

Wavelength-shifting molecular beacons

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We describe wavelength-shifting molecular beacons, which are nucleic acid hybridization probes that fluoresce in a variety of different colors, yet are excited by a common monochromatic light source. The twin functions of absorption of energy from the excitation light and emission of that energy in the form of fluorescent light are assigned to two separate fluorophores in the same probe. These probes contain a harvester fluorophore that absorbs strongly in the wavelength range of the monochromatic light source, an emitter fluorophore of the desired emission color, and a nonfluorescent quencher. In the absence of complementary nucleic acid targets, the probes are dark, whereas in the presence of targets, they fluoresce—not in the emission range of the harvester fluorophore that absorbs the light, but rather in the emission range of the emitter fluorophore. This shift in emission spectrum is due to the transfer of the absorbed energy from the harvester fluorophore to the emitter fluorophore by fluorescence resonance energy transfer, and it only takes place in probes that are bound to targets. Wavelength-shifting molecular beacons are substantially brighter than conventional molecular beacons that contain a fluorophore that cannot efficiently absorb energy from the available monochromatic light source. We describe the spectral characteristics of wavelength-shifting molecular beacons, and we demonstrate how their use improves and simplifies multiplex genetic analyses.

Keywords: hybridization probes, FRET, real-time PCR, homogeneous assays, haplotype determination

Molecular beacons are hybridization probes that can report the presence of complementary nucleic acid targets without having to separate probe–target hybrids from excess probes in hybridization assays^{1,2}. Because of this property, they have been used for the detection of RNAs within living cells^{3,4}, for monitoring the synthesis of specific nucleic acids in sealed reaction vessels^{2,5}, and for the construction of self-reporting oligonucleotide arrays⁶. They can be used to perform homogeneous one-tube assays for the identification of single-nucleotide variations in DNA^{7–10} and for the detection of pathogens^{11,12}. A molecular beacon probe is a hairpin-shaped, single-stranded oligonucleotide comprising a probe sequence embedded within complementary sequences that form a hairpin stem. A fluorophore is covalently attached to one end of the oligonucleotide, and a nonfluorescent quencher is covalently attached to the other end. In the absence of target, the stem of the hairpin holds the fluorophore so close to the quencher that fluorescence does not occur. When this probe binds to its target, the rigidity of the probe–target duplex forces the stem to unwind, causing the separation of the fluorophore and the quencher and the restoration of fluorescence. This permits the detection of probe–target hybrids in the presence of unhybridized probes.

The fluorophore and the quencher are in such close proximity at the end of the hairpin stem that they share electrons, transiently forming a nonfluorescent complex that absorbs the energy of light and then loses that energy as heat. The transfer of energy from the fluorophore to the quencher within this complex takes place by a mechanism that is distinct from, and more efficient than, fluorescence resonance energy transfer (FRET), which only occurs when the fluorophore and the quencher interact over a distance of 20–100 Å, and only when there is a significant overlap between the emission spectrum of the fluorophore and the absorption spectrum of the quencher¹³. Quenching by FRET is limited to only a few pairs of interacting compounds, and it is not particularly efficient. Quenching in molecular beacons, on the other hand, is extremely efficient. A single nonfluorescent chromophore, such as dabcyI, can serve as a universal quencher for a large variety of fluorophores,

whether or not its absorption spectrum overlaps the emission spectrum of the fluorophore².

Even though it is possible to construct molecular beacons that fluoresce in a variety of different colors, the number of different molecular beacons that can be used in the same solution to detect different targets simultaneously is limited by the capability of available instruments. In particular, instruments that perform programmed changes in temperature for polymerase chain reactions (PCRs) while simultaneously monitoring fluorescence in real time, fluorescence-activated cell sorters, and a variety of different microscopes, utilize a monochromatic light source, such as a laser or light-emitting diodes, for the excitation of fluorophores^{14–16}. Monochromatic light sources excite some fluorophores optimally but excite other fluorophores less well or not at all. To overcome this limitation, we describe a strategy to make a series of different molecular beacons in which each probe emits fluorescent light in a distinct range of the visible spectrum, yet each can be efficiently excited by the same monochromatic light source. In these probes, the functions of absorption of energy from light and the emission of that energy as fluorescent light are performed by two separate fluorophores. Because the emission spectrum is shifted from the characteristic emission range of the fluorophore that absorbs the incident light to the characteristic emission range of a second fluorophore, we refer to these probes as “wavelength-shifting” molecular beacons. This approach enhances the multiplexing capacity of nucleic acid detection assays and allows those assays to be performed on simpler devices.

Results

Design of wavelength-shifting molecular beacons. In a conventional molecular beacon, the functions of absorbing energy from light and of emitting that energy as fluorescent light of a longer wavelength reside in the same chemical moiety. In order to construct a series of molecular beacons that are excited by the same monochromatic light source, yet possess the capacity to emit strong fluorescent signals in many different colors, we assign these functions to two separate fluorophores. These modified molecular beacons contain a “harvester” fluorophore

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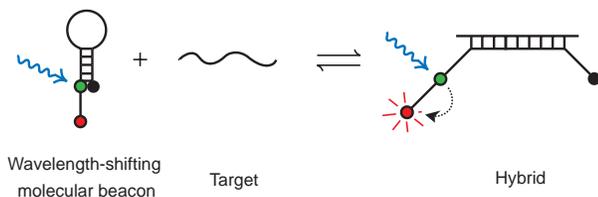


Figure 1. Principle of operation of wavelength-shifting molecular beacons. A wavelength-shifting molecular beacon contains three labels: a quencher moiety at the end of its 3' arm (black circle), a harvester fluorophore at an internal location in its 5' arm that is opposite to the quencher in the hairpin conformation (green circle), and an emitter fluorophore at the distal end of its 5' arm (red circle). The harvester fluorophore is chosen so that it efficiently absorbs energy from the available monochromatic light source. In the absence of targets, these probes are dark, because the energy absorbed by the harvester fluorophore is rapidly transferred to the quencher and is lost as heat. In the presence of targets, molecular beacons undergo a conformational reorganization caused by the rigidity of the probe–target duplex, which forcibly separates the 5' arm from the 3' arm. In the target-bound conformation, the energy absorbed by the harvester fluorophore is transferred by FRET to the emitter fluorophore, which then releases the energy as fluorescent light of its own characteristic color.

and an “emitter” fluorophore, in addition to a quencher (Fig. 1). The harvester fluorophore is chosen to have high absorbance in the wavelength range of an available monochromatic light source. It is placed at an internal location within the 5' arm of the oligonucleotide, so that in the hairpin conformation of the molecular beacon it is in close proximity to the quencher, which is located at the 3' end. The molecule contains a spacer arm, several nucleotides long, that extends past the position of the harvester fluorophore on the 5' arm of the oligonucleotide. The emitter fluorophore is covalently linked to the 5' end of the spacer arm. This design for wavelength-shifting molecular beacons is based on our hypothesis that the transfer of energy from a fluorophore to a quencher that is in close physical contact is more rapid than the transfer of energy between two fluorophores that are at a distance from each other and interact by FRET.

Like conventional molecular beacons, wavelength-shifting molecular beacons assume a hairpin conformation when they are not bound to their target, emitting little light in the emission ranges of either fluorophore. The energy of the light absorbed by the harvester fluorophore is efficiently channeled to the quencher moiety and is lost as heat, because the two moieties exist in close proximity. When the probe sequence in the loop binds to its target, the rigidity of the resulting probe–target duplex drives the dissociation of the stem hybrid, and the quencher is removed from physical contact with the harvester fluorophore. However, unlike what occurs in conventional molecular beacons, the fluorescence of the harvester fluorophore is not restored, because its stored energy is transferred by FRET to the emitter fluorophore. The distance between the harvester fluorophore and the emitter fluorophore is chosen so that efficient resonance energy transfer takes place between them. After storing the transferred energy for a few nanoseconds, the emitter fluorophore releases the energy as fluorescent light of its own characteristic color. Therefore, in the target-bound state, wavelength-shifting molecular beacons absorb light in the optimal excitation wavelength range of the harvester fluorophore and emit light in the characteristic emission wavelength range of the emitter fluorophore. Thus, molecular beacons can be designed so that they emit strong signals in a variety of desired colors by selecting emitter fluorophores possessing appropriate emission characteristics.

Depending upon the wavelength of the available monochromatic light source, an appropriate fluorophore can be chosen to serve as the harvester fluorophore. Concentrating on blue argon-ion lasers (488 nm) and blue light-emitting diodes (459–479 nm), which are

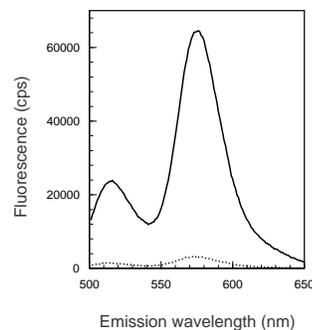


Figure 2. Emission spectrum of a wavelength-shifting molecular beacon with fluorescein as its harvester fluorophore and tetramethylrhodamine as its emitter fluorophore, when excited by monochromatic blue light (488 nm) in the presence of targets (continuous line), and in the absence of targets (dotted line).

the most commonly used light sources, we chose fluorescein as the harvester fluorophore. Fluorescein is excited optimally in this wavelength range. We chose dabcyI as the quencher because it is nonfluorescent and quenches the fluorescence of fluorescein extremely well when these two moieties are in close proximity². For emitter fluorophores, we chose a series of fluorophores that are excited poorly by blue light, but that are good acceptors of energy from fluorescein. The peak emission wavelengths of these fluorophores are well spaced from each other, making them suitable for use in multiplex detection experiments. The physical distance between the emitter fluorophore and the harvester fluorophore can be optimized by varying the number of nucleotides in the spacer arm that separates them. Glazer and his colleagues empirically determined that a seven-nucleotide spacer permits optimal energy transfer between fluorescein and rhodamine moieties that are covalently linked to a single-stranded DNA molecule^{17,18}. We therefore utilized a six- or seven-nucleotide oligodeoxythymidine spacer to separate the harvester fluorophore from the emitter fluorophore.

Spectral characteristics of wavelength-shifting molecular beacons. In order to determine if this design yields probes that possess the characteristics desired of wavelength-shifting molecular beacons, we synthesized three probes, each containing dabcyI as the quencher and fluorescein as the harvester fluorophore, and either 6-carboxyrhodamine 6G, tetramethylrhodamine, or Texas red as the emitter fluorophore. The fluorescence intensity of solutions of each of these probes was recorded as a function of emission wavelength, both in the absence of targets and in the presence of an excess of targets, while holding the excitation wavelength constant at 488 nm. The emission spectrum (in the presence and absence of targets) of the wavelength-shifting molecular beacon that contained tetramethylrhodamine as its emitter fluorophore is shown in Figure 2. These spectra show that the predominant emission is in the characteristic emission range of tetramethylrhodamine. A comparison of the spectrum in the presence of targets (continuous line) to the spectrum in the absence of targets (dotted line) indicates that target-bound probes are substantially more fluorescent than free probes. In the peak emission range of tetramethylrhodamine (575 nm), fluorescence increased 18-fold upon the addition of targets. When this experiment was repeated with wavelength-shifting molecular beacons containing either 6-carboxyrhodamine 6G or Texas red as their emitter fluorophore, and fluorescence was measured in the peak emission ranges of each fluorophore, a 13-fold and an 11-fold enhancement in fluorescence was observed, respectively, for each fluorophore. The overall shape of the spectrum for each molecular beacon was the same in the target-bound conformation as it was when the molecular beacons were free in solution.

The observation that most of the fluorescence occurs in the emission range of tetramethylrhodamine, rather than in the emission

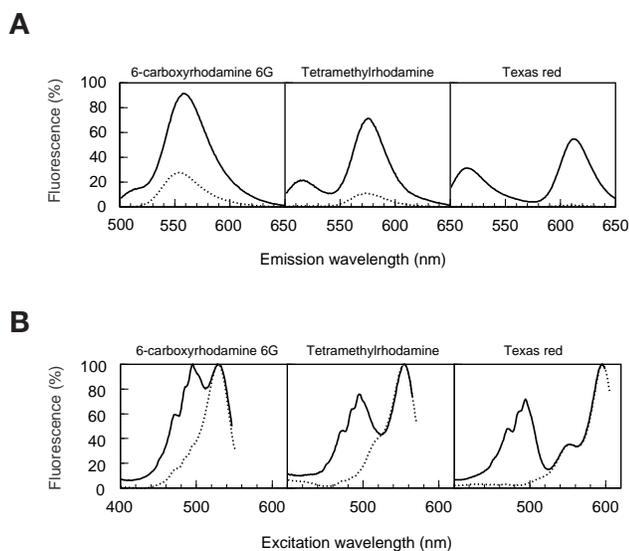


Figure 3. Enhancement in the brightness of molecular beacons. (A) Comparison of the emission spectrum of different wavelength-shifting molecular beacons (continuous lines) with the emission spectrum of corresponding conventional molecular beacons (dotted lines), when each molecular beacon is excited by monochromatic blue light (488 nm). **(B)** Comparison of the excitation spectrum of different wavelength-shifting molecular beacons (continuous lines) with the excitation spectrum of corresponding conventional molecular beacons (dotted lines). The excitation spectra were recorded at the optimal emission wavelength of the emitter fluorophore contained in each molecular beacon: 556 nm for 6-carboxyrhodamine 6G, 575 nm for tetramethylrhodamine, and 609 nm for Texas red.

range of fluorescein, indicates that the transfer of energy from fluorescein to tetramethylrhodamine is efficient. This was also reflected in the suppression of the fluorescein fluorescence. In order to measure the extent of this suppression, a solution containing the same wavelength-shifting molecular beacon and an excess of its oligonucleotide target was incubated in the presence of deoxyribonuclease I. This treatment resulted in the complete degradation of the DNA, abolishing the transfer of energy from fluorescein to dabcy1 and from fluorescein to tetramethylrhodamine. Deoxyribonuclease I treatment resulted in a 15-fold increase in the intensity of fluorescence at 515 nm (which is the emission maximum of fluorescein) from its level when the probes were intact and bound to their targets, indicating that fluorescence from the harvester fluorophore is suppressed, both in the target-free state and in the target-bound state.

Improvement in fluorescence yield. In order to estimate the improvement in the sensitivity of target detection obtained with wavelength-shifting molecular beacons, we compared the spectral characteristics of wavelength-shifting molecular beacons to the spectral characteristics of conventional molecular beacons. Figure 3A shows the emission spectra of wavelength-shifting molecular beacons possessing different emitter fluorophores (continuous lines) and compares them to the emission spectra of correspondingly labeled conventional molecular beacons (dotted lines). Each molecular beacon was bound to its target and was present at the same concentration. The emission spectra were recorded utilizing an excitation wavelength of 488 nm. These spectra are plotted on a normalized scale, where 100% is the highest level of fluorescence of each conventional molecular beacon when it is bound to its target and is excited at its optimal excitation wavelength. When a conventional molecular beacon possessing 6-carboxyrhodamine 6G was excited at 488 nm, its fluorescence intensity was only 28% of the fluorescence intensity that is obtained when it is stimulated at its optimal excitation wavelength (530 nm) (dotted line in the left panel of Fig. 3A). Similarly, when excited at 488 nm, conventional molecular beacons possessing tetramethylrhodamine yielded only 11% of

their maximum fluorescence, and those possessing Texas red yielded only 1.3% of their maximum fluorescence. However, when these fluorophores were used as the emitter fluorophore in wavelength-shifting molecular beacons possessing fluorescein as the harvester fluorophore, and when they were stimulated by 488 nm light, their fluorescence intensity increased substantially. The fluorescence of the wavelength-shifting molecular beacon possessing 6-carboxyrhodamine 6G reached 90% of its maximum fluorescence. Similarly, the fluorescence of the wavelength-shifting molecular beacon possessing tetramethylrhodamine was 71% of its maximum fluorescence, and the probe possessing Texas red achieved 55% of its maximum fluorescence.

These emission spectra also indicate the extent to which the green fluorescence of fluorescein is suppressed in each wavelength-shifting molecular beacon. Whereas wavelength-shifting molecular beacons possessing 6-carboxyrhodamine 6G exhibit almost no fluorescence in the emission range of fluorescein, wavelength-shifting molecular beacons possessing tetramethylrhodamine and wavelength-shifting molecular beacons possessing Texas red emit increasing levels of fluorescence in that range (Fig. 3A). The increasing intensity of these fluorescein emission peaks reflects the decreasing efficiency of FRET as emitter fluorophores that fluoresce further toward the red end of the visible spectrum are used.

Enhancement in the intensity of the fluorescence of the emitter fluorophore was also apparent when the excitation spectrum of each wavelength-shifting molecular beacon was compared to the excitation spectrum of the corresponding conventional molecular beacon. In these experiments, the fluorescence intensity of the emitter fluorophore was measured at its optimal emission wavelength, as the excitation wavelength was varied. The results are shown in Figure 3B, where 100% represents the fluorescence of each fluorophore at its optimal excitation wavelength. The excitation spectra of the conventional molecular beacons (dotted lines) show poor excitation at wavelengths below 500 nm, whereas the excitation spectra of the wavelength-shifting molecular beacons (continuous lines) exhibit additional excitation peaks in this range, which are attributable to the absorption of energy from the stimulating light by fluorescein. These results show that the excitation spectrum of wavelength-shifting molecular beacons has the combined absorption characteristics of both the harvester fluorophore and the emitter fluorophore, whereas the emission spectrum predominantly exhibits the emission characteristics of the emitter fluorophore.

In order to estimate the enhancement in the brightness of the emitter fluorophore in each wavelength-shifting molecular beacon, the peak fluorescence intensity of each wavelength-shifting molecular beacon when it was excited at 488 nm was divided by the peak fluorescence intensity of the corresponding conventional molecular beacon when it was excited at 488 nm. There was a 2.3-fold enhancement in the fluorescence intensity of 6-carboxyrhodamine 6G, a 7.2-fold enhancement in the fluorescence of tetramethylrhodamine, and a 33-fold enhancement in the fluorescence of Texas red. The larger gain in the intensity of fluorescence as one moves from emitter fluorophores that fluoresce in the yellow wavelength range to fluorophores that fluoresce in the red wavelength range is not due to an increased efficiency of energy transfer, but rather is a reflection of the decreased efficiency of excitation of the red fluorophores by 488 nm light in the conventional molecular beacons.

Increased signal strength in real-time PCRs. In order to demonstrate improvements that result from the use of wavelength-shifting molecular beacons on an instrument that utilizes a 488 nm light source, we compared the intensity of the fluorescence signals from wavelength-shifting molecular beacons to the intensity of the fluorescence signals from corresponding conventional molecular beacons in multiplex assays. In these experiments, each reaction contained a mixture of four molecular beacons, each of which was specific for a different target and each of which had an emission spectrum that was distinguishable from the others. These molecular beacons were designed to be "allele-discriminating," which ensures that the presence of even a

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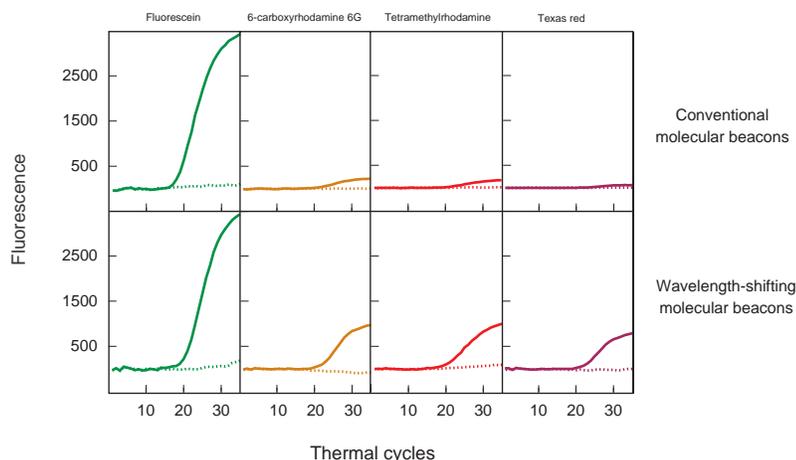


Figure 4. Multiplex detection of the products of PCRs containing conventional molecular beacons and wavelength-shifting molecular beacons. Every reaction contained an equimolar mixture of four different allele-discriminating molecular beacons, each specific for a different target sequence and each labeled with a differently colored fluorophore. All probes used in the first set of reactions (upper panels) were conventional molecular beacons labeled with either fluorescein, 6-carboxyrhodamine 6G, tetramethylrhodamine, or Texas red, whereas the second set of reactions (lower panels) contained a conventional molecular beacon labeled with fluorescein as their harvester fluorophore and three wavelength-shifting molecular beacons possessing fluorescein as their harvester fluorophore and either 6-carboxyrhodamine 6G, tetramethylrhodamine, or Texas red as their emitter fluorophore. Four of the reactions in each set were each initiated with a different template DNA (differing from each other only by the identity of a single nucleotide at the same position). The fifth reaction in each set did not contain any template DNA. The fluorescence of the molecular beacon that was perfectly complementary to the target added to initiate the reaction is plotted in each panel (continuous line) along with the fluorescence of the same molecular beacon in the control reaction to which no template had been added (dotted line).

single nucleotide substitution in the target strand will prevent the formation of a probe–target hybrid^{2,10}. In order to ensure that any observed differences in fluorescence would not be due to differential rates of amplification of the different template DNAs or differential accessibility of the resulting target amplicons to the molecular beacons, we used the same pair of primers in each reaction to amplify DNA templates that differed from each other only by the identity of a single nucleotide at the same position. Furthermore, each molecular beacon was present at the same concentration. We performed two sets of reactions on a PE Biosystems 7700 Prism spectrofluorometric thermal cycler with multiplex detection in real time. The first set of reactions each contained four conventional molecular beacons that were labeled with either fluorescein, 6-carboxyrhodamine 6G, tetramethylrhodamine, or Texas red. The second set of otherwise identical reactions each contained a conventional molecular beacon labeled with fluorescein and three wavelength-shifting molecular beacons possessing fluorescein as their harvester fluorophore and either 6-carboxyrhodamine 6G, tetramethylrhodamine, or Texas red as their emitter fluorophore. Inclusion of a conventional fluorescein molecular beacon in each set served as an internal standard against which the signal strengths of the other molecular beacons could be compared.

Five PCRs were performed in each set. Four reactions were initiated with one of the four different DNA templates, and the fifth reaction was a control that did not contain any template DNA. The intensity of the fluorescence of each molecular beacon was monitored during the course of each reaction. Figure 4 shows, for each reaction, the change in the fluorescence intensity of the particular molecular beacon in the mixture that was perfectly complementary to the target sequence in the DNA template that was added to initiate the reaction (continuous lines), and the change in the fluorescence intensity of the same molecular beacon in the absence of added template DNA (dotted lines). The top panels show that conventional molecular beacons labeled with 6-carboxyrhodamine 6G, tetramethylrhodamine, or Texas red yield

very low levels of fluorescence compared with the fluorescence of the molecular beacon that was labeled with fluorescein. The bottom panels show that when these fluorophores are used as the emitter fluorophore in corresponding wavelength-shifting molecular beacons, their fluorescence intensity is significantly enhanced and is comparable to the fluorescence intensity of fluorescein, which is the strongest fluorophore known and was excited at a wavelength close to its excitation maximum. There was a 4-fold, 6-fold, and 12-fold net gain, respectively, in the fluorescence intensity of 6-carboxyrhodamine 6G, tetramethylrhodamine, and Texas red when these fluorophores were used as labels in wavelength-shifting molecular beacons instead of in conventional molecular beacons.

Multiplex genetic analysis. In order to demonstrate the utility of wavelength-shifting molecular beacons for the solution of complex genotyping problems in which more than one type of target sequence may be present in the same reaction, we used these probes in assays that simultaneously detect two different single-nucleotide polymorphisms that occur at positions only a few nucleotides apart. Such polymorphisms are difficult to detect because allele-specific hybridization probes cannot be designed to detect each nucleotide substitution independently. Although closely spaced polymorphisms are sparse throughout the human genome, they are common in viral genomes and are often associated with drug resistance. As an example, we studied a pair of polymorphisms in the human gene encoding chemokine receptor 5, in which a cytidine residue can substitute for a thymidine residue at position 627, and a thymidine residue can substitute for a cytidine residue at position 630^{19,20}. Thus, four

different haplotypes can occur in this region (**627•630•) of the chemokine receptor 5 gene: •T•C•, •C•C•, •T•T•, and •C•T•. Because every person receives two copies of the chemokine receptor 5 gene (one from their mother and one from their father) and the gene from each parent can be any of the four possible haplotypes, ten different genotypes can occur (see Table 1). In order to uniquely determine which of these ten genotypes is present in an individual in a single assay, we constructed a set of four different allele-discriminating molecular beacons, each designed to hybridize to only one of the four haplotypes, and each designed to fluoresce in a different color. Two of these probes were conventional molecular beacons, one labeled with fluorescein and the other labeled with tetrachlorofluorescein, and the other two probes were wavelength-shifting molecular beacons, both possessing fluorescein as their harvester fluorophore, and one possessing tetramethylrhodamine as its emitter fluorophore and the other possessing Texas red as its emitter fluorophore. Using all four molecular beacons together, we performed multiplex PCRs on a collection of 24 different human DNA samples derived from randomly selected individuals, as well as on ten different artificial DNA templates with known genotypes.

The color of the fluorescence that developed at the end of each gene amplification reaction uniquely identified which of the ten possible genotypes was present. Table 1 shows the color of the fluorescence that developed in response to each of the ten artificial genotypes. The fractional fluorescence intensity of each molecular beacon in the mixture was calculated by dividing the fluorescence intensity from that molecular beacon by the sum of the fluorescence intensities of all four molecular beacons. Only one of the four molecular beacons fluoresced strongly when one of the four homozygous genotypes was present, and only two of the molecular beacons fluoresced strongly when one of the six heterozygous genotypes was present. The colors of the fluorescent signal uniquely identified the genotype of the DNA. Using the fractional fluorescence intensities observed for each artificial genotype as a guide to what is to be expected from human DNA of each genotype, we

Table 1. Genotyping two closely spaced human alleles with molecular beacons in multiplex polymerase chain reactions^a

Genotype	Fractional fluorescence observed with artificial DNA templates				Number of human DNA samples with this pattern
	Fluorescein	Tetrachloro-fluorescein	Tetramethyl-rhodamine	Texas red	
••T•C•• / ••T•C••	0.95	0	0.05	0	6
••C••T•• / ••C••T••	0	1.00	0	0	0
••C••C•• / ••C••C••	0	0	1.00	0	4
••T••T•• / ••T••T••	0	0	0	1.00	0
••T•C•• / ••C••T••	0.51	0.49	0	0	0
••T•C•• / ••C••C••	0.77	0	0.23	0	11
••T•C•• / ••T••T••	0.73	0	0	0.27	1
••C••T•• / ••C••C••	0	0.68	0.32	0	0
••C••T•• / ••T••T••	0	0.56	0	0.44	0
••C••C•• / ••T••T••	0	0	0.45	0.55	2

^aEach reaction contained a mixture of two conventional molecular beacons (one labeled with fluorescein and the other labeled with tetrachlorofluorescein) and two wavelength-shifting molecular beacons (one labeled with tetramethylrhodamine and the other labeled with Texas red). Each of the ten possible genotypes involving the two alleles at positions 627 and 630 in the chemokine receptor 5 gene are indicated in the left-hand column. Fractional fluorescence was calculated by dividing the fluorescence of a particular molecular beacon by the total fluorescence of all four molecular beacons, measured at the end of the reaction. The right-hand column shows the number of human DNA samples (out of 24 samples) whose fractional fluorescence values closely matched the fractional fluorescence values of each control genotype (variation was <2%).

tested 24 randomly selected human DNA samples (see right-hand column of Table 1). The fractional fluorescence intensities obtained from each of the human DNA samples perfectly matched the fractional fluorescence intensities obtained from one of the ten artificial genotypes. Significantly, the observed frequency of each genotype in the human DNA samples reflected the known frequency of occurrence of the two polymorphisms^{19,20}. These results also demonstrate that signals from wavelength-shifting molecular beacons do not spill over to a significant extent into the fluorescein emission range, since the fluorescein signal remained negative when the wavelength-shifting molecular beacon signals became positive. These results show that wavelength-shifting molecular beacons enable the detection of as many as ten different genotypes in a single-tube multiplex assay.

Discussion

Molecular beacons that utilize the arrangement of the fluorophores and the quencher shown in Figure 1 are dark in the absence of targets and exhibit wavelength-shifted fluorescence when bound to targets. We constructed other probes with similar characteristics, utilizing several different topological schemes. In these alternative arrangements, the harvester fluorophore was in intimate contact with the quencher in the hairpin conformation and was located at an optimal distance from the emitter fluorophore, in order for FRET to occur when the probe is bound to its target. For example, the emitter fluorophore can be placed at the junction of the 5' end of the hairpin loop and the 3' end of the 5' arm, eliminating the need for a spacer arm. We have also found that the positions of the harvester fluorophore and the emitter fluorophore can be reversed, both in the scheme just described and in the scheme shown in Figure 1. Furthermore, molecules that contain two quencher moieties in one arm and two fluorophore moieties in the other arm can function as wavelength-shifting molecular beacons. It should also be possible to introduce wavelength-shifting features into molecular beacons by utilizing energy transfer dyes that contain two interacting fluorophores linked by an aminomethylbenzoic acid linker, as described by

Lee and her colleagues²¹. However, an oligonucleotide spacer offers a simpler route for the automated synthesis of wavelength-shifting molecular beacons.

Although the emission spectra of wavelength-shifting molecular beacons that utilize fluorescein as their harvester fluorophore are dominated by the fluorescence of the emitter fluorophore, there is some residual fluorescence in the emission range of fluorescein. This residual emission can be reduced by choosing a cyanine dye, such as 3-(ϵ -carboxypentyl)-3'-ethyl-5,5'-dimethylxocarbocyanine, in place of fluorescein, because these fluorophores absorb more energy at 488 nm than fluorescein does, yet they have lower fluorescence quantum yields²².

One limitation of conventional molecular beacons is that the optimal emission wavelength is usually only a few nanometers longer than the optimal excitation wavelength (Stokes shift). Consequently, a portion of the excitation light can reach the detector by scattering and reflection, thus limiting detection sensitivity. The large Stokes shifts of wavelength-shifting molecular beacons, on the other hand, allow more effective filtering of the excitation light, thereby enhancing the sensitivity of target detection. Large Stokes shifts are

particularly significant in the detection of probe-target hybrids in living cells, where amplification of the primary target is not possible, and where autofluorescence of cellular components introduces a background signal. Because autofluorescence is characterized by relatively small Stokes shifts, it is possible to filter this background signal and to enhance the sensitivity of detection of nucleic acids in vivo through the use of wavelength-shifting molecular beacons.

Wavelength-shifting molecular beacons enable more reliable multiplex genetic analyses, because a comparable level of fluorescence is obtained from a variety of different molecular beacons, each emitting fluorescent light in a different color range. Using a combination of conventional molecular beacons and wavelength-shifting molecular beacons it will be possible to reliably perform six-plex or seven-plex PCRs. In addition to increasing the multiplexing capacity of existing instruments, wavelength-shifting molecular beacons will enable very simple devices (constructed from light-emitting diodes, optical filters, and cameras) to be used for multiplex real-time amplification reactions.

Experimental protocol

Synthesis of molecular beacons. Both conventional and wavelength-shifting molecular beacons were prepared by solid-phase DNA synthesis on a PE Biosystems (Foster City, CA) 394 DNA synthesizer. A controlled-pore glass column (Biosearch Technologies, Novato, CA) was used to incorporate a quencher moiety, 4-dimethylaminoazobenzene-4'-sulfonyl (dabcyl), at the 3' end of the oligodeoxyribonucleotides, and a fluorescein phosphoramidite (Glen Research, Sterling, VA) was used to incorporate a fluorescein moiety at an internal location within the oligonucleotides. Emitter fluorophores were introduced at the 5' end of the oligonucleotides in postsynthetic reactions. In order to make the oligonucleotides receptive to reactive fluorophores, either an amino or a thiol group was introduced at the 5' end of the oligonucleotides, using a hexalkyl-aminomodifier phosphoramidite or a hexalkyl-thiolmodifier phosphoramidite (both from Glen Research). The oligonucleotides, with their amino or thiol groups protected by trityl moieties, were purified by high-pressure liquid chromatography (HPLC) on a C-18 reverse-phase column (Waters Corp., Milford, MA). The trityl moieties were removed before coupling the emitter fluorophores. 6-carboxyrhodamine 6G was introduced by reacting its succinimidyl ester with a 5'-amino

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group, and fluorescein, tetramethylrhodamine, or Texas red was introduced by reacting their acetamides with a 5'-thiol group (all reactive fluorophores were purchased from Molecular Probes, Eugene, OR). Oligonucleotides that contained all three labels (5' fluorophore, internal fluorescein, and 3' dabcy) were purified by gel-exclusion chromatography on a NAP-5 Sephadex column (Amersham Pharmacia Biotech, Piscataway, NJ), followed by HPLC on a C-18 column. Details of the synthetic steps are available on the internet²³.

Two sets of allele-discriminating molecular beacons were synthesized to explore their spectral characteristics: one set of wavelength-shifting molecular beacons and one set of conventional molecular beacons. The sequence of the molecular beacons within each set differed from each other only by the identity of one nucleotide at the same position within the probe sequence. The nucleotide sequences of the wavelength-shifting molecular beacons were as follows: emitter fluorophore-5'-TTTTTT-fluorescein-CCACGCTTGTNGGTCAACCCCGTGG-3'-dabcy, where underlines identify the nucleotides that participate in the formation of the hairpin stem and N represents the distinguishing nucleotide in the probe sequence. The nucleotide sequences of the conventional molecular beacons were as follows: fluorophore-5'-CCACGCTTGTNGGTCAACCCCGTGG-3'-dabcy. The emitter fluorophore in both sets of molecular beacons was either 6-carboxyrhodamine 6G, tetramethylrhodamine, or Texas red, with the corresponding distinguishing nucleotide being adenosine, cytidine, or guanosine, respectively. A conventional molecular beacon with fluorescein as its reporter fluorophore and thymidine as its distinguishing nucleotide was the fourth member of each set. Four different synthetic targets for these molecular beacons were prepared that differed from each other only by the identity of one nucleotide situated at the same position. The nucleotide sequences of these target oligonucleotides were as follow: 5'-CTGTGGGGTTGACCN*ACAAGCGC-CG-3', where N* is the nucleotide that is complementary to nucleotide N in the probe sequence of one of the four molecular beacons in each set.

Spectral measurements. The emission spectra of wavelength-shifting molecular beacons were recorded by holding the excitation wavelength constant at 488 nm while varying the wavelength at which emission was recorded from 500 nm to 650 nm in a QuantaMaster spectrofluorometer (Photon Technology International, South Brunswick, NJ). Spectra were recorded at 25°C from a 150 µl solution that contained 30 nM molecular beacon, 1 mM MgCl₂, and 20 mM Tris-HCl (pH 8.0). These measurements were repeated after adding a 5-fold molar excess of perfectly complementary target oligonucleotide and waiting 30 min to enable hybridization to reach completion. The spectra of wavelength-shifting molecular beacons bound to their targets were compared with the spectra of corresponding conventional molecular beacons bound to targets under the same conditions. The excitation spectrum of each probe was recorded by determining its fluorescence intensity at the optimal emission wavelength of each emitter fluorophore while varying the excitation wavelength.

Monitoring PCRs. We carried out ten reactions, five with a set of conventional molecular beacons labeled with fluorescein, 6-carboxyrhodamine 6G, tetramethylrhodamine, or Texas red, and five with a set consisting of a conventional molecular beacon labeled with fluorescein and three wavelength-shifting molecular beacons labeled with 6-carboxyrhodamine 6G, tetramethylrhodamine, or Texas red. For each probe set, four reactions were initiated with 100,000 molecules of one of the four different templates¹⁰, and a fifth reaction contained no template. Each reaction contained the same pair of primers¹⁰. These 50 µl reactions contained 100 nM of each molecular beacon, 500 nM of each primer, 2 units of AmpliTaq Gold DNA polymerase (PE Biosystems), 250 µM dATP, 250 µM dCTP, 250 µM dGTP, 250 µM dTTP, 4 mM MgCl₂, 50 mM KCl, and 10 mM Tris-HCl (pH 8.0). The thermal cycling program consisted of 10 min at 95°C to activate the DNA polymerase, followed by 40 cycles of 30 s at 95°C, 45 s at 56°C, and 30 s at 72°C. Fluorescence was monitored during the 56°C annealing steps in a PE Biosystems 7700 Prism spectrofluorometric thermal cycler, which uses an argon-ion laser (488 nm) as its excitation source. Before initiating the PCRs, we determined the emission spectrum of each molecular beacon and stored it in the memory of the computer controlling the spectrofluorometric thermal cycler. Each reference spectrum was obtained from a solution of one of the molecular beacons in the presence of an excess of its oligonucleotide target, in which the concentration of the molecular beacon, the composition of the buffer, and the temperature at which the spectrum was read, were the same as were used in the PCRs. The computer utilized the individual reference spectra for the decomposition of the complex emission spectra generated during the PCRs into the spectral contributions from each of the four differently colored molecular beacons.

Genotyping. The sequences of the four allele-discriminating molecular beacons for the simultaneous genotyping of two different alleles in the

human gene encoding chemokine receptor 5 were as follows: fluorescein-5'-CGCACCTCTGGTCTGAAAGTTTATTGGTGGCG-3'-dabcy, tetrachlorofluorescein-5'-CGCACCTCTGGTCTAAAGTTTATTGGTGGCG-3'-dabcy, tetramethylrhodamine-5'-TTTTTT-fluorescein-CGCACCTCTGGTCT-GAAGGTTTATTGGTGGCG-3'-dabcy, and Texas red-5'-TTTTTT-fluorescein-5'-CGCACCTCTGGTCTAAAGTTTATTGGTGGCG-3'-dabcy, where underlines identify the nucleotides that participate in the formation of the hairpin stem and bold letters identify the distinguishing nucleotides in each probe sequence. The sequence of the forward primer was 5'-AGATGAATG-TAAATGTCTCTAG-3', and the sequence of the reverse primer was 5'-CTTTTAAAGTTGAGCTTAAATAAGC-3'.

Artificial DNA templates corresponding to the four different haplotypes were prepared by PCR-mediated in vitro mutagenesis²⁴. They were used as artificial DNA templates that mimicked each of the four homozygous genotypes. They were also mixed in equimolar ratios to obtain templates that mimicked each of the six heterozygous genotypes. These ten artificial DNAs were each used as template in a PCR in the presence of all four molecular beacons, in order to determine the response of each molecular beacon in the mixture to a given genotype. Then, DNA samples from 24 randomly selected people (Coriell Cell Repositories, Camden, NJ) were analyzed in the same manner to determine their genotype.

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