

# Multicolor molecular beacons for allele discrimination

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**Molecular beacons are hairpin-shaped oligonucleotide probes that report the presence of specific nucleic acids in homogenous solutions. When they bind to their targets they undergo a conformational reorganization that restores the fluorescence of an internally quenched fluorophore. We found that their hairpin conformation enables the use of a wide variety of differently colored fluorophores. Using several molecular beacons, each designed to recognize a different target and each labeled with a different fluorophore, we demonstrate that multiple targets can be distinguished in the same solution, even if they differ from one another by as little as a single nucleotide. A comparison of "hairpin probes" with corresponding "linear probes" confirms that the presence of the hairpin stem in molecular beacons significantly enhances their specificity.**

Keywords: fluorescent hybridization probes, homogeneous real-time assays, mutation detection

When detecting nucleic acids by hybridization, there are many situations in which it is not desirable or possible to isolate probe-target complexes from the excess of probes that are used. We have developed single-stranded oligonucleotide probes, called molecular beacons, that can be used to indicate the presence of specific nucleic acids in homogenous solutions<sup>1</sup>. These probes can monitor nucleic acid syntheses in real-time, without interrupting the reactions, and they can be used to locate specific RNAs within living cells without killing the cells<sup>2</sup>. Molecular beacons undergo a fluorogenic conformational change when they hybridize to their target. They possess a stem-and-loop structure (Fig. 1), where the loop portion of the molecule is a probe sequence that is complementary to a target sequence in the nucleic acid to be detected, and the stem is formed by the annealing of complementary arm sequences that are on the ends of the probe sequence. A fluorescent moiety is covalently linked to the end of one arm and a quenching moiety is covalently linked to the end of the other arm. The stem keeps these two moieties in close proximity to each other, causing the fluorescence of the fluorophore to be quenched by energy transfer. Because the quencher is a nonfluorescent chromophore that emits the energy that it receives from the fluorophore as heat, fluorescence does not occur. When the probe encounters a target molecule, it forms a probe-target hybrid that is longer and more stable than the stem hybrid. The rigidity and length of the probe-target hybrid precludes the simultaneous existence of the stem hybrid. Consequently, the molecular beacon undergoes a spontaneous conformational reorganization that forces the stem hybrid to dissociate and the fluorophore and the quencher to move away from each other, restoring fluorescence. Fluorescence increases as much as 900-fold when these probes bind to their target.

In this report we show that molecular beacons can be constructed that fluoresce in a wide variety of different colors, and we demonstrate that these molecular beacons can be used for the detection of several different targets in the same solution. Moreover, we demonstrate that the presence of the hairpin stem markedly enhances the specificity of molecular beacons, enabling them to distinguish targets that differ from one another by as little as a single nucleotide.

## Results and discussion

**Real-time detection of PCR amplicons.** As target nucleic acids are rare components of biological samples, molecular beacons are best used in conjunction with target amplification, such as for real-time detection of amplification products in polymerase chain reactions (Fig. 2). Six reactions were carried out in sealed tubes, each initiated with a different quantity of target nucleic acid, and each containing molecular beacons specific for a sequence in the center of the expected amplicon. The molecular beacons were present in each reaction at a concentration similar to the concentration of the primers and the length of their probe sequence was chosen so that the resulting hybrids would be stable at the annealing temperature. The intensity of the fluorescence at the annealing stage in each amplification cycle was a direct measure of amplicon concentration. The number of amplification cycles needed before fluorescence from the probe-target hybrids became detectable was inversely proportional to the logarithm of the number of target molecules initially placed in each reaction tube. This relationship permits quantitative determinations to be made over a wide range of target concentrations. Because these assays are carried out in sealed tubes, the risk of contaminating untested samples is eliminated.

**Differently colored molecular beacons.** In many applications it is important to detect multiple targets in the same solution. This can be accomplished by using a number of different molecular beacons simultaneously, each specific for a different target and each emitting light of a different color. We constructed a series of molecular beacons, each containing the quencher DABCYL (4-[4'-dimethylaminophenylazo]benzoic acid) linked to the same oligonucleotide sequence but possessing a different fluorophore. As a group, the emission spectra of these fluorophores spanned the entire visible range from blue to red. The fluorescence of all of these molecular beacons was quenched when no targets were present, but was restored to its full intensity and natural color when they were bound to target (Fig. 3). To obtain a quantitative measure of the extent of quenching, the fluorescence of a solution of each molecular beacon was determined in a spectrofluorometer before and after the addition of target, stimulating each fluorophore at its maximal excitation wavelength and measuring its fluorescence at its maxi-

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mal emission wavelength. The results show that DABCYL serves as a universal quencher, efficiently suppressing the fluorescence of all fluorophores (Table 1).

We had not expected DABCYL to serve as a universal quencher. It was originally chosen for use in molecular beacons<sup>1</sup> because it was known to quench the fluorescence of EDANS over short distances via fluorescence resonance energy transfer (FRET)<sup>3</sup>. An important criterion for FRET to occur is that the emission spectrum of the fluorophore significantly overlaps the absorption spectrum of the quencher<sup>4</sup>. However, DABCYL has little or no spectral overlap with the emission spectra of some of the fluorophores that we used, particularly the fluorophores that emit in the red color range, yet we found that DABCYL efficiently quenches their fluorescence. In FRET, there is a linear relationship between spectral

overlap and quenching efficiency<sup>5</sup>, yet we observed that quenching efficiency was independent of spectral overlap (Table 1). Moreover, the quenching efficiencies that we measured were higher than are usual for FRET. Taken together, these observations indicate that the fluorophore and the quencher are held so close together by the hairpin stem in the molecular beacon that direct transfer of energy is possible<sup>6,7</sup>. This conclusion was confirmed by comparing the visible absorption spectra of fluorophore-quencher pairs in molecular beacons in the target-bound conformation to the absorption spectra that occur when the molecular beacons are in the closed conformation (Fig. 4). The results show that the spectra are substantially different, indicating that the closed conformation brings the fluorophore and quencher sufficiently close to one another to perturb their electronic structure. This experiment was carried out with a

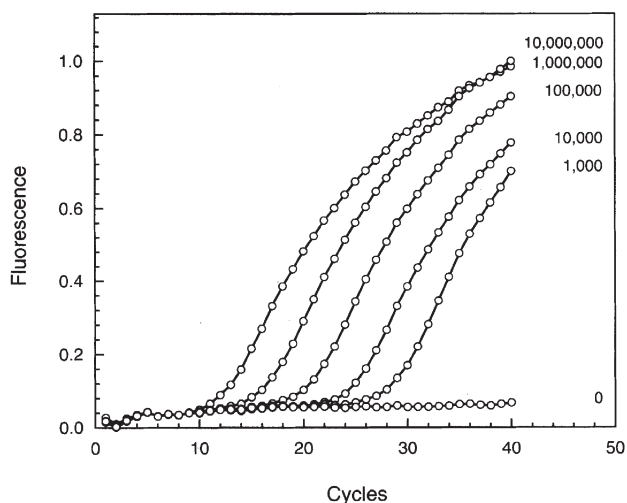


**Figure 1. Operation of molecular beacons.** On their own, these molecules are nonfluorescent, because the stem hybrid keeps the fluorophore close to the quencher. When the probe sequence in the loop hybridizes to its target, forming a rigid double helix, a conformational reorganization occurs that separates the quencher from the fluorophore, restoring fluorescence.

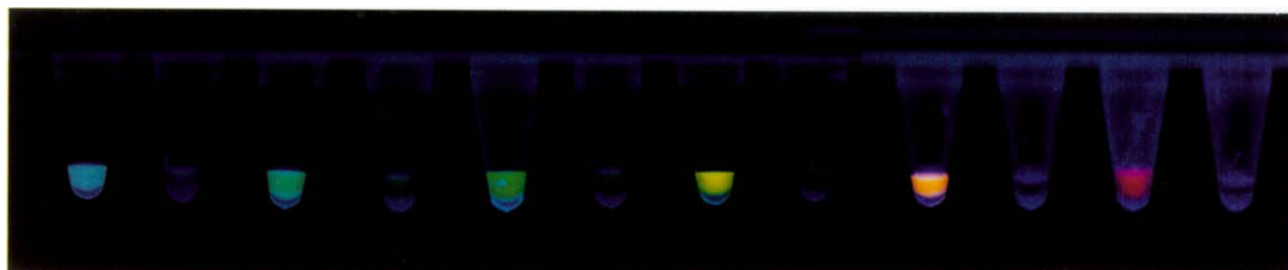
**Table 1. Quenching of different fluorophores by DABCYL in molecular beacons.**

| Fluorophore          | Emission maximum (nm) | Spectral overlap ( $10^{18} \text{ M}^{-1} \text{ cm}^2$ ) | Quenching efficiency (%) |
|----------------------|-----------------------|--|--------------------------|
| Coumarin             | 475                   | 1.28   | 99.3                     |
| EDANS                | 491                   | 1.12   | 99.5                     |
| Fluorescein          | 515                   | 1.02   | 99.9                     |
| Lucifer yellow       | 523                   | 0.87   | 99.2                     |
| BODIPY               | 525                   | 0.85   | 95.0                     |
| Eosine               | 543                   | 0.55   | 98.2                     |
| Tetramethylrhodamine | 575                   | 0.15   | 98.7                     |
| Texas red            | 615                   | 0.01   | 99.1                     |

Quenching efficiency is  $100 \times (1 - [F_{\text{closed}}/F_{\text{open}}])$ , where  $F_{\text{closed}}$  is the fluorescence of a molecular beacon in the absence of target and  $F_{\text{open}}$  is its fluorescence when bound to target. Spectral overlap is a measure of the extent to which the electronic transitions of each fluorophore are in resonance with the transitions of DABCYL<sup>3</sup>, and is defined as  $\int F(\lambda)\epsilon(\lambda)\lambda^2 d\lambda / \int F(\lambda)d\lambda$ , where  $F(\lambda)$  is the normalized fluorescence emission at wavelength  $\lambda$ , and  $\epsilon(\lambda)$  is the molar extinction coefficient of DABCYL at wavelength  $\lambda$ .



**Figure 2. Real-time measurement of amplicon synthesis during polymerase chain reactions using molecular beacons.** Six reactions, each initiated with a different number of template molecules (indicated), were incubated simultaneously in a spectrofluorometric temperature cycler. The concentration of amplicons that were present after each cycle of amplification was determined by measuring fluorescence during the last few seconds of the annealing step. During the denaturation step, the molecular beacons assume a random coil configuration and they fluoresce brightly. As the temperature is lowered to allow annealing of the primers, stem hybrids form rapidly in the molecular beacons, preventing fluorescence. At the annealing temperature, molecular beacons also bind to any amplicons that are present and generate fluorescence. When the temperature is raised to allow primer extension, the molecular beacons dissociate from their targets and do not interfere with polymerization. Thus, a new hybridization takes place in the annealing step of every cycle, and the intensity of the resulting fluorescence indicates the amount of accumulated amplicon.



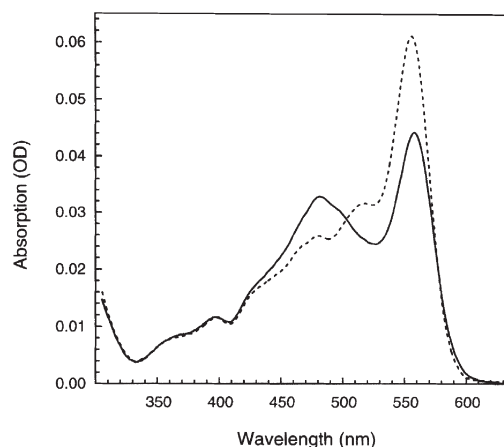
**Figure 3. Fluorogenic response of differently colored molecular beacons to the addition of target.** A solution of each molecular beacon was placed in a pair of test tubes. The molecular beacons contained (left to right) coumarin (blue), EDANS (blue-green), fluorescein (green), Lucifer yellow, tetramethylrhodamine (orange), and Texas red. Complementary single-stranded target oligonucleotides were added to the left tube of each pair, and the tubes were illuminated with a broad-wavelength ultraviolet lamp.

variety of fluorophore-quencher pairs, including tetramethylrhodamine-DABCYL, EDANS-DABCYL, eosine-DABCYL, fluorescein-tetramethylrhodamine, and tetramethylrhodamine-tetramethylrhodamine, and in every case the spectral changes indicated that the fluorophore and quencher are so close together in the hairpin conformation that they form a nonfluorescent complex.

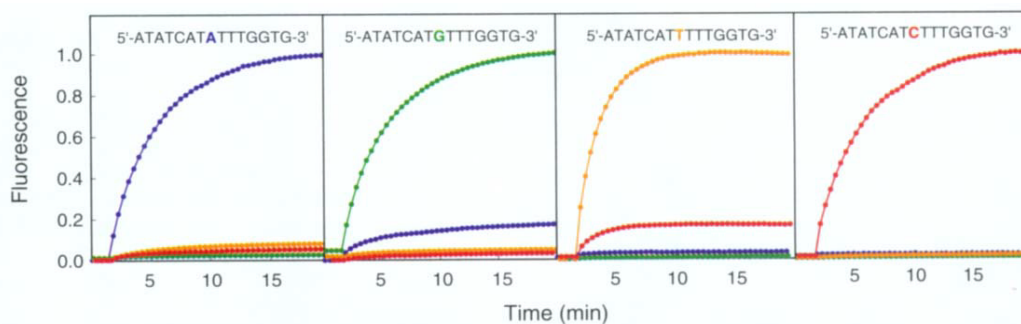
**Allele discrimination.** Exploiting the capacity of DABCYL to quench fluorophores of any color, we constructed a set of four differently colored molecular beacons for use in multiplex hybridization experiments. We chose coumarin, fluorescein, tetramethylrhodamine, and Texas red because in a mixture the fluorescence of each of these fluorophores is completely distinguishable from the fluorescence of the other three when each fluorophore is stimulated at its maximal excitation wavelength and its fluorescence is measured at its maximal emission wavelength. In order to demonstrate the specificity of molecular beacons, we carried out hybridization experiments with four oligonucleotide targets that differed from each other by only a single nucleotide. The molecular beacons were complementary to these targets and were identical in all respects, except that each was labeled with a different fluorophore and each possessed a different nucleotide at the center of its probe sequence; with coumarin labeling the molecular beacon that had thymidine at this position, fluorescein labeling cytidine, tetramethylrhodamine labeling adenosine, and Texas red labeling guanosine. An equimolar mixture of these molecular beacons was placed in four cuvettes. A different target was added to each cuvette and the fluorescence of each of the four molecular beacons in each cuvette was monitored over time. The addition of a target resulted in a marked increase in the fluorescence of the molecular beacon whose probe sequence was perfectly complementary to the target, but did not result

in an increase in the fluorescence of the other molecular beacons that were present (Fig. 5). The color of the fluorescence in each cuvette identified which nucleotide was present in the target. These results indicate that only perfectly complementary targets can form a hybrid with a molecular beacon, and a single nucleotide mismatch between the target and the probe prevents hybridization. A small amount of hybridization was observed, however, when the mismatch was a G:T basepair, as this is the least destabilizing of all mismatches<sup>8</sup>. When more than one target was added, the colors in the resulting fluorescence spectrum confirmed the identity of the targets. Thus, multicolor molecular beacons present in the same solution can distinguish alleles that differ from one another by as little as a single nucleotide.

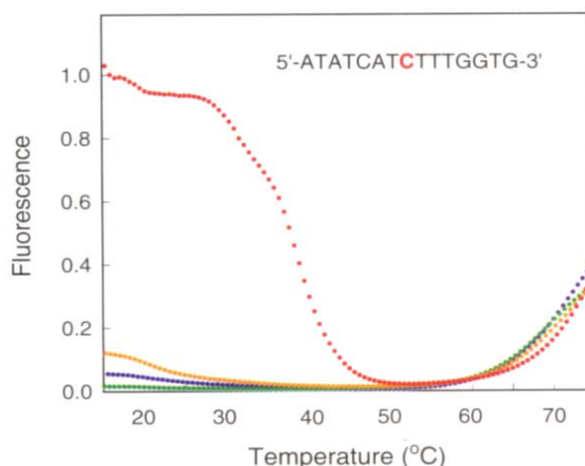
To understand the basis of this extraordinary specificity, we determined the stability of probe-target hybrids as a function of temperature. Using the four probe-target mixtures, we measured the fluorescence of each of the four molecular beacons as the temperature was slowly raised from 15°C to 75°C. At low temperatures perfectly complementary probe-target hybrids fluoresce brightly (Fig. 6). As the temperature was raised, a point was reached at which these hybrids dissociate. This transition was accompanied by a marked decrease in fluorescence due to the reformation of hairpin stems in the dissociated molecular beacons. The stability of the



**Figure 4.** Alteration in the absorption characteristics of the fluorophore and quencher when a molecular beacon is in its closed conformation. The visible absorption spectrum of a molecular beacon (0.5 mM solution) labeled with tetramethylrhodamine and DABCYL was measured in the absence (continuous line) and in the presence (broken line) of an equimolar quantity of target.

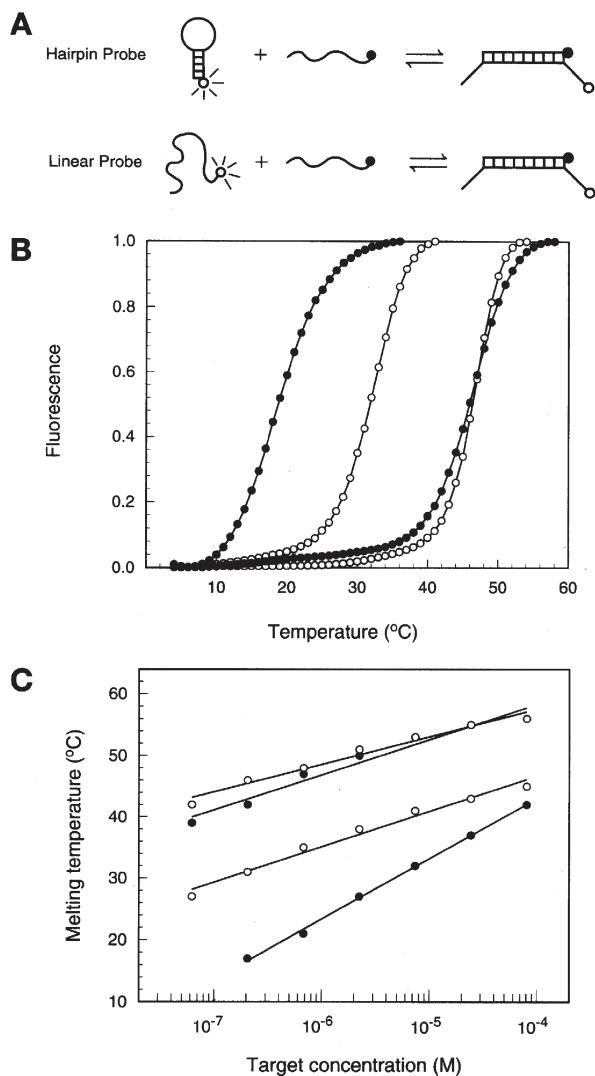


**Figure 5.** Detection of single-nucleotide differences using a mixture of four differently colored molecular beacons. When a target oligonucleotide (sequence shown in each panel) was added to the mixture, only the molecular beacon whose probe sequence was perfectly complementary to that target formed a hybrid and emitted its characteristic fluorescent color. Each kinetic curve is printed in the color of the molecular beacon's fluorescence (blue:coumarin, green:fluorescein, orange:tetramethylrhodamine, and red:Texas red).



**Figure 6.** Comparison of the stability of perfectly complementary probe-target hybrids to the stability of mismatched probe-target hybrids. Utilizing the hybridization mixture from the experiment shown in the right-hand panel of Figure 5, the fluorescence intensity of each of the four molecular beacons in the mixture was measured as a function of temperature. The melting temperatures of the mismatched probe-target hybrids were substantially lower than the melting temperature of the perfectly complementary probe-target hybrid.

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**Figure 7.** (A) Labeling scheme for comparing hairpin probes to linear probes. A fluorophore was linked to the end of each probe and a quencher was linked to the end of the target. (B) Comparison of the thermal stabilities of hairpin-probe hybrids (filled circles) to the thermal stabilities of linear-probe hybrids (open circles). Both the hairpin probe and the linear probe formed hybrids of equal stability with perfectly complementary targets ( $T_m = 46^\circ\text{C}$ ). Hairpin probes formed less stable hybrids with mismatched targets ( $T_m = 18^\circ\text{C}$ ) than linear probes did with mismatched targets ( $T_m = 31^\circ\text{C}$ ). (C) Stability of probe-target hybrids as a function of target concentration. The upper two lines show the similar stabilities of perfectly complementary hairpin-probe hybrids (filled circles) and perfectly complementary linear-probe hybrids (open circles), while the lower two lines show the different stabilities of mismatched hairpin-probe hybrids and mismatched linear-probe hybrids.

bimolecular probe-target hybrids decreases as the temperature is increased, while the stability of the intramolecular stem hybrids is affected to a much lesser extent. At even higher temperatures, the hairpin stems unravel, leading to an increase in fluorescence. Although mismatched probe-target hybrids form at very low temperatures, they fall apart at substantially lower temperatures than perfectly complementary hybrids. Furthermore, their fluorescence intensity is significantly lower than the intensity of perfectly complementary hybrids, a surprising phenomenon that is currently under investigation. Because alleles can be distinguished at temperatures that are in between the temperature at which mismatched

probe-target hybrids dissociate and the higher temperature at which perfectly complementary probe-target hybrids dissociate, and as this temperature difference is quite large, molecular beacons easily discriminate alleles that differ by a single nucleotide.

**Enhanced specificity of hairpin probes.** When conventional oligonucleotides are used as hybridization probes instead of molecular beacons, the difference in stability between a mismatched hybrid and a perfectly complementary hybrid is rather small<sup>9,10</sup>. To determine whether the hairpin stem of molecular beacons is responsible for their extraordinary specificity, we compared the stability of a “hairpin probe” to an otherwise identical probe that could not form a hairpin stem (a “linear probe”). The only difference between the probes was that the arms of the hairpin probe were complementary to each other while the arms of the linear probe were not. In order to observe the hybridization of both of these probes to targets, we utilized a different labeling scheme than we use for molecular beacons (Fig. 7A). Both probes were labeled at their 5' ends with fluorescein, but neither probe possessed a quencher at its 3' end, so both fluoresced brightly in the absence of target. Instead, the target oligonucleotides were labeled with DABCYL at their 3' ends, so that the binding of either probe to a target caused a marked reduction in fluorescence. The stability of hybrids formed by these probes was determined by monitoring fluorescence as a function of temperature. As the temperature was slowly raised, the dissociation of the probe-target hybrid was accompanied by a marked increase in fluorescence. Hybrids formed with perfectly complementary targets were equally stable, irrespective of whether the probe was a hairpin probe or a linear probe (Fig. 7B). However, when targets containing a single nucleotide substitution were used in place of perfectly complementary targets, the mismatched hybrids formed by the hairpin probes were considerably less stable than the mismatched hybrids formed by the linear probes. Mismatched hairpin-probe hybrids dissociated at a temperature 28°C lower than perfectly complementary hairpin-probe hybrids, whereas mismatched linear-probe hybrids melted apart at a temperature only 15°C lower than perfectly complementary linear-probe hybrids. This experiment was repeated over a wide range of target concentrations (Fig. 7C). Mismatched hairpin-probe hybrids were less stable than mismatched linear-probe hybrids at all target concentrations, confirming that the presence of the hairpin stem markedly enhances the specificity of molecular beacons.

Our original reason for including hairpin stems in molecular beacons was to obtain probes that only fluoresce when bound to targets. It was entirely fortuitous that the hairpin stem also enhanced specificity, enabling single-nucleotide differences to be distinguished. Furthermore, the hairpin stem brings the label moieties so close to each other that an entire palette of colored fluorophores can be used. Thus, mixtures of multicolored molecular beacons can be formulated that identify closely related allelic variants of a target by the color of the fluorescent signal. Assays that use molecular beacons for allele discrimination are now being carried out<sup>11,12</sup>, and they will find broad application in clinical diagnostics.

### Experimental protocol

**Synthesis of molecular beacons.** Molecular beacons were synthesized from oligonucleotides that contained a primary amino group at their 3' end and a sulfhydryl group protected by a trityl moiety at their 5' end (Midland Certified Reagents, Midland, TX). DABCYL was covalently linked to the 3'-amino group, utilizing an amino-reactive derivative of DABCYL (Molecular Probes, Eugene, OR). The oligonucleotides that were coupled to DABCYL were purified by high-pressure liquid chromatography. The protective trityl moiety was then removed from the 5'-sulfhydryl group, and a fluorophore was introduced in its place. A maleimide derivative of coumarin, a bromomethyl derivative of BODIPY, a sulfonylchloride derivative of Texas red, and iodoacetamide derivatives of all other fluorophores, were used for 5'-terminal coupling (all fluorophore derivatives were purchased from Molecular

Probes). Finally, oligonucleotides containing both a fluorophore and DABCYL were purified by high-pressure liquid chromatography. A detailed protocol for synthesizing molecular beacons is available on the worldwide web at [http://www.phri.nyu.edu/molecular\\_beacons](http://www.phri.nyu.edu/molecular_beacons).

**Polymerase chain reactions.** Each 50- $\mu$ l reaction contained a different number of nucleic acid template molecules, 0.34  $\mu$ M molecular beacon, 1  $\mu$ M of each primer, 0.25 mM dATP, 0.25 mM dCTP, 0.25 mM dGTP, 0.50 mM dUTP, 2.5 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Foster City, CA), 50 mM KCl, 3.5 mM MgCl<sub>2</sub>, and 10 mM Tris-HCl, pH 8.3. Forty cycles of amplification (94°C denaturation for 30 sec, 50°C annealing for 60 sec, and 72°C polymerization for 30 sec) were carried out in sealed tubes in an Applied Biosystems 7700 Prism spectrofluorometric thermal cycler (Perkin-Elmer). The nucleotide sequence of the molecular beacon was fluorescein-5'-GCGAGCTAGGAAACACCAAAGATGATATTTGCTCGC-3'-DABCYL, where underlines identify the arm sequences. The 24-nucleotide-long probe sequence hybridized to a segment in the middle of the 84-nucleotide-long amplicon.

**Viewing multicolor molecular beacons.** Molecular beacons containing coumarin, EDANS, fluorescein, Lucifer yellow, tetramethylrhodamine, or Texas red were placed in pairs of test tubes. Each test tube contained 10  $\mu$ l of 10  $\mu$ M molecular beacon dissolved in 5 mM MgCl<sub>2</sub> and 20 mM Tris-HCl, pH 8.0. The EDANS molecular beacon was present at 30  $\mu$ M and the fluorescein molecular beacon was present at 2.5  $\mu$ M in order that the intensity of their fluorescence would be about the same as the intensity of the other molecular beacons. Complementary single-stranded target oligonucleotides (at a concentration equal to that of each molecular beacon) were added to one test tube of each pair, and fluorescence was viewed by illuminating the tubes with an ultraviolet lamp.

**Determination of quenching efficiency with different fluorophores.** The nucleotide sequence of all of the molecular beacons was fluorophore-5'-CCAAGCGCAAAGTATCATCCCTCCAGGCTTGG-3'-DABCYL, where underlines identify the arm sequences. The fluorescence of a 200- $\mu$ l solution containing 30 nM molecular beacon in 5 mM MgCl<sub>2</sub> and 20 mM Tris-HCl, pH 8.0, was determined at 25°C ( $F_{\text{bound}}$ ) with a QuantaMaster spectrofluorometer (Photon Technology International, South Brunswick, NJ), using maximal excitation and emission wavelengths. A twofold molar excess of target was added and the rise in fluorescence was monitored until it reached a stable level ( $F_{\text{free}}$ ). The excitation and emission maxima did not change when the molecular beacons were bound to targets. We confirmed that the restoration of fluorescence that occurs upon binding was complete by showing that digestion of the hybrids into nucleotides by incubation with deoxyribonuclease did not increase fluorescence further. Absorption spectra were determined with a Cary 219 spectrophotometer.

**Multiplex detection.** Four molecular beacons were used: coumarin-5'-GCGAGCCACCAAATATGATATGCTCGC-3'-DABCYL, fluorescein-5'-GCGAGCCACCAAATATGATATGCTCGC-3'-DABCYL, tetramethylrhodamine-5'-GCGAGCCACCAAATATGATATGCTCGC-3'-DABCYL, and Texas red-5'-GCGAGCCACCAAATATGATATGCTCGC-3'-DABCYL, where the underlined nucleotides identify the only differences among their sequences. The probe sequence in each molecular beacon was 15 nucleotides long. The targets were 5'-AAAGAAAATATCATNTTTGGTGTTCCTAT-3', where N was either A, G, T, or C. Four cuvettes were prepared, each containing a 200- $\mu$ l solution of 8.5 nM of each of the four molecular beacons in 5 mM MgCl<sub>2</sub> and 20 mM Tris-HCl, pH 8.0, thermostated at 25°C. Five microliters of a 14- $\mu$ M solution of one of the four oligonucleotide targets dissolved in the same buffer was added to each cuvette and the fluorescence of each of the four molecular beacons was monitored over time with a QuantaMaster spectrofluorometer. The reaction temperature and the concentration of the molecular beacons and the targets were kept low, in order to observe the kinetics of hybridization. Four fluorescence intensity measurements were repeated every 20 sec: exciting at 400 nm, the fluorescence of coumarin was measured at 475 nm for 5 sec; exciting at 491 nm, the fluorescence of fluorescein was measured at 515 nm for 5 sec; exciting at 555 nm, the fluorescence of tetramethylrhodamine was measured at 575 nm for 5 sec; and exciting at 595 nm, the fluorescence of Texas red was measured at 615 nm for 5 sec. The fluorescence

intensities of each fluorophore were normalized so that 0 represents fluorescence in the absence of target and 1 represents the final level of fluorescence achieved in response to the addition of an excess of perfectly complementary target. To obtain thermal transition profiles, the volume of the hybridization mixture in each cuvette was increased to 400  $\mu$ l by the addition of 5 mM MgCl<sub>2</sub> and 20 mM Tris-HCl, pH 8.0, and the solution was thermostated at 15°C for 2 h. The fluorescence of each molecular beacon was then monitored as the temperature in the cuvette was raised from 15°C to 75°C at a rate of 0.5°C per min. Temperature was determined with a thermistor immersed directly in the solution and was regulated with a programmable circulating water bath (Neslab, Portsmouth, NH).

**Comparison of hairpin probes to linear probes.** Four hybridization mixtures were prepared: hairpin probe with perfectly complementary target, linear probe with perfectly complementary target, hairpin probe with mismatched target, and linear probe with mismatched target. Each mixture consisted of 50  $\mu$ l of 125 nM probe, 500 nM target, 1 mM MgCl<sub>2</sub>, and 20 mM Tris-HCl, pH 8.0. The fluorescence of the mixtures was monitored in sealed tubes, in parallel, as a function of temperature in an Applied Biosystems 7700 Prism spectrofluorometric thermal cycler. The temperature was increased from 4°C to 65°C in 1°C steps, with each step lasting 5 min to ensure that equilibrium was achieved. The experiment was repeated utilizing different target concentrations. The melting temperature of each probe-target hybrid was the temperature at which the derivative of the fluorescence-temperature profile reached a maximum. The nucleotide sequence of the hairpin probe was fluorescein-5'-GCGAGAAGTTAAGACCTATGCTCGC-3' and the nucleotide sequence of the linear probe was fluorescein-5'-CTGTGAAGTTAAGACCTATGCCATC-3', where underlines identify the arm sequences. The nucleotide sequence of the perfectly complementary target was 5'-CATAGGTCTTAACCT-3'-DABCYL and the nucleotide sequence of the mismatched target was 5'-CATAGGTGTTAACCT-3'-DABCYL, where the underlined nucleotide in each sequence identifies the difference between them.

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