

Recent Patents on Self-Quenching DNA Probes

Jens-Peter Knemeyer^{*1} and Nicole Marmé²

¹Department of Functional Genome Analysis, German Cancer Research Center, Im Neuenheimer Feld 580, 69120 Heidelberg, Germany

²Department of Physical Chemistry, University of Heidelberg, Im Neuenheimer Feld 229, 69120 Heidelberg, Germany

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Abstract: In this review, we report on patents concerning self-quenching DNA probes for assaying DNA during or after amplification as well as for direct assaying DNA or RNA, for example in living cells. Usually the probes consist of fluorescently labeled oligonucleotides whose fluorescence is quenched in the absence of the matching target DNA. Thereby the fluorescence quenching is based on fluorescence resonance energy transfer (FRET), photoinduced electron transfer (PET), or electronically interactions between dye and quencher. However, upon hybridization to the target or after the degradation during a PCR, the fluorescence of the dye is restored. Although the presented probes were originally developed for use in homogeneous assay formats, most of them are also appropriate to improve surface-based assay methods. In particular we describe patents for self-quenching primers, self-quenching probes for TaqMan assays, probes based on G-quartets, Molecular Beacons, Smart Probes, and Pleiades Probes.

Key words: Self-quenching probes, TaqMan assays, Molecular Beacons, Smart Probes, Pleiades Probes, FRET, PET, day aggregation.

INTRODUCTION

DNA detection methods are becoming increasingly important in many fields such as criminology, environmental studies, biochemical research, and especially health care.

Generally, DNA assay methods can be divided into heterogeneous and homogeneous assay formats. Conventional, heterogeneous hybridization assays, typically comprise the following steps: (i) immobilization of at least the target nucleic acid on paper, beads, or surfaces, with or without using capture probes, (ii) addition of an excess of labeled probes that are complementary to the sequence of the target, (iii) hybridization, (iv) removal of unhybridized probes, and (v) detection of the probes remaining bound to the immobilized target. Meanwhile, the target itself is often fluorescently labeled and thus adding of an additional labeled probe is unnecessary. However, unhybridized probes or fluorescent targets have to be removed by extensive washing of the hybrids, which is generally the most time-consuming part of the procedure. Furthermore, often complex formats like sandwich hybridization are applied. The use of solid surfaces lengthens the time it takes for hybridization due to restricting the mobility of or access to the target. The large area presented by the solid surfaces nonspecifically retains unhybridized probes, leading to background signal. One major advantage of surface-based assays is that several different sequences can be identified simultaneously. In modern chip-technology, 100.000 sequences can be easily detected on a 1 cm²-chip. However, the requirement that the probe-target hybrids must be isolated precludes *in vivo* detection and concurrent detection of nucleic acids during synthesis reactions like polymerase chain reaction (PCR). Especially since the development of PCR, fast and simple DNA assays are needed, for example in medical diagnostics. In this regard, homogeneous assays play an important role. By "homogeneous" we mean assays that are performed in solution and do not need any separation steps in order to remove unhybridized probe molecules. This specific detection of the probe-target hybrid beside unhybridized probes is often achieved by applying fluorescent labels with fluorescence properties that are affected by their molecular environment. For example, one of the first assays about monitoring a PCR by

detection of specific DNA sequences without opening the reaction tube was described by Higuchi *et al.* in 1992 [1,2]. They added ethidium bromide (EtBr) to the PCR. Since the fluorescence of EtBr increases in the presence of double-stranded DNA, a fluorescence intensity increase indicates a positive amplification. The fluorescence can be easily measured externally and the amplification can be continuously monitored in order to follow its progress. Usually, more specific probes consist of oligonucleotides and preferably fluorescence labels are applied.

Already in the 1980s Heller *et al.* [3] and Cardullo *et al.* [4] described assays that apply a pair of oligonucleotide probes labeled with different fluorescence dyes which are able to form a FRET pair. Both probes are complementary to adjacent regions on the target DNA. After hybridizing to the target, both chromophores are in close proximity to each other and thus the dye absorbing light at shorter wavelengths (donor) can transfer its energy to the second dye (acceptor) *via* a fluorescence resonance energy transfer (FRET). Consequently, the acceptor fluoresces and a bathochrome spectral shift occurs so that the fluorescence light can be distinguished from light arising from unhybridized probes. However, the sensitivity of these assays is often limited by relatively high background signals. Another disadvantage is that two probes must bind to one target sequence. Since the kinetics of a tri-molecular hybridization is relatively slow, this technique is limited for real-time detection.

First DNA assays using self-quenching probes were developed at the end of the 1980's. The forerunner of today's self-quenching DNA probes was developed by Morrison *et al.* [5, 6]. Their probes consist of two doubly labeled oligonucleotides that are complementary to one another. Each oligonucleotide includes a fluorophore attached to its 3'-end and a quenching moiety attached to its 5'-end, or the other way around. When the two oligonucleotide probes are annealed to each other, the fluorophore of each probe is held in close proximity to the quenching moiety of the other probe. If the fluorescence label is then excited, the fluorescence is quenched by the quenching moiety. However, when either probe is bound to a target, the quenching effect of the complementary probe is absent. Because the probes are sufficiently long, they do not self-quench when target-bound.

Meanwhile, many different self-quenching DNA probes and assay methods have been developed. Thereby, the underlying quenching mechanisms can be divided in three main fields - fluorescence resonance energy transfer (FRET), photoinduced electron

*Address correspondence to this author at the Department of Functional Genome Analysis, German Cancer Research Center, Im Neuenheimer Feld 580, 69120 Heidelberg, Germany; Tel: (+49) 6221-54-4648; Fax: (+49) 6221-42-4682; E-mail: knemeyer@single-molecule-spectroscopy.de

transfer (PET), and electronically interaction of fluorophores (aggregation).

Fluorescence quenching via FRET. The phenomenon of fluorescence resonance energy transfer (FRET) was already observed by Perrin at the beginning of the century. Förster proposed his theory in 1948 and described FRET by long-range molecule interaction. He derived an equation that relates the energy transfer rate to the spectroscopic properties, for example the overlap between the emission spectrum of the donor and absorption spectrum of the acceptor chromophore and to the distance as well as orientation between the chromophores. The distance influences the transfer rate on the sixth power and is therefore a very sensitive parameter. Later, Stryer *et al.* verified this theory experimentally [7, 8]. Depending on the chosen donor-acceptor pair, FRET typically occurs at distances between 10 Å and 100 Å. The mechanism of FRET and many applications are described in several review articles [9, 10].

The energy transfer from an excited donor dye to an acceptor leads to a reduction of the fluorescence intensity and lifetime of the donor dye. If a chromophore with a high quantum yield is used as acceptor, the fluorescence of the acceptor appears whenever energy is transferred. Compared to the donor fluorescence, the acceptor fluorescence is shifted to longer wavelength and thus both signals are usually distinguishable. Especially for the probes described in this review, the acceptor chromophores do not have high quantum yields but the energy that is received from the donor dye is released by radiationless processes. Consequently, a strong FRET results in an efficient quenching of the donor dye and no other fluorescence appears.

Due to electronically interactions between the chromophores, the Förster theory is not valid for distances below approximately 10 Å (depending on the donor-acceptor pair). Such electronically interactions can also cause strong fluorescence quenching (e.g. formation of dimers) and can be observed by changes in the absorption spectra.

Fluorescence quenching via photoinduced electron transfer (PET). Photoinduced electron transfer reactions can occur from a strong electron donor to a fluorescent dye while it is in an excited state. In contrast to FRET, PET requires very close proximity or even van der Waals contact between dye and quencher for fast and efficient conversion of electronic excited-state energy. Generally, quenching of fluorophores in the first excited singlet state by electron donors leads to the formation of a radical ion pair $A^{\bullet}D^{+}$, which returns to the ground state *via* radiationless charge recombination. The efficiency of charge separation is controlled by the free energy of the reaction, the reorganization energy, and the distance between donor and acceptor [11]. Weller's equation [12] can be used to estimate the free energy change for the charge separation (ΔG_{cs}), which has to be negative for efficient quenching *via* PET.

$$\Delta G_{cs} = E_{ox} - E_{red} - E_{0,0} + C$$

E_{ox} and E_{red} are the first one-electron oxidation of the donor and the first one-electron reduction potential of the acceptor in the solvent under consideration. $E_{0,0}$ is the energy of the zero-zero transition to the lowest excited singlet state of the electron acceptor (fluorophore), and C is the solvent-dependent Coulombic attraction energy, which is small in polar environments and can be usually neglected if aqueous solvents are used.

In contrast to other nucleotides, guanosine is known as a strong intrinsic donor with an oxidation potential of 1,25 V *vs* SCE [13]. The free energy change for the photoinduced charge separation from the ground state guanosine to the excited oxazine dye MR121 can be estimated to be approximately -0.2 eV applying the reduction potential ($E_{red} = -0.5$ V *vs* SCE) and the transition energy ($E_{0,0} = 1.9$ eV) of MR121 [14]. This oxazine derivative has been used for the first DNA probes based on fluorescence quenching by guanosine [15].

A PET reaction between guanosine and a fluorescent dye has been reported, for example, for coumarin [13], rhodamine [16-18], oxazine [15, 19], and bora-diaza-indacene [20] dyes.

Quenching via dye aggregation. With increasing concentration, many fluorescent dyes form dimers that are classified as so-called J-type ("face-to-edge" conformation) and H-type ("face-to-face" conformation) dimers. Usually, only H-type dimers are observed in aqueous solution, due to their higher stability [21]. Dimerization has been described for a wide variety of fluorescent dyes, e.g. the xanthene [22], phenothiazine [23], oxazine [24], squaraine [25], BODIPY [26, 27], and cyanine [28] dyes. As predicted by excitation theory [29], H-type dimers show a blue-shifted absorption band and the fluorescence intensity is strongly decreased compared to the monomers. One of the most popular dyes forming non-fluorescent dimers is tetramethylrhodamine (TMR) which has already been used in many analytical applications, for example, detection of proteolytic enzyme activity [30]. It shows a dimerization constant of 7300 M⁻¹ and 18000 M⁻¹ for the 5'-isomer and the 6'-isomer, respectively [31]. In addition to homodimers, most of the chromophores are also able to form heterodimers with other dyes or quencher molecules, which also results in an efficient fluorescence quenching. However, in contrast to FRET, both chromophores have to be in direct contact to each other to form non-fluorescent complexes.

In this review different self-quenching probes and DNA assays are presented. Some of them (e.g. TaqMan assay or self-quenching primers) can only be used in combination with DNA amplification methods like PCR and enable "real-time" PCR. Others (e.g. Molecular Beacons, Smart Probes, Pleiades Probes) are developed for a more direct identification of a DNA sequence and can be applied for PCR monitoring as well as direct DNA or RNA detection, for example in living cells. Although all of the presented probes were originally developed for use in homogeneous assay formats, most of them are also appropriate to improve surface-based assay methods.

PATENTS ON SELF-QUENCHING DNA PROBES

Self-quenching primers. Nadeau *et al.* present in their patents (US5846726; US6054279) a novel class of self-quenching signal primers comprising two regions [32, 33]. The working mechanism of this assay is shown in Fig. 1. On the 5'-end, a reporter (fluorophore) and a fluorescence quencher flank a restriction endonuclease recognition site (RERS), whereas the sequence of the 3'-end is complementary to the target DNA and represents the functions of a standard primer. In the single-stranded primer, the RERS sequence corresponds to one strand of the double-stranded RERS. Since the signal primer is initially single-stranded and remains single-stranded in the absence of target, RERS is not cleavable by the restriction endonuclease. In the presence of target, however, signal primer and the RERS are rendered double-stranded due to the PCR. Afterwards, a restriction endonuclease is added to the reaction mixture and the double-stranded RERS is cleaved, resulting in a separation of the two chromophores and an increase in fluorescence intensity. Similar assays using signal primers containing a RERS have been filed for patent before. They apply the separation of a lipophilic dye by extraction into an organic phase [34] and fluorescence polarization detection [35], respectively. Furthermore, Nadeau *et al.* developed a primer containing a hairpin motif (US5928869), which leads to a close proximity between the two chromophores and thus efficient quenching (similar to Molecular Beacons that are described in a separate paragraph) [36]. The working mechanism is quite similar to the linear self-quenching primer and is therefore not described in detail in this manuscript.

Keys *et al.* present a DNA probe comprising two individual dye-labeled oligonucleotides (US6593091). One of them is labeled with a donor dye and the other with an acceptor dye or quencher.

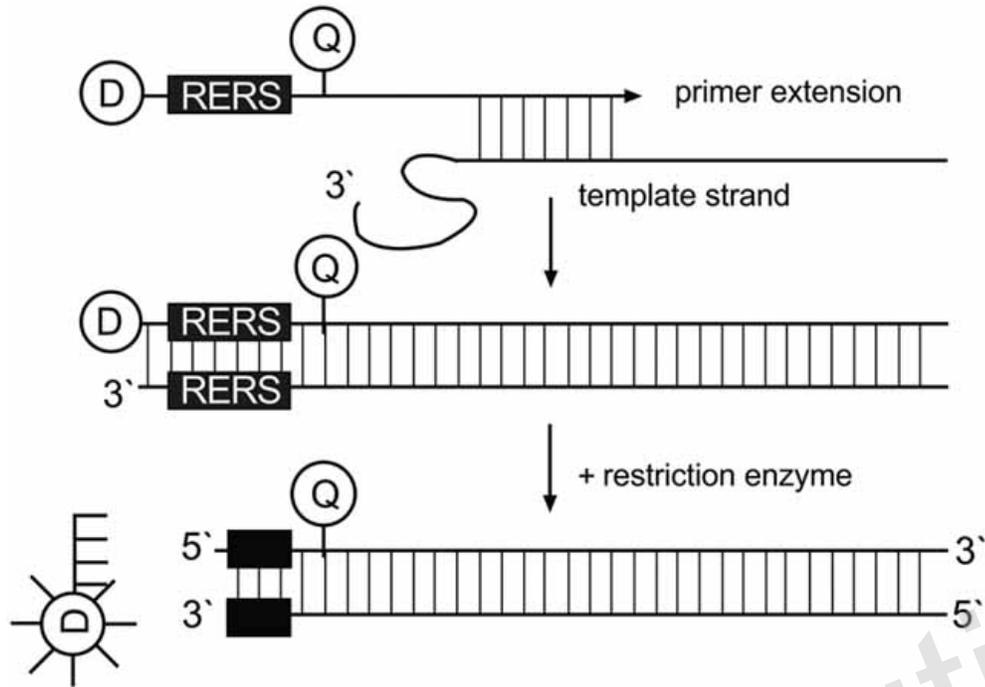


Fig. (1). Self-quenching signal primer reaction scheme for the detection of DNA target after amplification *via* PCR, patented by Nadeau *et al.*

Because both oligonucleotides have regions (e.g. at the 5'-end) with sequences complementary to each other, they can hybridize and thus an efficient FRET occurs, resulting in a reduction in the fluorescence of the donor dye (Fig. 2) [37]. In the presence of the target DNA which is complementary to the full length of the DNA probe, each probe oligonucleotide rather hybridizes to the target than to the other probe strand. Consequently, the interaction between the chromophores is prevented and the fluorescence of the donor dye increases. The probe can be applied as primers of PCR and thus they are appropriate for online monitoring of the amplification. Furthermore, Keys *et al.* describe the use of these probes for the detection of specific DNA sequences in homogeneous solutions as well as on surfaces without amplification

of the target DNA. The main advantage of this probe is the easy and inexpensive synthesis of the probes.

TaqMan assay. One of today's state of the art assays for monitoring PCR reactions is the TaqMan assay. The first generation of these assays did not work with self-quenching or even fluorescently labeled probes but with radioactive ^{32}P markers. This assay developed by Holland *et al.* [38] exploits the 5'-3' exonuclease activity of *Thermus aquaticus* DNA polymerase and was filed for patent in 1990 (US5210015) [39]. The assay is based on adding an oligonucleotide probe to a PCR reaction. The probe that is not extendable at the 3'-end is labeled on the 5'-end. It anneals to the PCR product strand and is degraded by the DNA polymerase during the amplification step (Fig. 3). Afterwards, the shorter fragments of

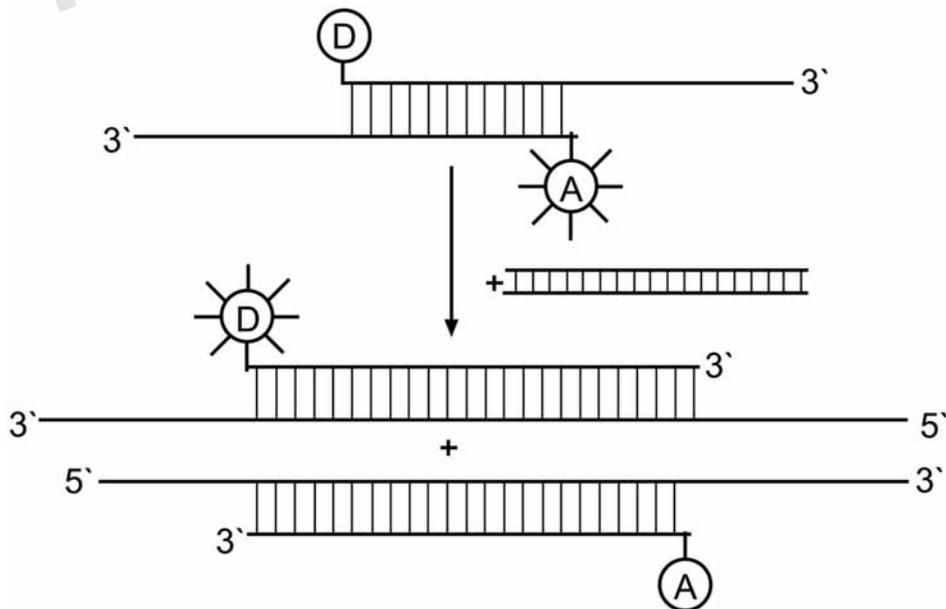


Fig. (2). Working mechanism of the DNA probes described by Keys *et al.* in the patent US6593091 consisting of two individual fluorescently labeled oligonucleotides.

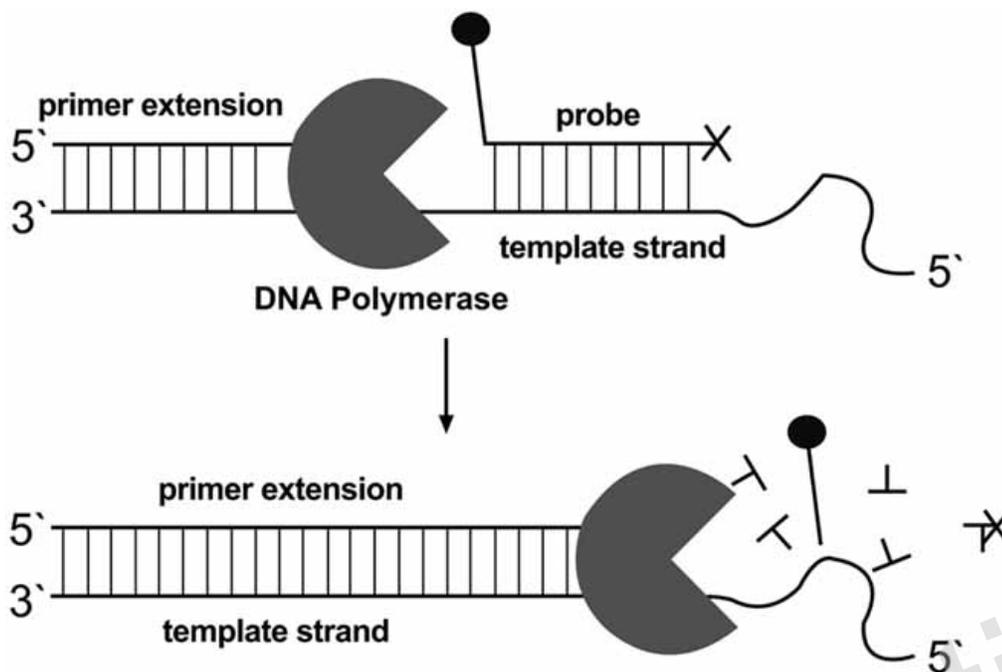


Fig. (3). Reaction scheme of the first TaqMan assay using radioactive-labeled oligonucleotides that are degraded by the DNA polymerase during PCR. X at the 3'-end of the probe is a modification that prevents an extension of the probe by the DNA polymerase.

the radioactive-labeled probe are detected on a gel. At the point of invention this method makes the PCR product detection much faster and easier. However, the assay in this particular form still requires separation steps after finishing the PCR and is not appropriate for "real-time" PCR.

Indeed, in this patent and in its continuation (US5487972) [40] it is already proposed and in a further continuation also shown (so far last continuation: US20067141377) [41] to apply probes that are labeled with a fluorescent dye and a quencher moiety. Both labels are in very close proximity (only a few nucleotides are located between them) so that the fluorescence of the probe is significantly reduced, whereby it does not matter whether the probe is hybridized to a target or not. Only after digestion by the DNA polymerase during the PCR reaction does the fluorescence intensity increase due to separation of the fluorescence quencher from the dye. Gelfand and Holland *et al.* already mentioned in their first patent that these fluorescent-labeled, self-quenching probes should be qualified for a "real-time" PCR. It has been known from many publications that in a DNA double helix, the distance between the donor and the acceptor dyes has to be between 6 and 16 nucleotides in order to achieve an efficient FRET [42]. Such fluorescence-quenched probes have been introduced into the literature by Lee *et al.* in 1993 [43]. They attached the donor dye at the 5'-end and labeled the probe internally with the acceptor dye, resulting in a close proximity and thus an efficient FRET. This probe design has two major disadvantages. First, labeling internally requires a more complicated synthesis than attaching moieties like fluorophores or quenchers at the end of a DNA strand. And second, labeling of internal nucleotides adversely affects hybridization efficiency.

The TaqMan assay becomes much more powerful when well-designed, self-quenching probes are used (US5538848). The principle of the assay is similar but Livak *et al.* [44] describe a self-quenching oligonucleotide probe having a reporter molecule (e.g. a fluorescence dye) and a quencher molecule that substantially quenches any fluorescence from the reporter whenever the probe is in a single-stranded state. Thereby, both moieties are typically attached to the ends of the probe. In contrast to the probes suggested by Gelfand and Holland *et al.*, the fluorescence increases upon hybridization to a target and formation of a double-stranded

state. After degradation by the DNA polymerase, the fluorophore is separated from the quencher moiety and the fluorescence of the dye is completely restored. In the next cycle all amplicons can act as a "template" for further probes again. In a continuation (US2001 6258569) of the patent, Livak *et al.* describe the application of these probes in combination with a solid support [45]. In this case, the self-quenching probe is covalently linked to a surface, preferably using linkers of at least 50 atoms, and the fluorescence is decreased whenever the probe is single-stranded. This regime can be used either as an ordinary hybridization assay meaning that the fluorescence of the probe, which is detectable on the surface increases significantly upon hybridization to the specific target DNA, or it can be used for monitoring PCR. Therefore the probe is attached in a way that the fluorophore remains on the surface after the probe is degraded by the DNA polymerase. Additionally to the fluorescence of the reporter molecule (e.g. donor dye of a FRET system), also the fluorescence of the quencher (e.g. acceptor dye of the FRET system) can be monitored in solution.

An alternative TaqMan probe with improved fluorescence quenching properties is described in US20066727356. These probes have a minor groove binding moiety in addition to the fluorophore and the quencher. Their working mechanism is similar to the Pleiades Probes that are described later in this review.

Molecular Beacons. One fundamental patent, which covers most of the self-quenching probes used nowadays, was filed by S. Tyagi, F. R. Kramer and P. M. Lizardi (US5925517) in May 1995 [46,47]. It describes detectable labeled oligonucleotides that undergo conformational changes upon hybridization to target DNA. Since fluorescence dyes, in combination with a fluorescence quencher, are the most used and preferred labels, we write only about fluorescence, although the patent covers also other labels e.g. enzymes in combination with suppressors. Two major structures (classes of probes) are claimed (Fig. 4). Tyagi *et al.* name them bimolecular (Fig. 4A) and unimolecular (Fig. 4B) probes. The bimolecular probe consists of two separate DNA strands (we only write DNA, although also DNA analogs like PNA are claimed), that have sequences (bold) complementary to each other at the 3'-end and 5'-end, respectively. The formation of a double helix leads to the illustrated Y-form. Furthermore, both oligonucleotides are

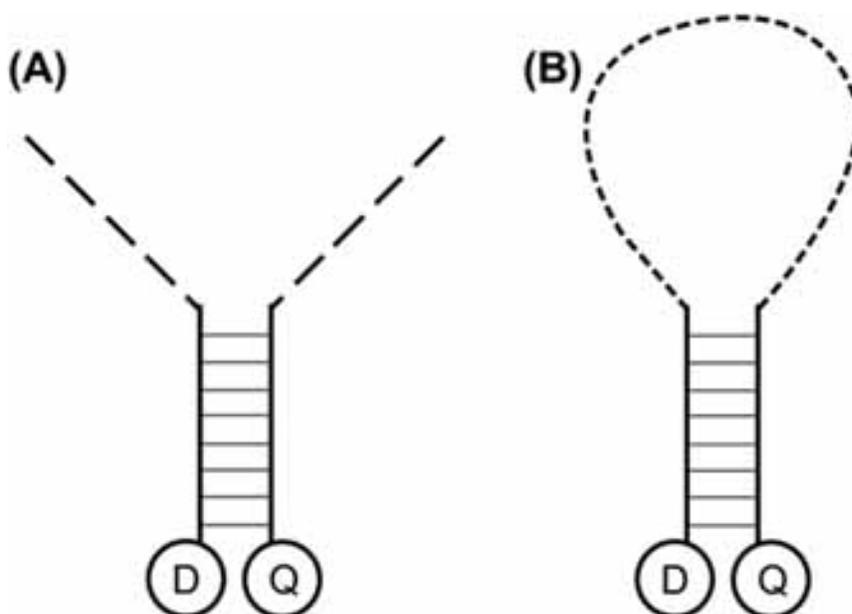


Fig. (4). Structure of (A) bimolecular and (B) unimolecular probes described by Tyagi *et al.* in the patent US5925517. D is the fluorescent dye that is the donor of a FRET system and Q is a quencher moiety.

labeled with a fluorescent dye and a quencher moiety Q. The labels can be attached anywhere at the bold sequences, so that they are in close proximity, resulting in a reduced fluorescence signal if the probe shows the so-called “closed conformation”. The dashed sequences of the probe are complementary to the target DNA. Due to hybridization to the target, the Y-form opens and the quencher is separated from the fluorescent dye, resulting in an increase in fluorescence intensity (also other measurable parameters like fluorescence lifetime or polarization are claimed). In order to achieve working probes, the probe-target hybrid has to be more stable than the double helix of the probe, usually meaning that the dashed sequences have to be longer than the bold sequences.

The second, unimolecular probe (Fig. 4B) is much more important in today’s DNA analysis. This probe consists of a single oligonucleotide that is labeled at its ends with a dye and a suitable quencher, respectively. Due to complementary sequences at both ends (bold), the probe is forced into a hairpin-structure and thus dye and quencher are directly adjacent and the fluorescence of the chromophore is strongly reduced. The loop sequence (dashed) is complementary to the target sequence, so that probe and target DNA can form a rigid double helix which opens the probe and removes the quencher from the dye, resulting in a fluorescence increase. Typical lengths for the loop sequences are 10-40 nucleotides and 5-11 base pairs for the stem. These probes are also known as Molecular Beacons and their applications are described meanwhile in several hundred publications.

Originally, the Molecular Beacons were labeled with a fluorescent dye and a quencher molecule forming a FRET pair. Thereby the energy of the dye is transferred to the acceptor molecule, which releases the energy *via* radiationless processes (heat). An efficient FRET and thus strong fluorescence quenching requires a large overlap between the emission spectrum of the fluorescence dye (donor) and the absorption spectrum of the quencher molecule (acceptor) [8]. Therefore, the number of fluorophore-quencher pairs is limited and additionally such pairs could be highly desirable for a number of reasons, e.g. flexibility in assay design.

For Molecular Beacons (and similar probes described above), also other “non-FRET” quenching processes can be used, which are described in a separate patent (US20006150097). Furthermore, this patent describes the possibilities to use several probe sequences labeled with different colored fluorescent dyes but the same

quencher molecule in a multiplex application. Tyagi and Russel claimed that instead of a FRET pair in which the energy is transferred over a distance between 10 Å and 100 Å, two chromophores that “touch” each other and show electronic interactions can be applied to achieve fluorescence quenching [48]. Thereby the second chromophore can be a quencher molecule, another fluorescent dye or the identical dye. The electronic interaction can be observed in the absorption spectra. For example, two identical chromophores are able to form non-fluorescent dye dimers that show a blue-shifted absorption band. These phenomena were already described in the excitation theory by Michael Kasha [29]. This patent also covers measuring the changes of the absorption properties after hybridization to the target DNA instead of detecting the fluorescence increase. Indeed, measuring fluorescence is preferable for example in terms of sensitivity. Tyagi and Russel proved that this “non-FRET” quenching mechanism is appropriate to build up a multiplex assay. They demonstrated that the common quencher DABCYL ($\lambda_{\text{abs}} \sim 475 \text{ nm}$) is able to quench six different dyes (EDANS, Fluorescein, Lucifer Yello, BODIPY, Tetramethylrhodamine, and Texas Red) showing emission maxima ranging from 450 nm - 600 nm with quenching efficiencies over 94 %.

Furthermore, Tyagi *et al.* also developed self-quenching probes showing bigger Stokes shifts (wavelength difference between the optimal excitation wavelength and the optimal emission wavelength of a dye) and named them “wavelength-shifted probes (or primers)” (US20006037130). These probes have three moieties. A fluorescence quencher (Q) is located at one end of the oligonucleotide like in normal Molecular Beacons. On the other end two chromophores forming a FRET system are attached [49]. The donor (D) (harvester in the patent) is separated by usually 5 -18 nucleotides (also other linkers are possible) from the acceptor dye (A) (the emitter in the patent). Thereby the emission spectrum of the donor overlaps well with the absorption spectrum of the acceptor dye in order to obtain a nearly 100% FRET, so that in spite of a short excitation wavelength only bathochrome-shifted fluorescence light from the acceptor dye is emitted. The distance between both dyes should not be shorter than five nucleotides, which corresponds to approximately 15 Å, in order to avoid fluorescence quenching due to electronically interactions. While the hairpin is closed, the two chromophores are in close proximity to the quencher moiety and thus the fluorescence is efficiently quenched. Thereby, the quencher can be chosen in a way that it quenches the fluorescence of the

harvester or the emitter moiety, or both. Tyagi *et al.* note in the patent that the quenching mechanisms are not fully understood yet. Although the synthesis of these probes is more complex, there are some advantages compared to normal self-quenching probes or primers.

Fluorescence assay instruments that operate with a single excitation wavelength are much less complicated and thus much less expensive than instruments that operate with multiple excitation wavelengths. Therefore, the number of different suitable chromophores is limited. Especially multiplex assays for multiple targets using multiple hairpin probes or primers having differently colored fluorophores are very difficult to realize if only one excitation wavelength should be used. Either the excitation of some dyes is very poor or the overlap of the emission spectra is very large which makes it difficult to distinguish different probes. In such cases the wavelength-shifted probes have great potential because they can be designed in a way that they all have the same harvester with optimal excitation properties but different emitter dyes with significantly different emission spectra.

Self-quenching probes like Molecular Beacons improve the detection of DNA in cells and can also be used in living cells. Linear fluorescent probes, as are used in fluorescence in-situ hybridization (FISH) [50], are always fluorescent, and thus unbound probes must be removed by washing to detect positive signals after hybridization, which prevents the application of this method to gene detection in living cells. Molecular Beacons do not require washing steps and should be directly usable in living cells [51-53]. However, interaction between Molecular Beacons and certain intracellular factors can cause fluorescence in the absence of target hybridization and lead to false-positive signals [54] because the fluorescent signals that result from target hybridization cannot be distinguished from any other events (e.g. degradation by intracellular nucleases) that spatially separate reporter from quencher [53, 55, 56].

Tsuji *et al.* have shown that a specific hybridization event in living cells can be detected by two linear oligonucleotide probes labeled respectively with donor and acceptor fluorophores that hybridize directly adjacent to the target DNA and thus an efficient

FRET occurs, which can be detected *via* fluorescence intensities [57] or fluorescent lifetimes [58]. However, the sensitivity of intracellular DNA or RNA detection using such probes suffers from strong background signals due to unbound probes. Bao *et al.* describe in their patent (US7081336) a method that combines both approaches [59]. They apply two Molecular Beacons that hybridize in close proximity to each other on the target. Both Molecular Beacons are labeled with a fluorescence dye and a suitable quencher. The two fluorophores are chosen in such a manner that they can form a FRET system (Fig. 5). One probe comprises the fluorescent label on its 3'-end, the other on its 5'-end, which allows a close distance when both probes hybridize to the target so that a FRET can occur. Only emission from the acceptor fluorophore serves as positive signal. To prevent crosstalk and therefore false positive signals, acceptor and donor fluorophores have to be chosen in such a way that excitation of the donor can be achieved at a wavelength that has little or no capacity to excite the acceptor. Emission of the acceptor will therefore only be detected if both Molecular Beacons are hybridized to the same target and a FRET occurs. Molecular Beacons that are degraded or open due to protein interactions will result in the presence of unquenched fluorophores, however, fluorescence emitted from these species is different from the signal obtained from donor/acceptor FRET pairs, making background and true positive signals more readily differentiated. Of course, the lower signal-to-background ratio of this detection method is not only advantageous for living cells but also in other applications like monitoring PCR because also the background of well designed Molecular Beacons can be different and increases due to the quality of the oligonucleotide synthesis and purification [60, 61].

Furthermore, the invention of Bao *et al.* provides a design variant for Molecular Beacons where one arm of the stem participates in both, hairpin formation and target hybridization, which is named "shared-stem" Molecular Beacons. To our mind, this approach is not completely new because Smart Probes, which are also hairpin-structured probes (they are described later) have been used in a manner that the stem sequence partly hybridizes to the target [15] and is claimed (for Smart Probes) in the respective patent (US20030003486) [62]. However, Bao *et al.* describe in

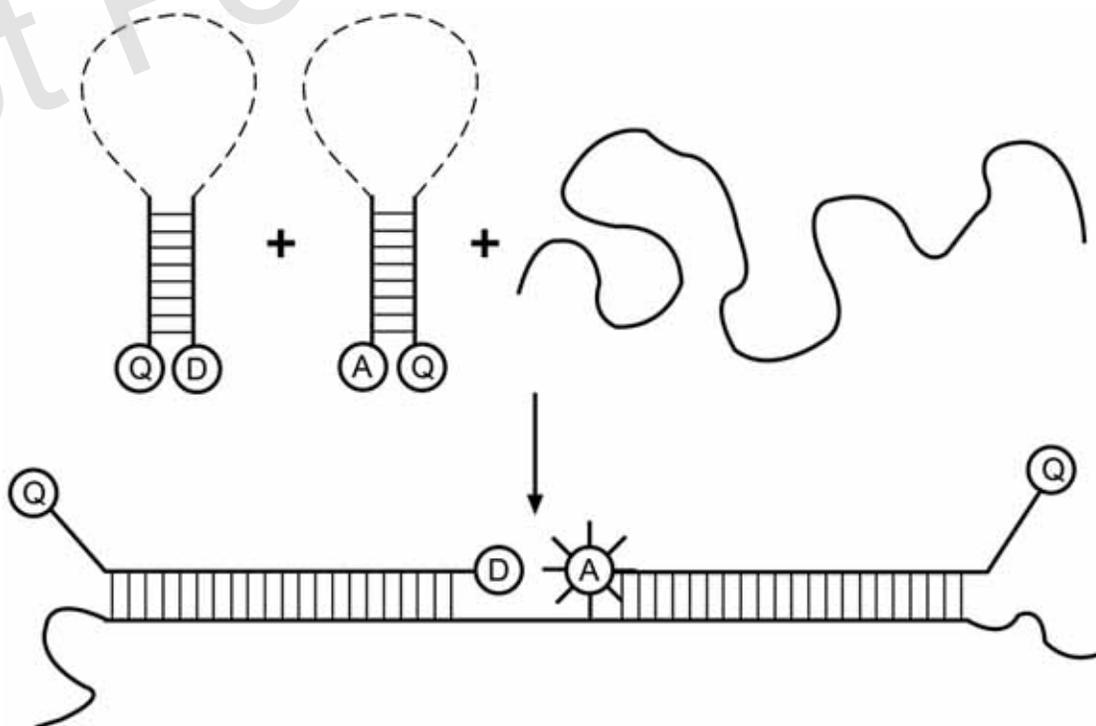


Fig. (5). Working mechanism of two Molecular Beacons that form a FRET system together, if they hybridize adjacent at the target DNA.

detail the advantages, e.g. concerning hybridization kinetics, of this design compared to classical Molecular Beacons.

G-Quartets. While investigating randomly generated single-stranded DNA aptamers, which are able to bind thrombin, it has been found that they contain the conserved consensus sequence GGNTGGN₂₋₅GGNTGG [63]. Analysis of the molecular structure has revealed a symmetrical structure containing two tetrads of guanine base pairs connected by three loops [64,65]. This characteristic structure is commonly referred to as a "G-quartet" or "G-quadruplex." E. Dias *et al.* [66] reported a similar sequence in which the G-quartet structure is maintained if the length of the oligonucleotide between the G pairs is increased. In these structures the 5'-end and the 3'-end are in very close proximity. Pitner *et al.* (US5691145) used this property for developing a DNA assay system based on double-labeled probe oligonucleotides that form G-quartets [67]. The labels are two chromophores building a FRET pair. Due to the close proximity, the fluorescence of the donor is often not only quenched *via* FRET but also due to electronic interactions (aggregation). However, in the presence of the target DNA the probe hybridizes to the complementary sequence and is unfolded, resulting in a fluorescence increase. One serious disadvantage of this method is the limitation in the choice of the sequences. The probe sequence has to be GGN_xGGN_yGGN_zGG, where x, y, and z indicate a variable number of nucleotides (x, y and z are typically at least 2, preferably about 2-10 nucleotides). Furthermore, the variable N sequences should not be self-complementary and should not contain G residues which would result in alternative G-quartet structures within the molecule.

Smart Probes. Sauer *et al.* described self-quenching, hairpin-structured DNA probes (so-called Smart Probes in the literature) that require only one label (US20030003486) [62]. The probes consist of dye-labeled oligonucleotides that have complementary sequences at both ends leading to a loop-stem structure (Fig. 6). The fluorophore (e.g. rhodamine and phenoxazine dyes) is attached in the stem region, preferably at the 5'-end. At the other end several guanosines are located and thus the dye is in contact with at least one guanine base when the hairpin is closed. This results in an efficient fluorescence quenching *via* a photoinduced electron transfer reaction. The guanosines are removed from the dye and the fluorescence is restored, if the probe hybridizes to the respective target sequence that is complementary to the loop and optional also partly to the stem sequence. Indeed, the dye should not be located close to other guanosines (of the target DNA) after hybridization in order to achieve a maximal fluorescence increase. Alternatively, to the guanosine residues also 7-deazaguanosine and 7-deazaadenosine can be used, which leads to a more efficient fluorescence quenching due to lower oxidation potentials. Moreover, the limitation in the choice of sequences is reduced. Beside a reduction of the fluorescence intensity the contact to guanosines shortens also

the fluorescence lifetime of the dye-labeled probes. Furthermore, the probes are usually labeled with red-absorbing fluorophores that can be easily excited by inexpensive diode lasers. These features make Smart Probes ideal candidates for single-molecule experiments that enable very sensitive and specific detection of DNA sequences with and without amplification of the target DNA [15, 68, 69].

Another mono-labeled DNA probe that exploits the quenching properties of guanine has been published [20] and filed for patent by Kurane *et al.* (US20046699661) [70]. Although these probes are not classical self-quenching probes they are mentioned in this review because their synthesis is very easy and inexpensive like for the Smart Probes described above. The probe consists of a single oligonucleotide that has at one end (position 1-3) no guanines, at least one cytosine and a fluorescence label (they prefer Bodipy FL). If this probe hybridizes to the complementary target sequence, the dye is now directly adjacent to a guanine and consequently the fluorescence is reduced. This patent shows also the use of these probes in surface-based DNA assays.

Pleiades Probes. Recently, Lukhtanov *et al.* introduced a novel class of self-quenching DNA probes (called "Pleiades" after a very bright star collection) that are based on oligonucleotides labeled at both ends with a fluorophore and a quencher, respectively [71]. In contrast to TaqMan probes or stemless Molecular Beacons, they have additionally a minor groove binder (MGB), which brings dye and quencher close together due to hydrophobic interaction, and thus enhances the fluorescence quenching (US20077205105) [72]. Preferably the MGB and the fluorophore are labeled to the 5'-end and the quencher to the 3'-end of the oligonucleotide probe (Fig. 7). Thereby, the fluorescence reduction is not only to the close proximity of the quencher moiety but also the interaction between the MGB and the fluorophore leads to an additional quenching. However, the fluorescence increases significantly after hybridization to the respective target DNA. Due to formation of the double helix, the dye-quencher interaction is prevented and the MGB moiety conceals inside the minor groove. This particular probe cannot be digested by polymerase during PCR because the 5'-end is blocked by the MGB.

The sensitivity and signal-to-background ratios of Pleiades Probes are comparable to Molecular Beacons. Although the specificity is slightly worse (but better than of other linear probes), Pleiades Probes have two major advantages compared to Molecular Beacons. Firstly, they have a relatively stable temperature-independent fluorescence background, which is important, for example, for accuracy and sensitivity of post-PCR melting curve analysis. Furthermore, the probes enable DNA assays at higher temperatures at which Molecular Beacons are already in an opened conformation. The second advantage is that the hybridization kinetics of Pleiades Probes are significantly faster than of hairpin-

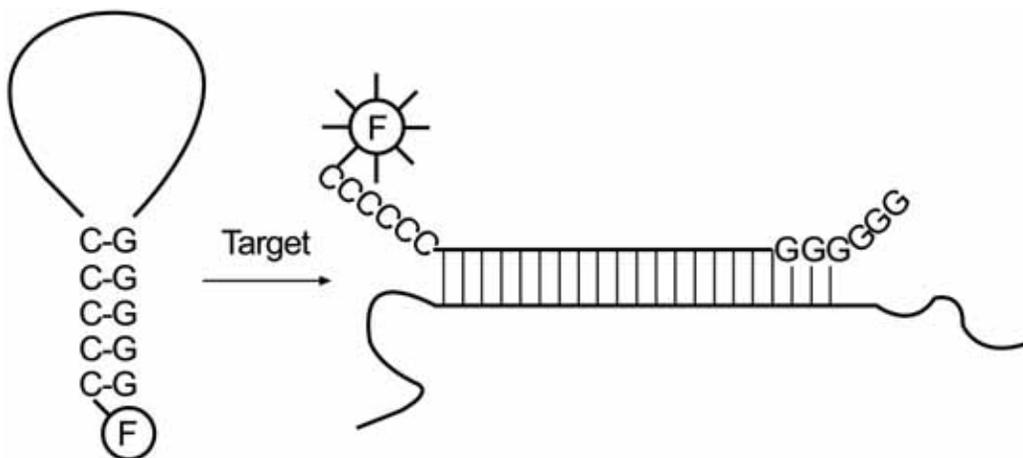


Fig. (6). Structure and working mechanism of Smart Probes described in the patent US20030003486 by Sauer *et al.*

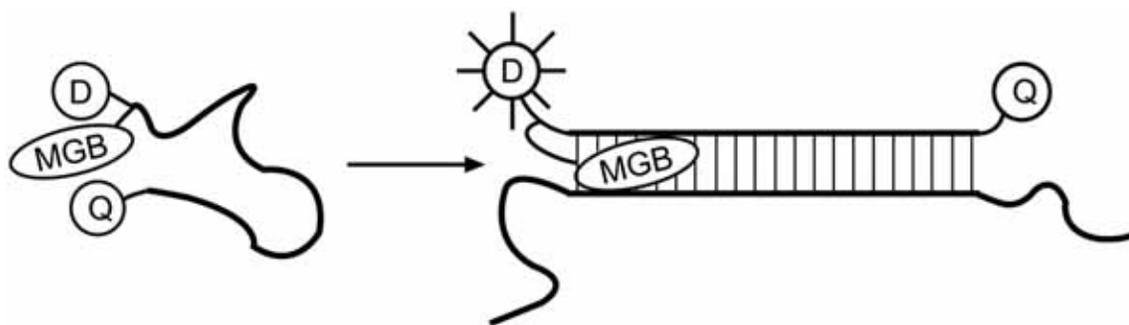


Fig. (7). Structure of Pleiades Probes claimed in patent US7205105 by Lukhtanov *et al.*

structures probes. One disadvantage of this new class of DNA probes is the relatively complicated synthesis of the probes. For example, the probes were synthesized on special MGB-modified DNA synthesis supports, which are also applied for patent (US Patent Application, 20050214797A1) [73].

Furthermore, Lukhtanov *et al.* have shown that the probes can be used as TaqMan probes, if the MBG is attached to the 3'-end (so-called MGB-TaqMan) [74], which are also claimed in a patent (US20046727356) [75].

Applications of self-quenching probes in combination with PCR. Self-quenching probes like Molecular Beacons and TaqMan probes can be applied in very sensitive and quantitative PCR methods. The patent US20026461817 describes non-competitive,

quantitative amplification methods [76]. Besides the most common PCR also amplification methods like nucleic acid sequence-based amplification (NASBA), transcription-mediated amplification (TMA), self-sustained sequence replication (3SR) [77], and strand displacement amplification (SDA) [78] are mentioned. The PCR is well known and is the most widely used technique for amplifying DNA or RNA. This method is often used for assaying DNA in many fields including, genetic analysis, forensics, food and agriculture testing, or medical diagnostics. Especially for the latter, quantitative PCR assays are necessary. One major problem of quantification is that the efficiency of PCR reactions can vary dramatically and are hard to control or to predict. One type of

Table 1. Patents Concerning Self-Quenching Primers

Patent Number (Year)	Inventors	Title
US5846726 (1998)	Nadeau, J. G. Pitner, J. B. Schram, J. L. Linn, C. P. Vonk, G. P. Walker, G. T.	Detection of nucleic acids by fluorescence quenching
US6054279 (1998)	Nadeau, J. G. Pitner, J. B. Schram, J. L. Linn, C. P. Vonk, G. P. Walker, G. T.	Detection of nucleic acids by fluorescence quenching
US5928869 (1999)	Nadeau, J. G. Pitner, J. B. Linn, C. P. Schram, J. L.	Detection of nucleic acids by fluorescence quenching
US20036593091 (2003)	Keys, D. A. Farooqui, F. Reddy, M. P.	Oligonucleotide probes for detecting nucleic acids through changes in fluorescence resonance energy transfer

Table 2. Patents Concerning the TaqMan Assay

Patent Number (Year)	Inventors	Title
US5210015 (1993)	Gelfand, D. H. Holland, P. M. Saiki, K. R. Watson, R. M.	Homogeneous Assay System using the Nuclease Activity of a Nucleic Acid Polymerase
US5487972 (1996)	Gelfand, D. H. Holland, P. M. Saiki, R. K. Watson, R. M.	Nucleic acid detection by the 5'-3' exonuclease activity of polymerases acting on adjacently hybridized oligonucleotides
US20067141377 (2006)	Gelfand, D. H. Holland, P. M. Saiki, R. K. Watson, R. M.	Process for discrimination of nucleic acid sequence variants
US5538848 (1996)	Livak, K. J. Flood, S. J. A. Marmaro, J.	Method for detecting nucleic acid amplification using self-quenching fluorescence probe
US20016258569 (2001)	Livak, K. J. Flood, S. J. A. Mamoro, J. Mullah, K. B.	Hybridization assay using self-quenching fluorescence probe
US20046727356 (2004)	Reed, M. W. Lukhtanov, E. A. Gall, A. A. Dempsy, R. O.	Fluorescent quenching detection reagents and methods

quantitative PCR assays involves simultaneous amplification of the sample containing the target sequence and a control. In addition to the pair of primers for the target, a second pair of primers for the control is required. After finishing the amplification the amounts of the amplified products are compared [79]. A better method is the quantitative-competitive PCR (QC-PCR) using a control which is similar to but ultimately distinguishable from the target sequence. The control competes with the target sequence for the same pair of primers. Firstly, two different products (amplicons) were distinguished, for example, by size using gel electrophoresis [80]. Such post-amplification manipulations complicate the assay, decreases throughput and seriously limits sensitivity and quantification. Meanwhile, these post-amplification methods are usually replaced by real-time PCR utilizing the self-quenching probes described above. Thereby, Molecular Beacons and TaqMan probes are widely used. These probes report for each cycle during the PCR the amount of target DNA (if it is higher than the detection limit) by increasing their fluorescence, which can be detected without opening the reaction tube or interrupting the PCR reaction. A further advantage of real time PCR is a significantly wider dynamic range. Nevertheless, accuracy is still limited due to variations in amplification efficiency. For example, two separate experiments testing the same amount of unknown target sequence

can easily differ by thirty percent [81]. The non-competitive, quantitative amplification described in the patent (US20026461817) by D. Alland *et al.* achieves a much higher accuracy. The major progress compared to the QC-PCR is the use of a control sequence that is very similar and differ sometimes only by one nucleotide compared to the target sequence. Therefore, "cross hybridization" occurs, meaning that the amplicons of each sequence hybridize not only to themselves but also to amplicons of the other sequences. Consequently, they act as a single amplicon and thus necessarily follow the same reaction kinetics. The development of this technique was strongly supported by new self-quenching probes, like Molecular Beacons, which are also able to distinguish between DNA sequences that differ by as little as one nucleotide. Alland *et al.* discovered that the ratio of the target sequence and the control sequence (ratio of the fluorescence of the respective probes) is constant during the whole time of the PCR reaction. With this method it is possible to detect less than 100 DNA copies quantitatively and with high accuracy. Because of the specificity of the applied DNA probes, this method is also appropriate for the identification of alleles and mutants, for example, drug-resistant mutant pathogens. They have shown that one can distinguish as little as two percent of mutant DNA in an otherwise wild-type DNA population.

Table 3. Patents Concerning Molecular Beacons

Patent Number (Year)	Inventors	Title
US5925517 (1999)	Tyagi, S. Kramer, F. R. Lizardi, P. M.	Detectably labeled dual conformation oligonucleotide probes, assays and kits
US20006103476 ¹ (2000)	Tyagi, S. Kramer, F. R. Lizardi, P. M.	Detectably labeled dual conformation oligonucleotide probes, assays and kits
US20006150097 (2000)	Tyagi, S. Kramer, F. R.	Nucleic acid detection probes having non-FRET fluorescence quenching and kits and assays including such probes
US20006037130 (2000)	Tyagi, S. Kramer, F. R. Marras, S. A. E.	Wavelength-shifting probes and primers and their use in assays and kits
US20067081336 (2006)	Bao, G. Tsourkas, A. Xu, Y.	Dual resonance energy transfer nucleic acid probes
US20026461817 ² (2002)	Alland, D. Kramer, F. R. Piatec, A. Tyagi, S. Vet, J.	Non-Competitive Co-Amplification Methods
EP1230387 ² (2002)	Tyagi, S. Kramer, F. R. Alland, D.	Assays for short sequence variants

As shown above, self-quenching probes like Molecular Beacons are appropriate for very specific DNA assays. On the other hand, sometimes probes with much lower specificity are required. Tyagi *et al.* describe (patent EP1230387B1) an application of such unspecific probes for the identification of short sequence variants, for example in different mycobacteria [82]. With classical Molecular Beacons or TaqMan probes, only a limited number of alleles or mutations can be analyzed, because only a limited number probes with different fluorophores can be distinguished. Furthermore, the sequences of all variants must be known and the probes must be carefully constructed to operate at the same reaction conditions. The approach described in this patent is based on probes that can hybridize not only to a specific target but to many target sequences (multiple variants or alleles) and therefore they are called "sloppy" probes. This behavior of the probes is achieved by longer loop sequences of 25-50 nucleotides and relatively short stems of 4-6 complementary base pairs. Using one or more of such probes in combination allows the detection of one variant amongst multiple

possible variants. Due to differences in the DNA sequence, each variant shows a different affinity to each applied sloppy probe and consequently various ratios between the fluorescence intensities of the different probes are detected. Therefore, the fluorescence ratios are significant fingerprints of a certain variant. The patent gives an example for the discrimination of eight mycobacteria with only four different sloppy probes that are designed for the hypervariable region A of the mycobacterial 16s rRNA genes. In this particular 40-nucleotide containing region, the different mycobacteria differ by 2-9 nucleotides from the sequence of *M. tuberculosis*. The sequences of the TMR, TET, FAM, and RhD labeled probes and of the different mycobacteria are given in the patent. Four probes means six different fluorescence ratios for each different *Mycobacterium* which are unique for each mycobacterium although, for example, the sequences of *M. tuberculosis* and *M. marinum* differ in only two alleles.

This invention provides an inexpensive and rapid diagnostic method that is able to identify a broad range of microorganisms (e.g. bacteria, viruses, or parasites). One advantage over classical self-quenching probes is the easy design of the sloppy probes due to less optimization work. Indeed, the most important advantage is that also variants with unknown sequences can be distinguished, because the sloppy probes do not target a highly specific DNA

¹ This application is a continuation of the patent mentioned previously.

² This patent deals with an application of molecular beacons but does not concern exclusively molecular beacons and is therefore described in the last paragraph of the article.

Table 4. Further Patents

Patent Number (Year)	Inventors	Title
US5691145 (1997)	Pitner, J. B. Vonk, G. P. Nadeau, J. G.	Detection of nucleic acids using G-quartets
US20030003486 (2003)	Sauer, M. Wolfrum, J.	Dye-labeled oligonucleotide for labeling a nucleic acid molecule
US20046699661 (2004)	Kurane, R. Kanagawa, T. Kamagata, Y. Kurata, S. Yamada, K. Yokomaku, T. Koyama, O. Furusuo, K.	Method for determining a concentration of target nucleic acid molecules, nucleic acid probes for the method, and method for analyzing data obtained by the method
US20026461817 (2002)	Alland, D. Kramer, F. R. Piatek, A. Tyagi, S. Vet, J.	Non-competitive co-amplification methods
EP1230387B1 (2002)	Tyagi, S. Kramer, F. R. Alland, D.	Assays for short sequence variants
US20077205105 (2007)	Afonina, I. A. Belousov, Y. S. Dempcy, R. O. Kutyavin, I. V. Lokhov, S. G. Lukhtanov, E. A.	Real-time linear detection probes: sensitive 5'-minor groove binder-containing probes for PCR analysis
US Patent Application, 20050214797A1 (2005)	Lokhov, S. Lukhtanov, E. A.	Fluorescent probes for DNA detection by hybridization with improved sensitivity and low background
US20046727356 (2004)	Reed, M. W. Lukhtanov, E. A. Gall, A. A. Dempcy, R. O.	Fluorescent quenching detection reagents and methods

sequence but report any differences in the sequence by changing their affinity and thus the fluorescence intensity.

CURRENT & FUTURE DEVELOPMENTS

Nowadays most applications need target DNA amplification, e.g. via PCR. Consequently, the most important property of DNA probes is that they are optimized for online monitoring of PCR (real-time PCR). Furthermore, it is advantageous if the probes are highly specific and useful for multiplex assays, which allow good discrimination between different alleles. Both, linear probes developed for the TaqMan assay and hairpin-structured Molecular Beacons fulfill these requirements. The two methods were developed simultaneously at the beginning of the 1990's and filed for patents in 1993 and 1994, respectively. These probes and their modifications dominate the market of real-time PCR. Other later developed probes and methods like Smart Probes or Pleiades

Probes that are also generally usable for PCR online monitoring are rarely used for gene analysis. Although they have some advantages (e.g. the mono-labeled Smart Probes are easier to synthesize) but also disadvantages (e.g. Smart Probes have usually a worse signal-to-background ratio than Molecular Beacons), it remains to be seen whether they will compete against the well-established Molecular Beacons and TaqMan probes. However, we envision applications of these probes in new and emerging areas, for example, detecting DNA or RNA without amplification in living cells or tissues. Hereby all probes that require degradation by a DNA polymerase are excluded. We think that for each new technique all generally suitable probes will need to be carefully evaluated and different probes will be used in different applications, because they all have inherent advantages and disadvantages. Also the application of other modern detection techniques like single-molecule detection may change the requirements on the probes and may lead to a new

evaluation. Another interesting future application of self-quenching DNA probes is their use in chip technology. Until today, mostly unlabeled probes are bound to the chip surface and the target DNA itself is fluorescently labeled (e.g. incorporation of fluorescently labeled nucleosides during PCR). The use of self-quenching probes on the surface could be advantageous if the sensitivity of chip technology can be further increased, so that PCR can be avoided.

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