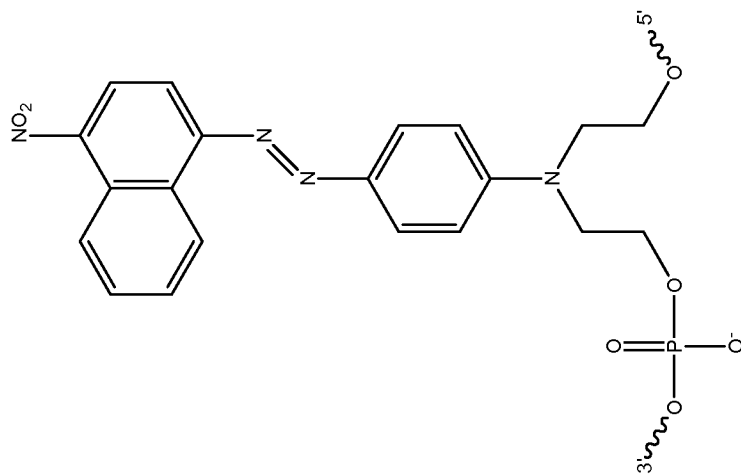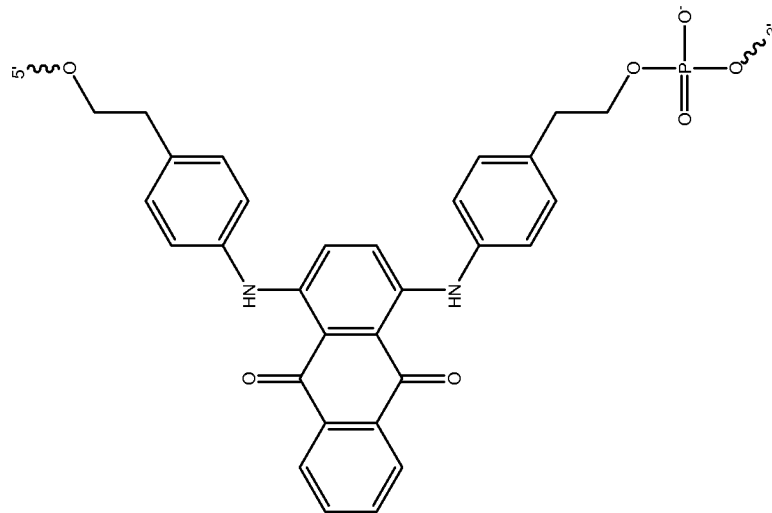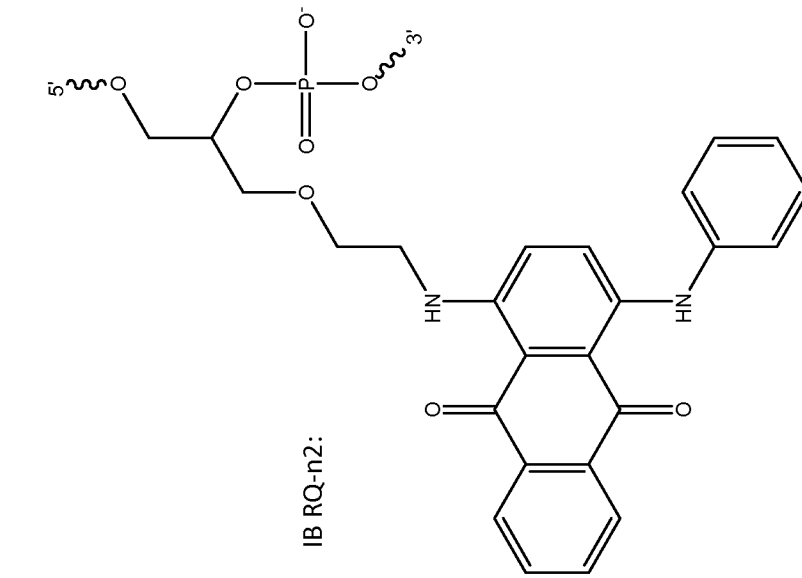
FIG. 1B (cont'd)

Epoch Eclipse:
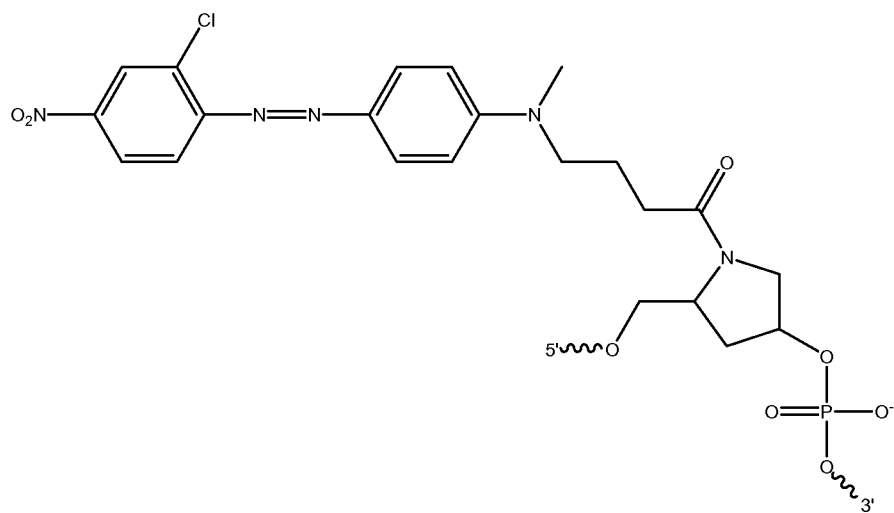
Black Hole 2:
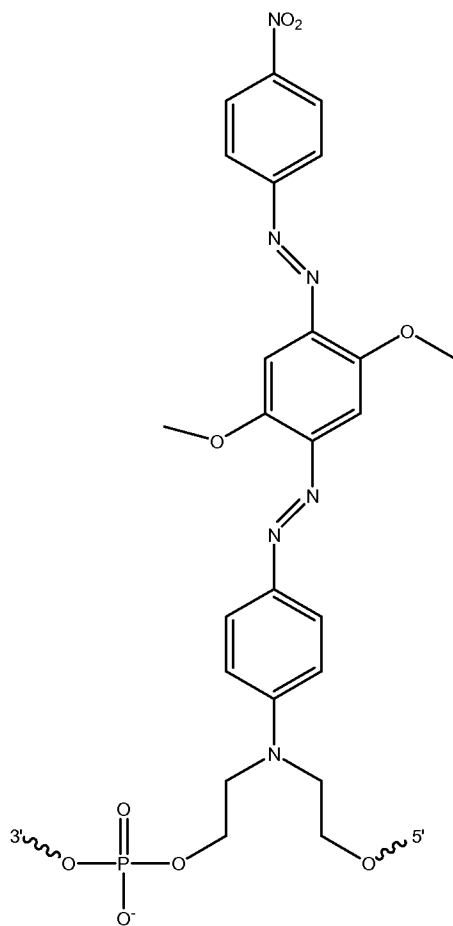
FIG. 1C
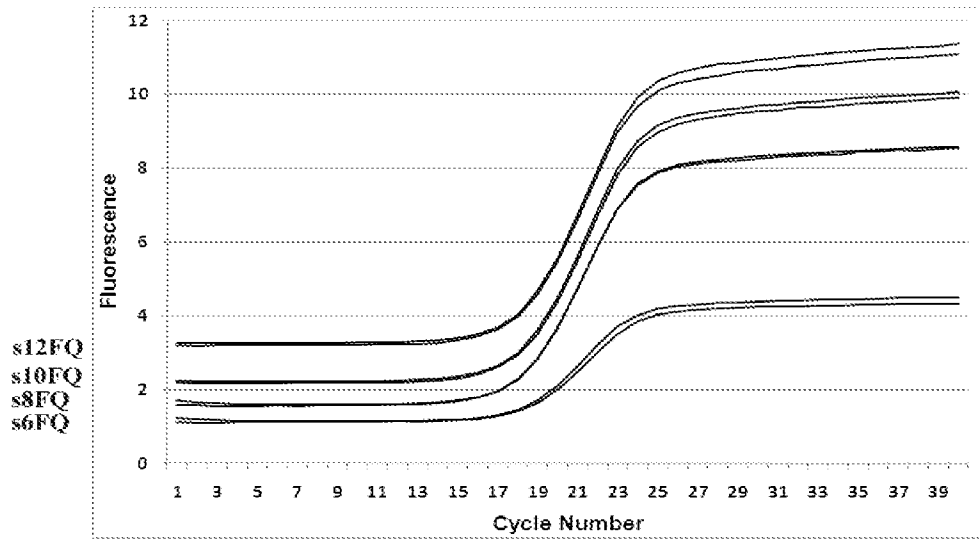
FIG. 2A
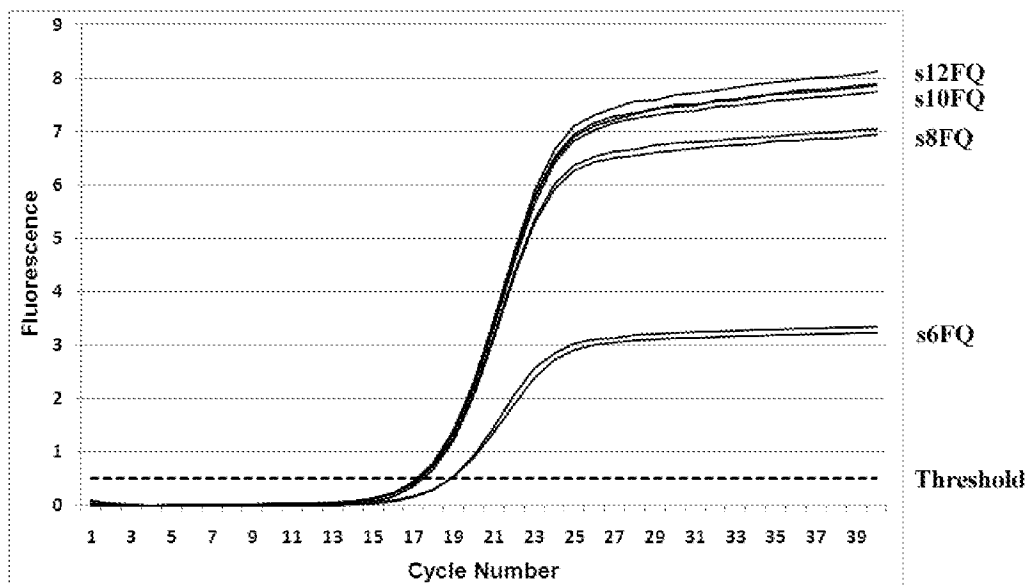
FIG. 2B
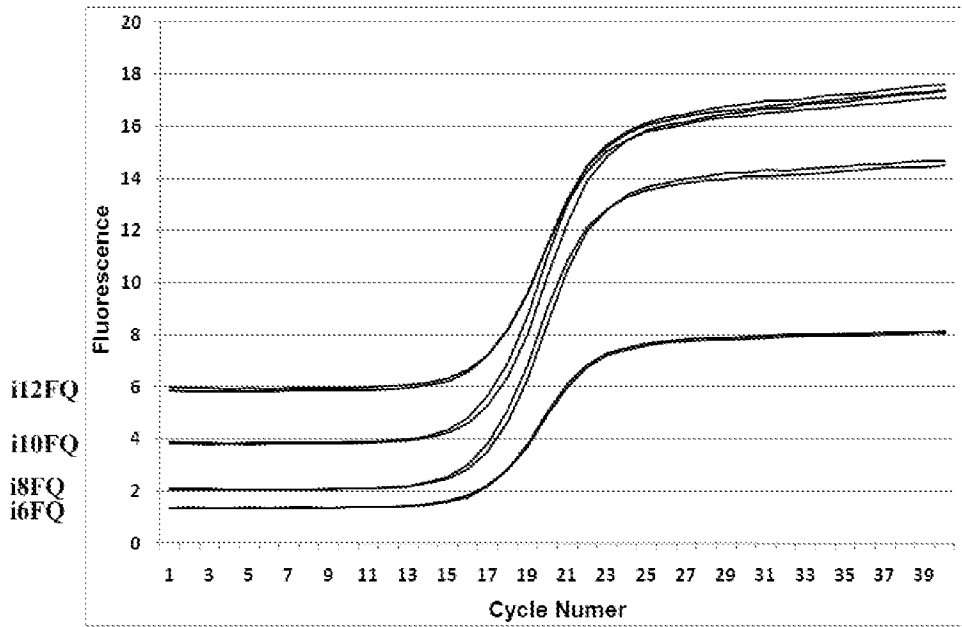
FIG. 3A
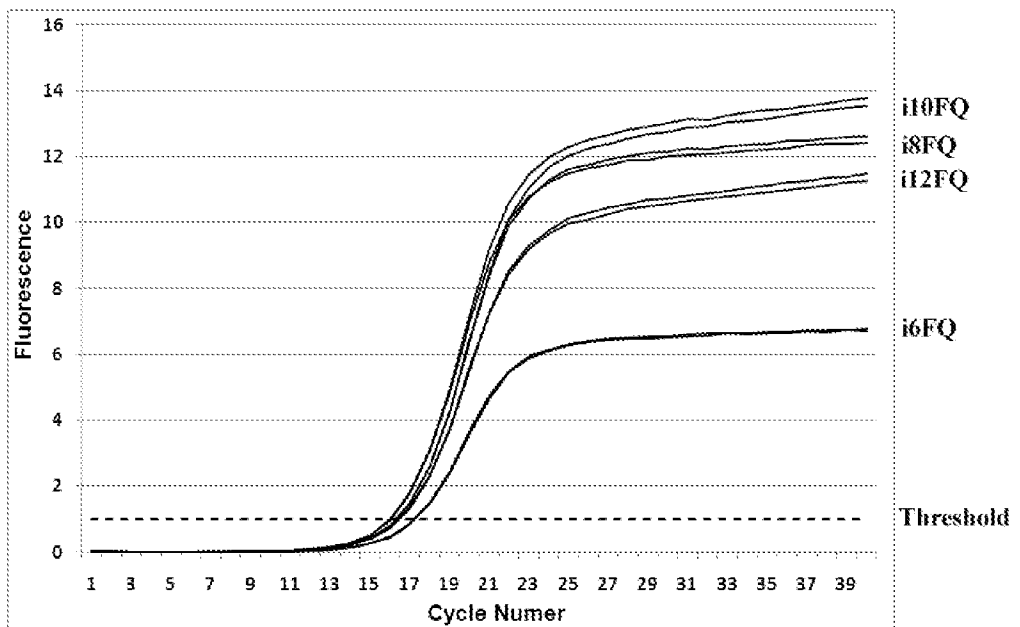
FIG. 3B
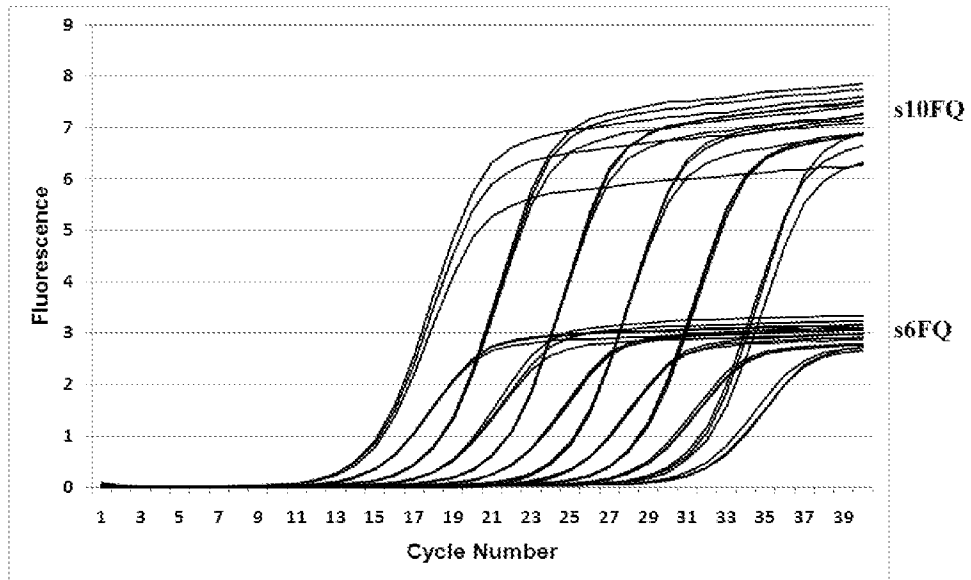
FIG. 4A
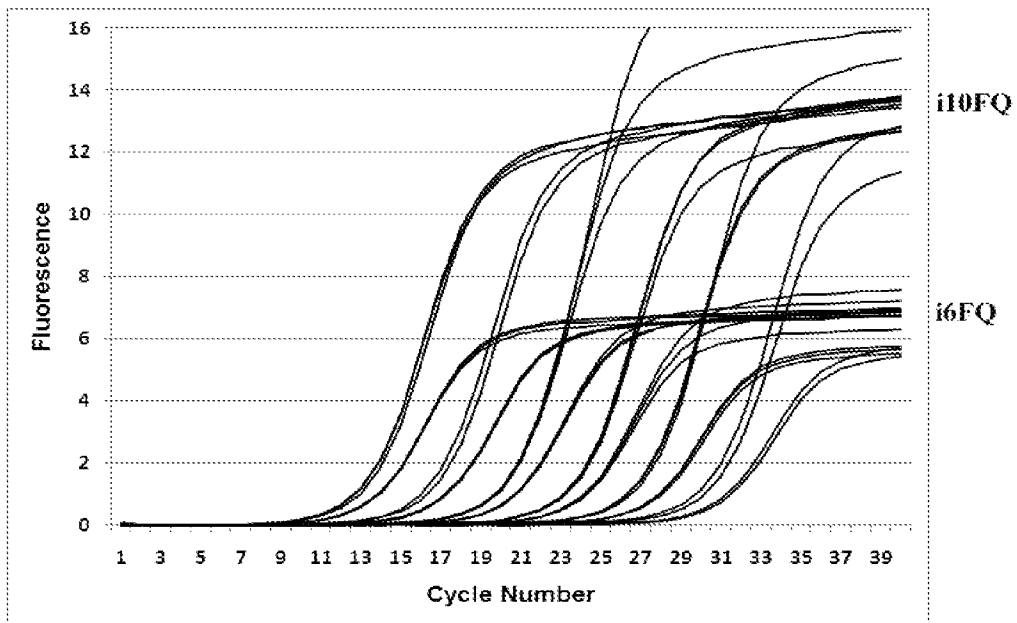
FIG. 4B
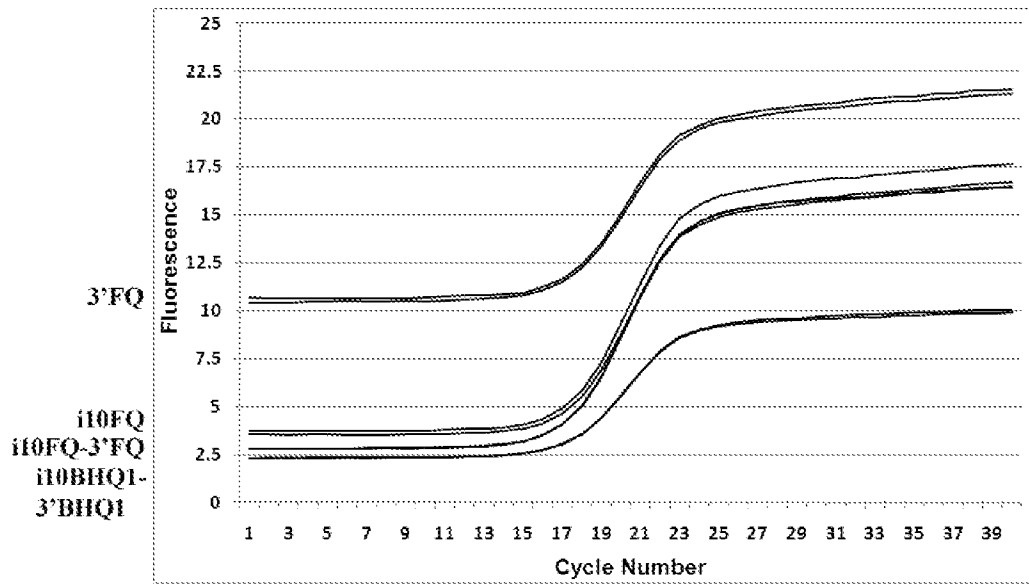
FIG. 5A
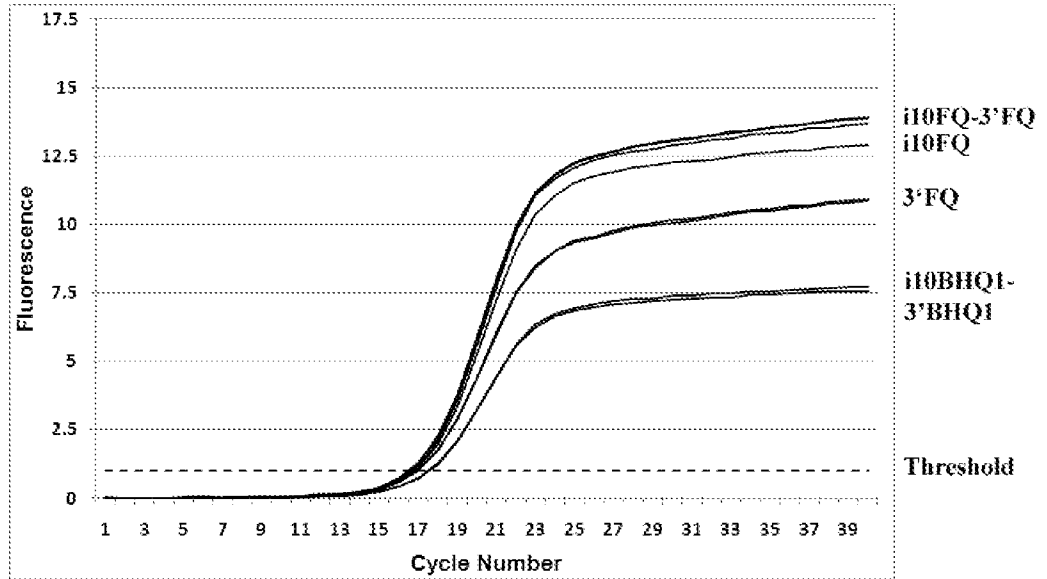
FIG. 5B
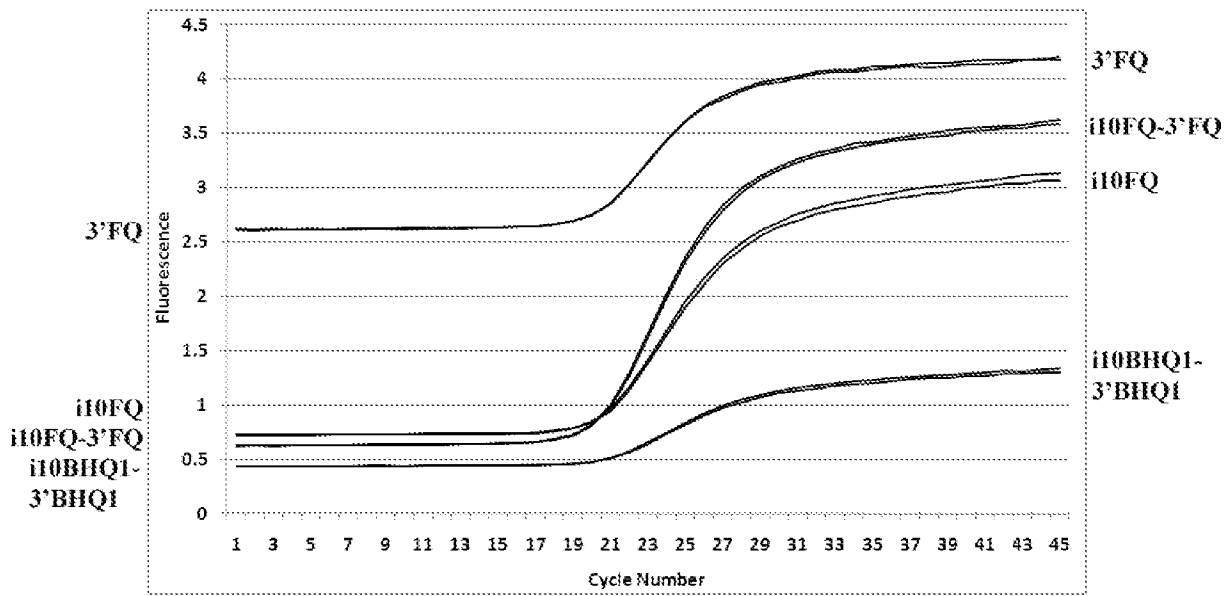
FIG. 6A
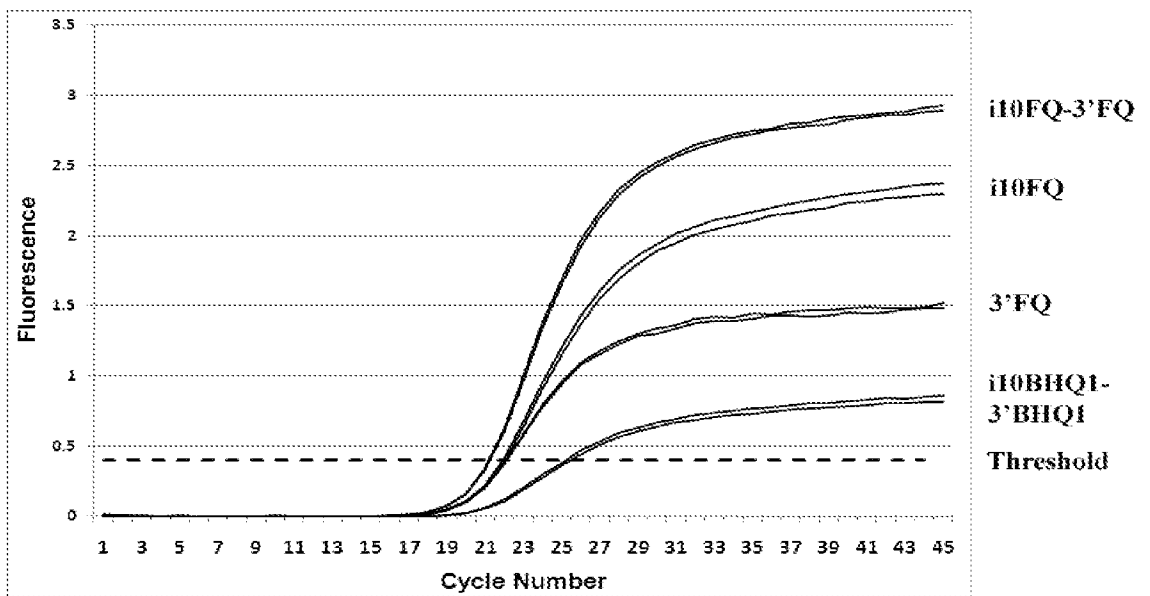
FIG. 6B
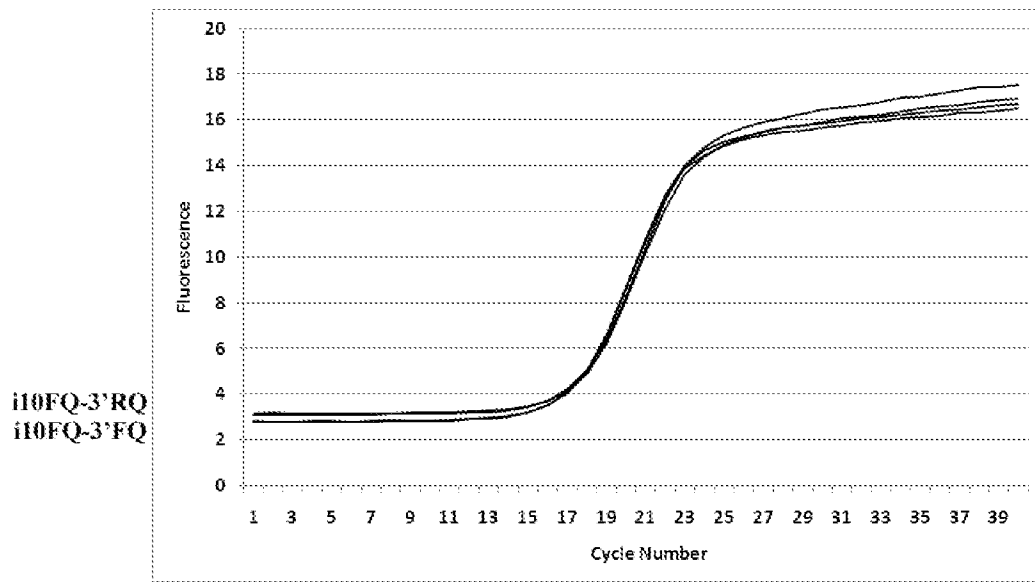
FIG. 7A
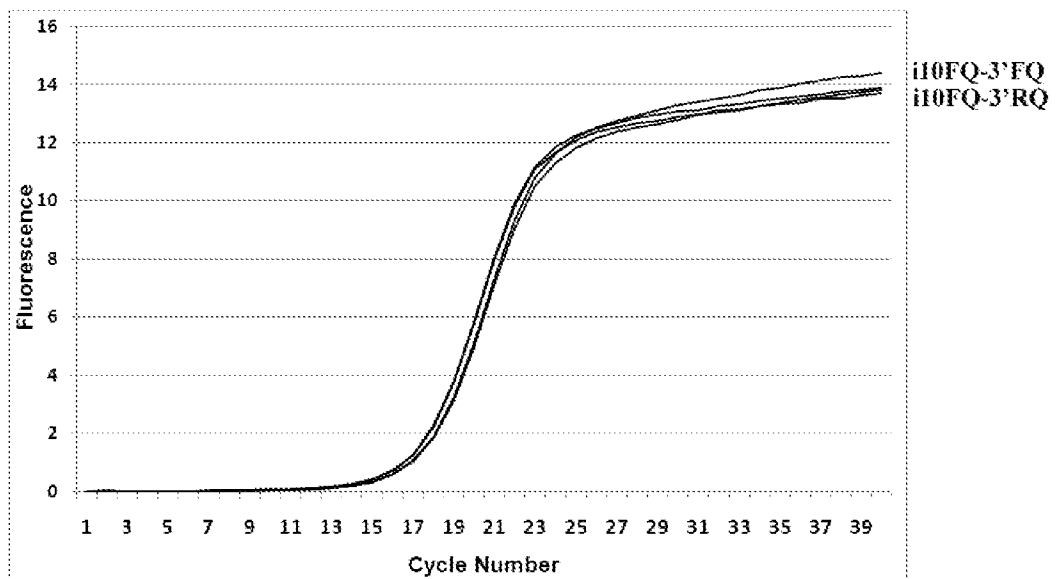
FIG. 7B
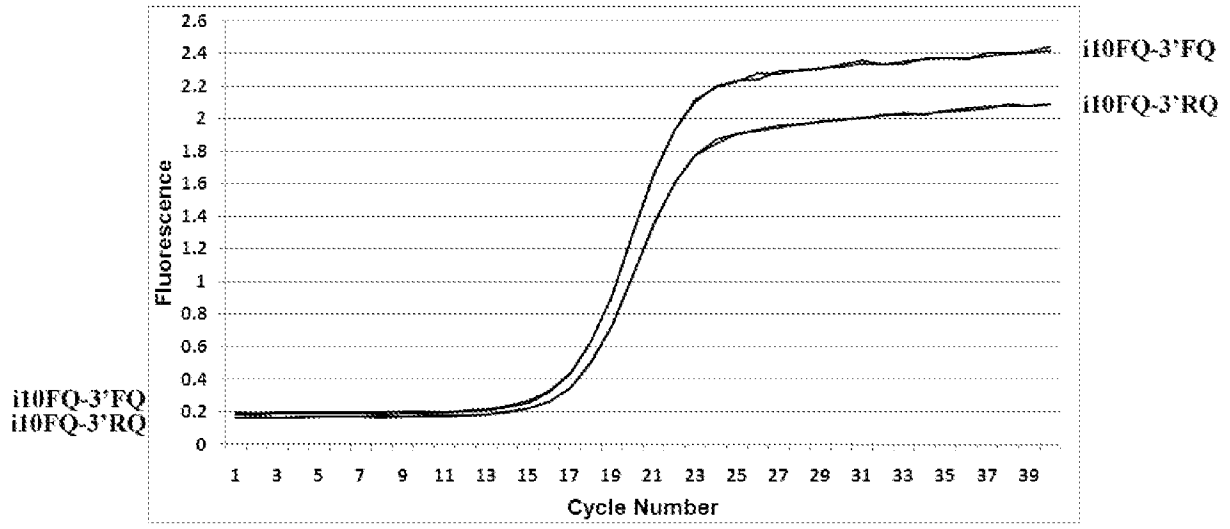
FIG. 8A
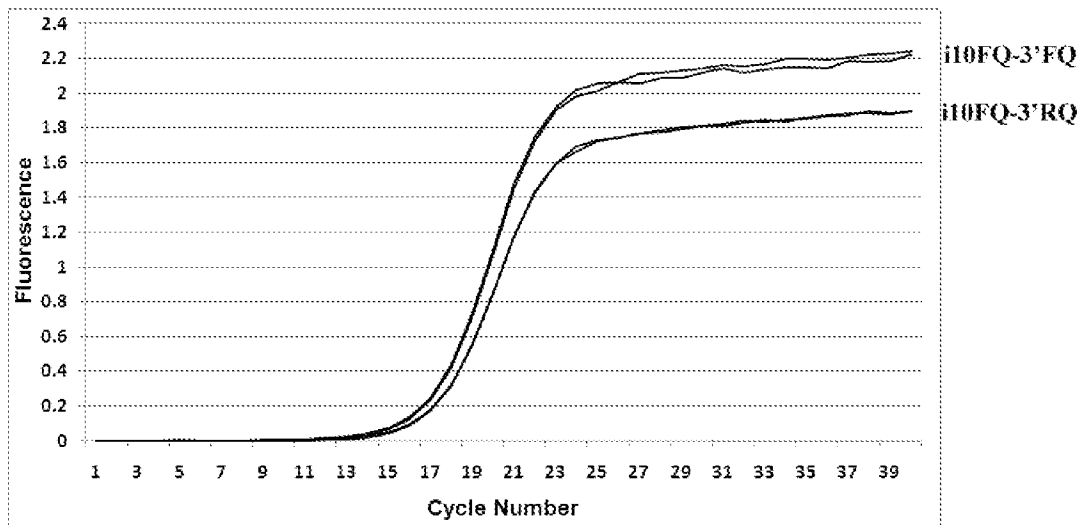
FIG. 8B
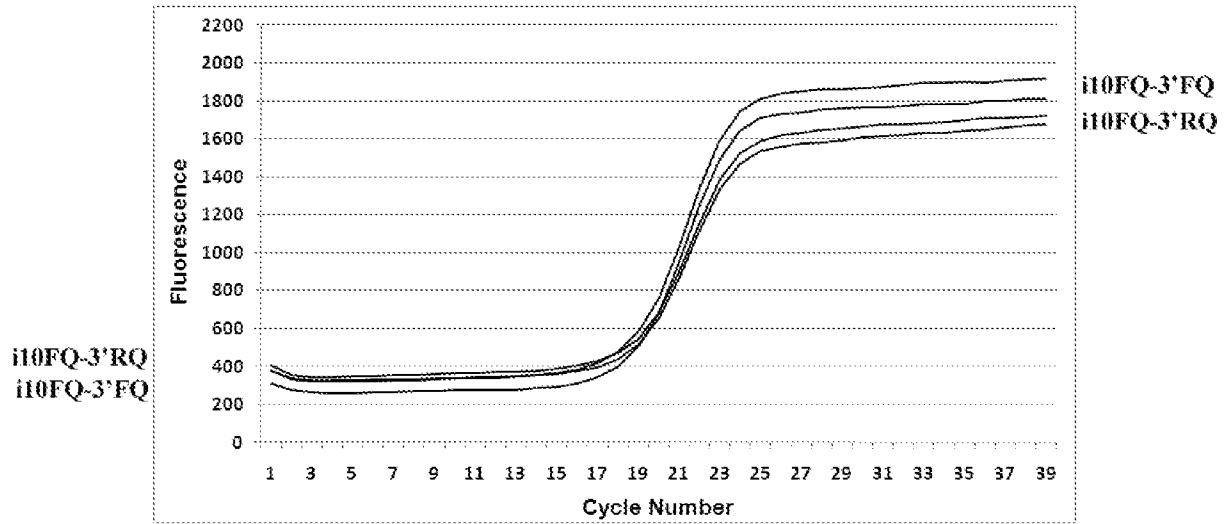
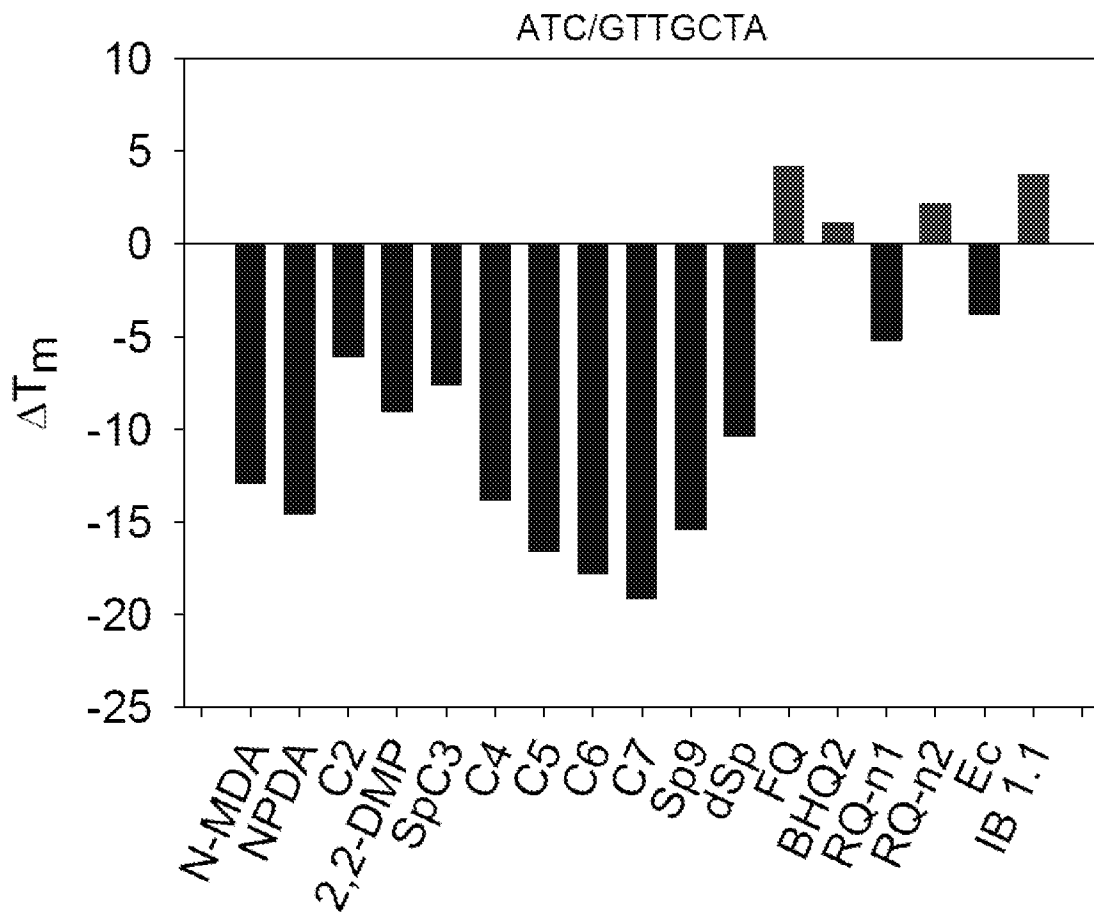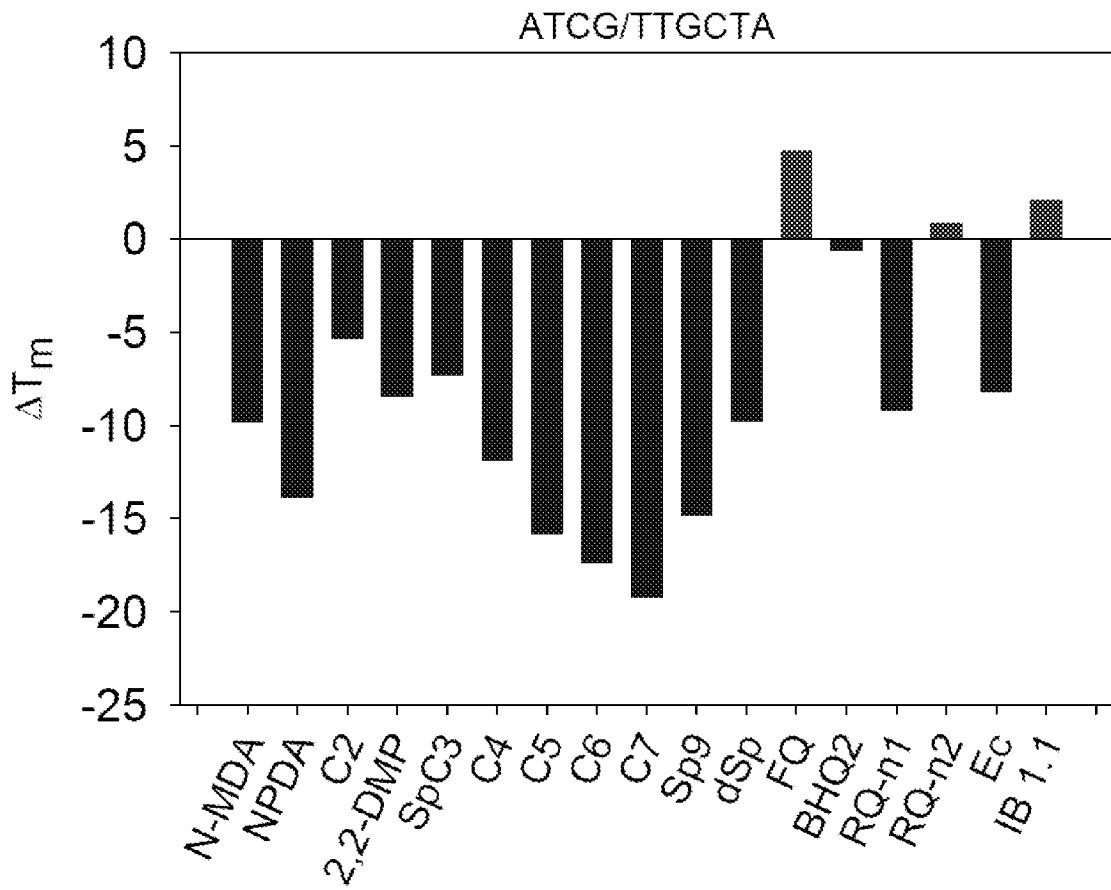**FIG. 9A**
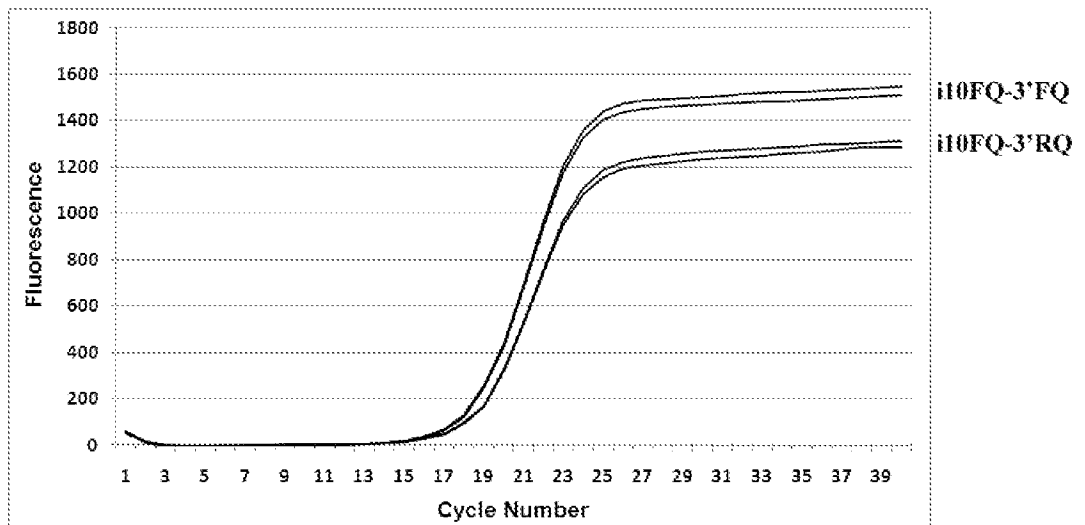
FIG. 9B
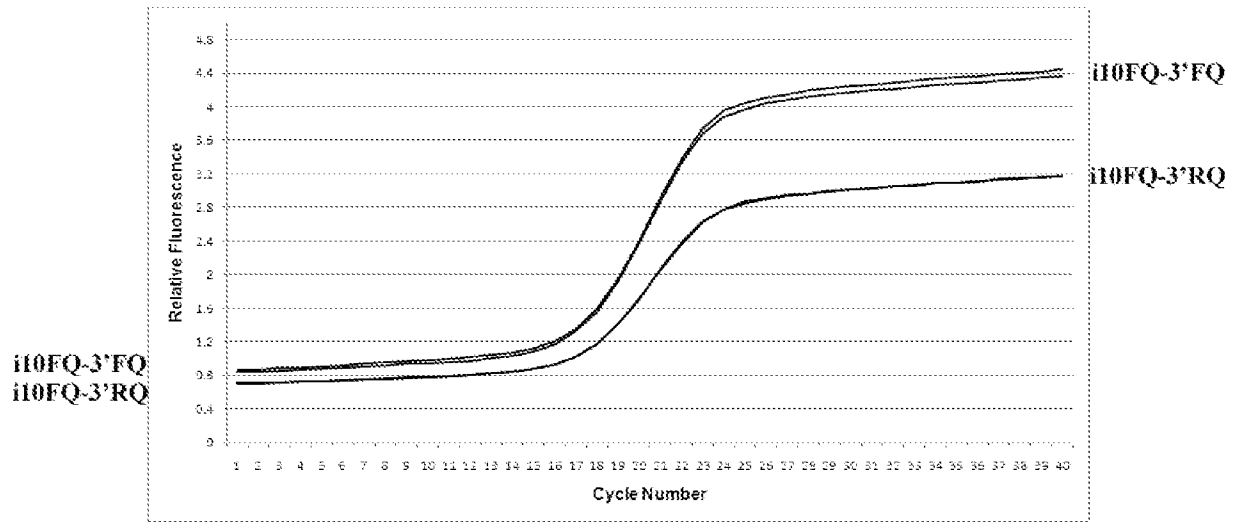
FIG. 10A
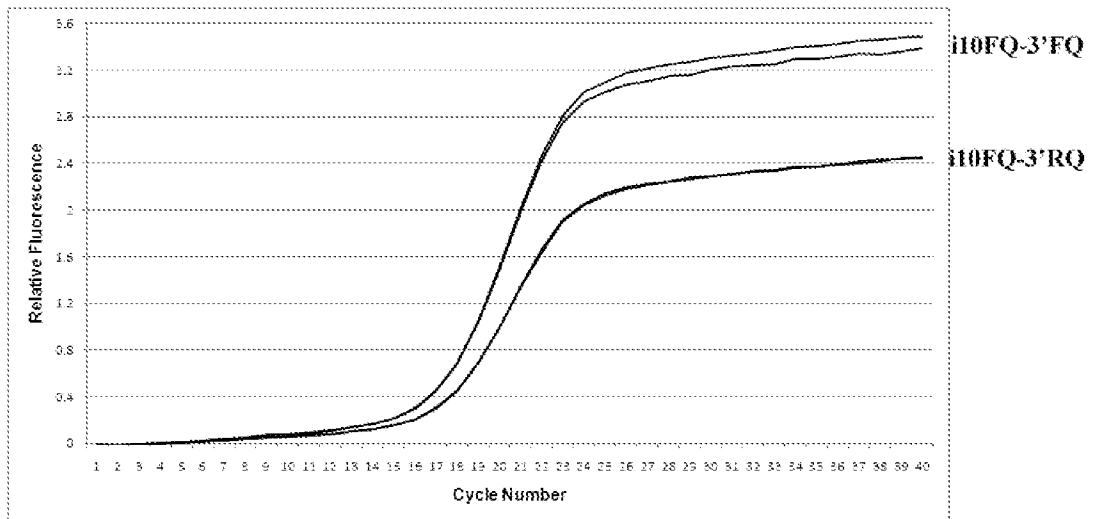
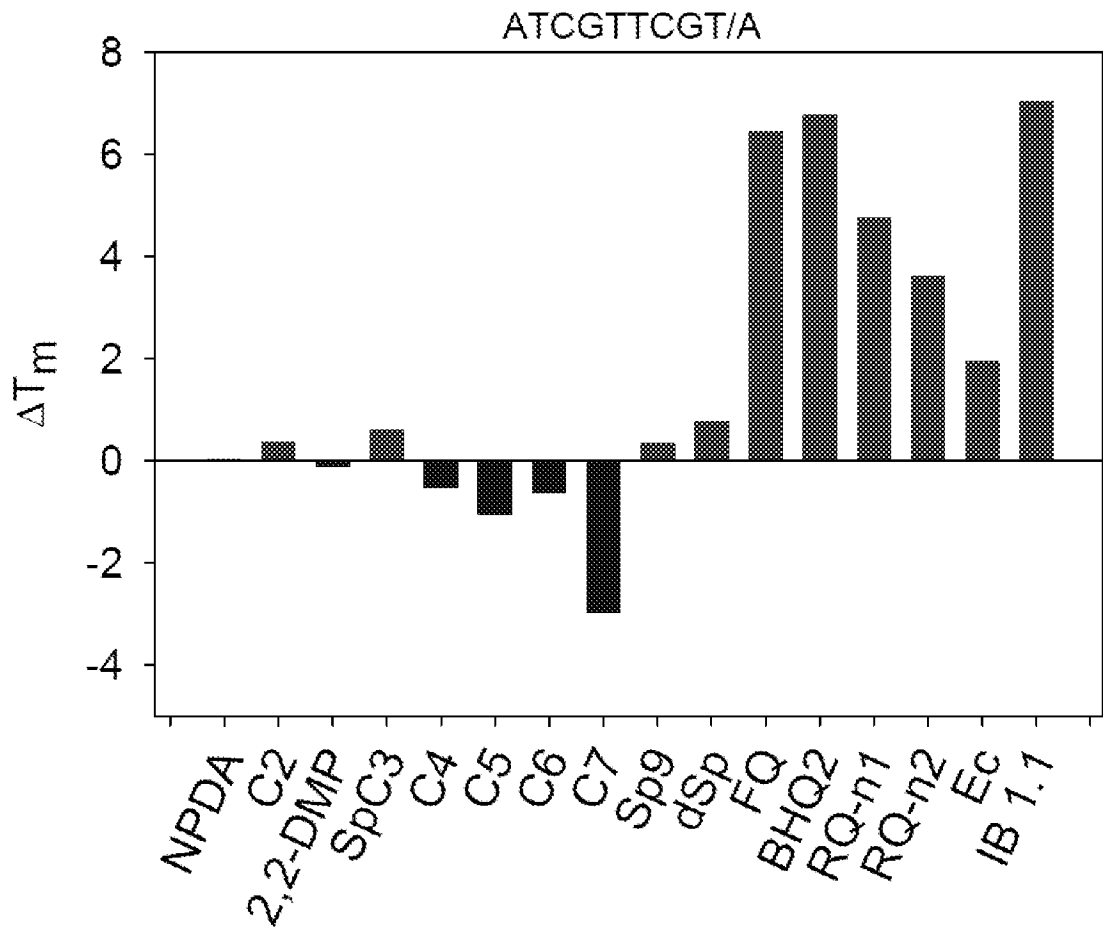
FIG. 10B
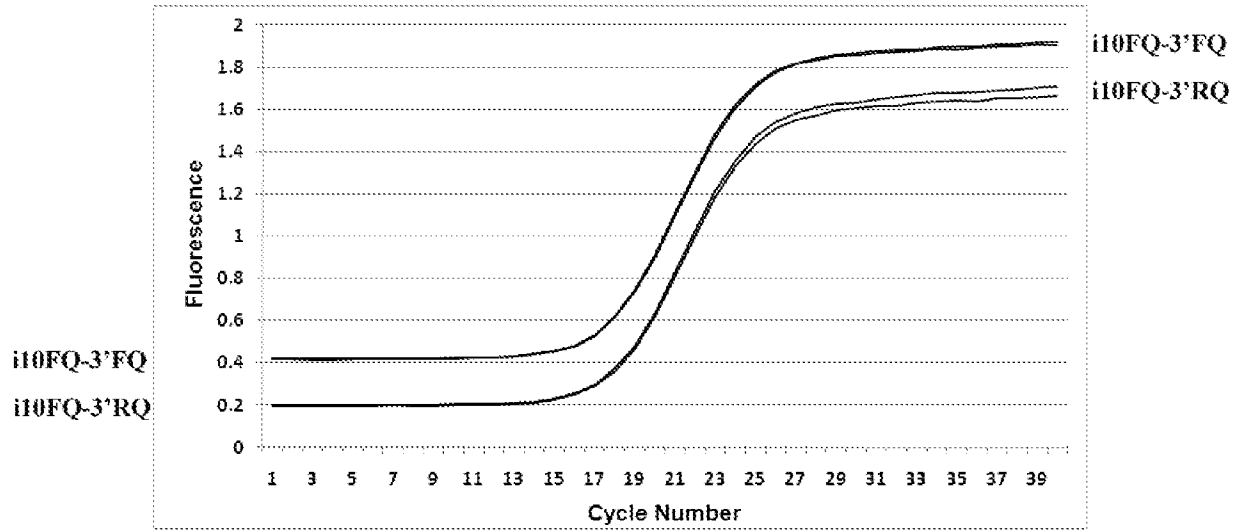
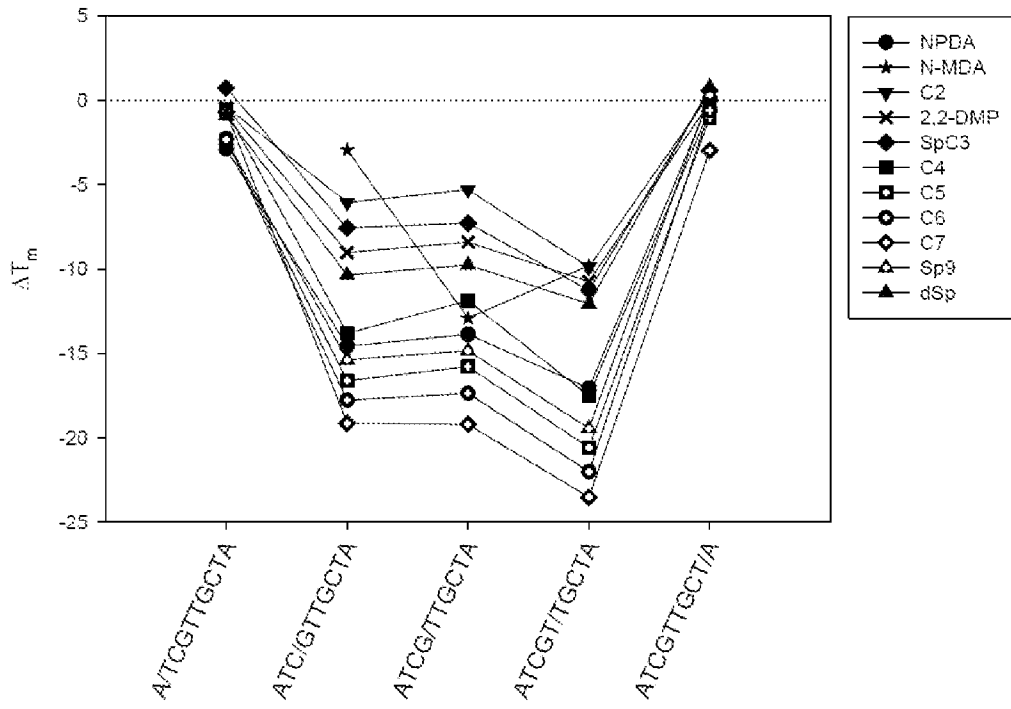**FIG. 11A**
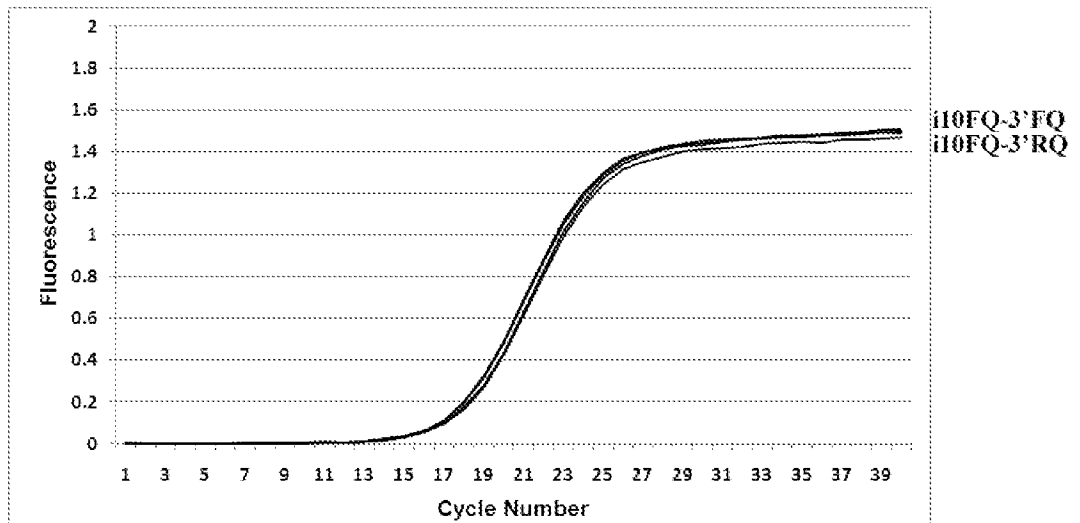
FIG. 11B
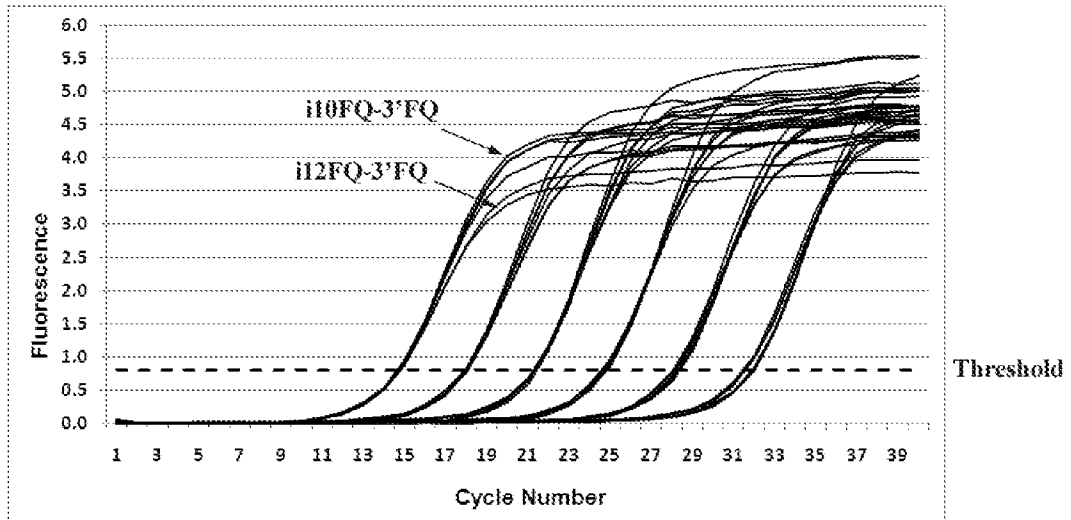
FIG. 12
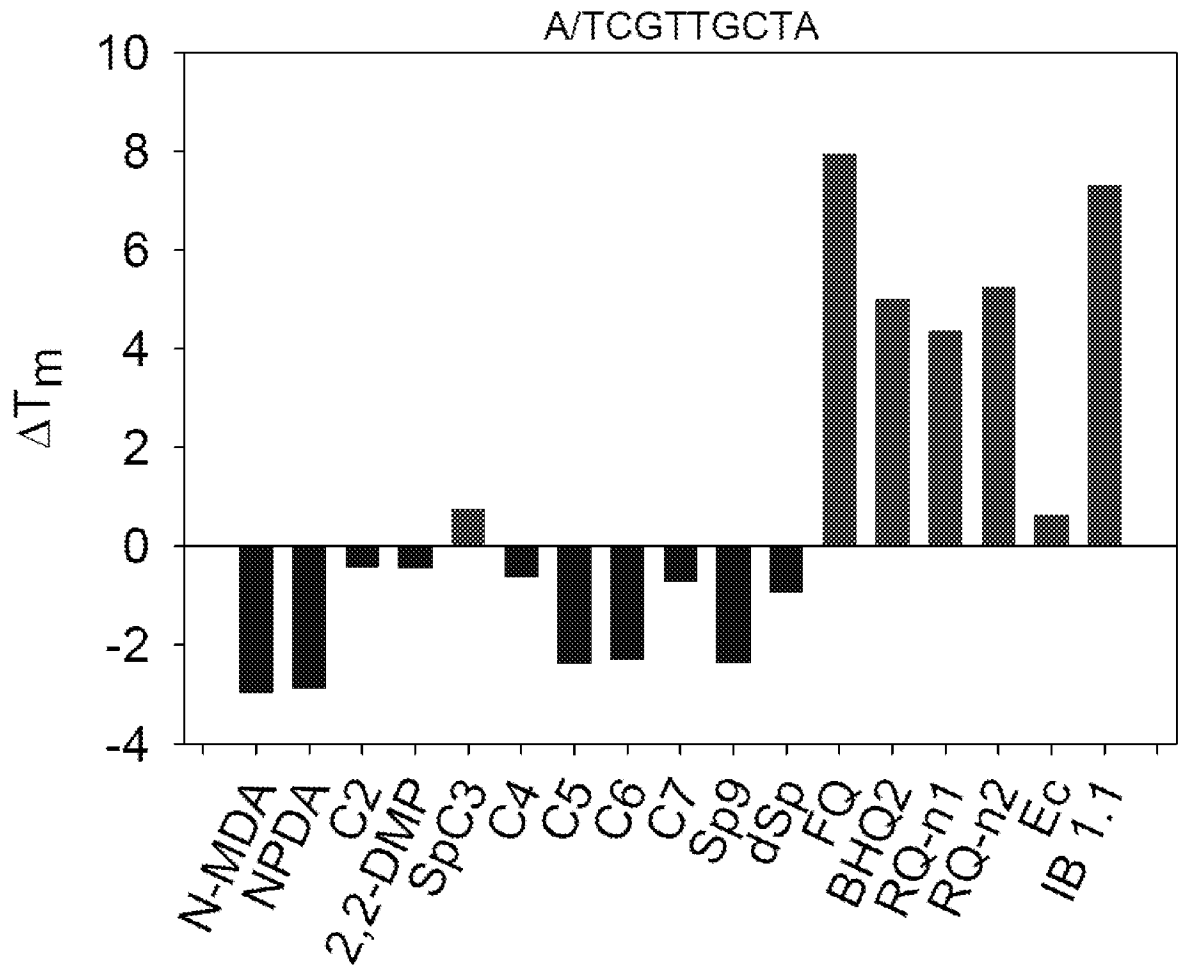
FIG. 13A

FIG. 13B

FIG. 13C
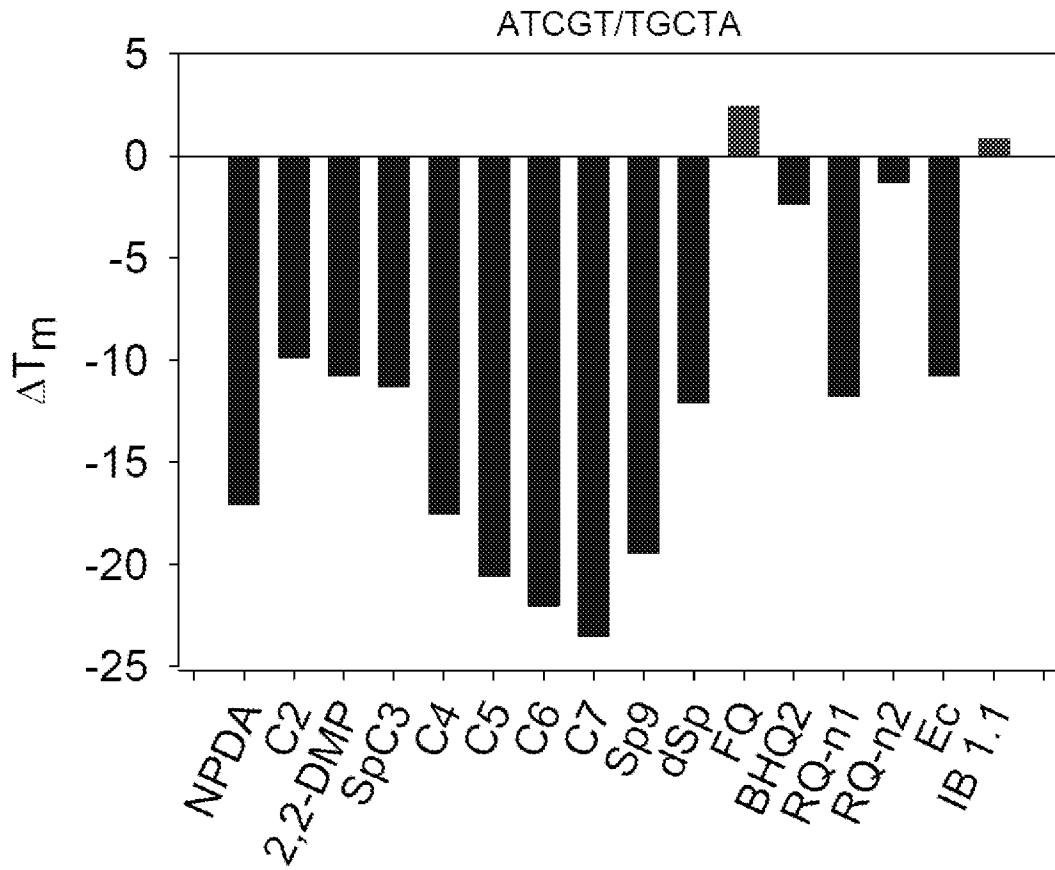
FIG. 13D

FIG. 13E

FIG. 14A
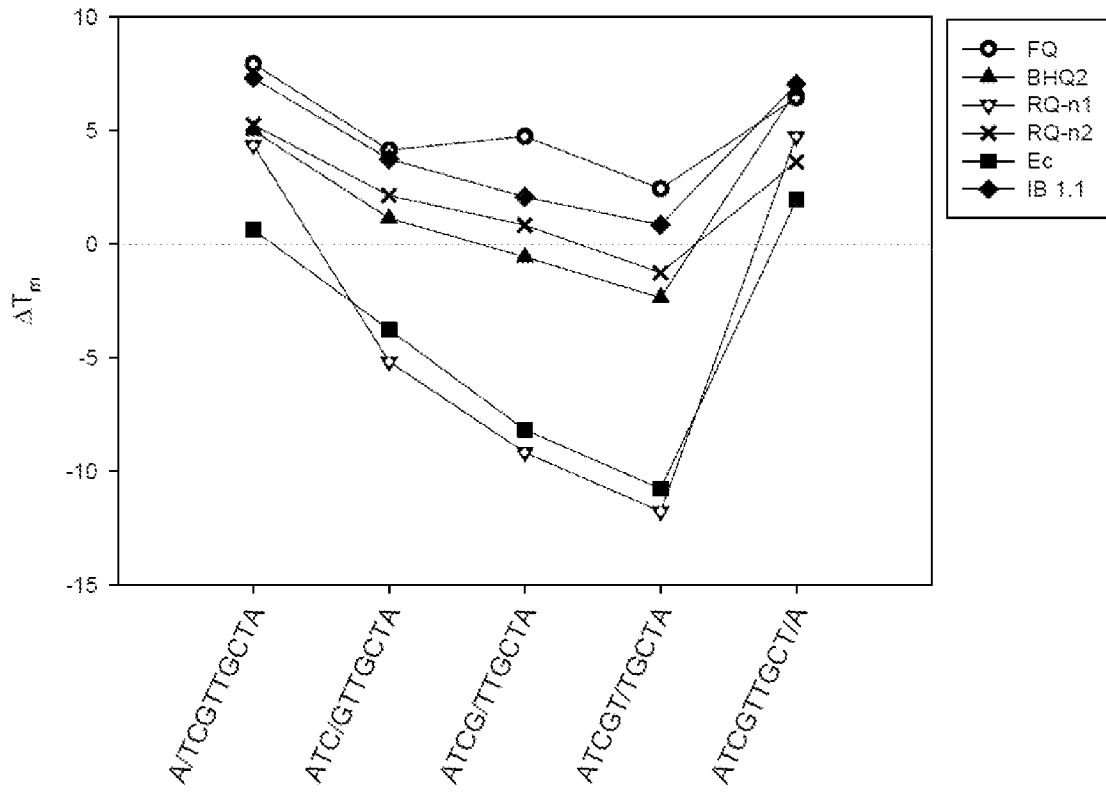
FIG. 14B

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68; A61K 31/11 (2011.01) USPC - 435/6; 514/700; 514/705 According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols) USPC: 435/6; 514/700; 514/705
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched USPC: 514/732 (keywords below)
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) PubWEST (USPT, PGPB, EPAB, JPAB), Google Patents/Scholar: oligonucleotide probe, internal linker, fluorescent, quencher

C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2005/0142598 A1 (Heindl et al.) 30 June 2005 (30.06.2005) para [0021]-[0022], [0050], [0069], [0092], [0097]-[0098], SEQ ID NO:6	1-4, 12-15, 24-27, 35-38, 44-47, 56-59
Y	US 2008/0085837 A1 (Coull et al.) 10 April 2008 (10.04.2008) para [0029]-[0030], [0089]	1-4, 12-15, 24-27, 35-38, 44-47, 56-59
Y	US 2004/0110308 A1 (Laikhter et al.) 10 June 2004 (10.06.2004) para [0018]-[0020], [0023]	12-15, 35-38, 56-59

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 May 2011 (10.05.2011)	2 3 MAY 2011

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450	Lee W. Young
Facsimile No. 571-273-3201	PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 11/30215

| Box No. II | Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet) |

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-11, 16-23, 28-34, 39-43, 48-55, 60-89
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

| Box No. III | Observations where unity of invention is lacking (Continuation of item 3 of first sheet) |

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2009)