Genotyping Single Nucleotide Polymorphisms
with Molecular Beacons

Introduction

Single-nucleotide substitutions represent the largest source of diversity in the human genome. Some of these variations have been directly linked to human disease, though the vast majority are neutral. Even neutral variations are important because they provide guideposts in the preparation of detailed maps of the human genome, serving as essential elements in linkage analyses that identify genes responsible for complex disorders (1). Although sequencing is adequate for the initial discovery of single-nucleotide variations, simpler, faster, and more automated genotyping methods are needed for routine clinical diagnostics and population studies. High-throughput methods are essential for understanding the distribution of genetic variations in populations, as well as for identifying the genes responsible for genetic disorders. Current alternatives to sequence analysis either miss some single-nucleotide substitutions or are too complex to enable high-throughput assays (2).

We have developed a simple method for typing single nucleotide polymorphisms in which alleles are identified by fluorescent colors generated in sealed amplification tubes. In this technique, amplification is carried out in the presence of two different molecular beacons and each allele is identified by the development of fluorescence of a unique color. Molecular beacons are single-stranded oligonucleotide probes that become fluorescent when they bind to perfectly complementary nucleic acids (3). Because they are non-fluorescent when they are not bound to their target, they can be used in hybridization reactions without having to separate the probe-target hybrids from the non-hybridized probes. Molecular beacons possess a stem-and-loop structure. A fluorophore is covalently linked to one end of the molecule and a quencher is covalently linked to the other end. When not bound to target, the hairpin stem keeps the fluorophore so close to the quencher that fluorescence does not occur. The energy absorbed by the fluorophore is transferred to the quencher and released as heat. However, when the probe sequence in the loop anneals to its target sequence, the rigidity of the probe-target hybrid forces the hairpin stem to unwind, separating the fluorophore from the quencher, and restoring fluorescence (Figure 1). Because molecular beacons can possess a wide variety of differently colored fluorophores, multiple targets can be distinguished in the same solution, using several different molecular beacons, each designed to detect a different target, and each labeled with a different fluorophore (4-6).
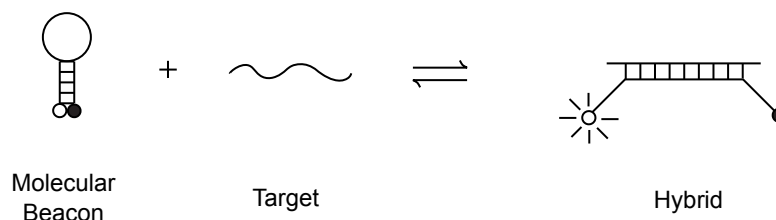

Molecular Beacon   Target   Hybrid

Figure 1. Principle of operation of molecular beacons. When the probe sequence in the loop of a molecular beacon binds to a target sequence a conformational reorganization occurs that restores the fluorescence of a quenched fluorophore.

The ability of molecular beacons to report the presence of their targets without having to isolate the probe-target hybrids makes them useful in a wide array of applications. They can be used to monitor the progress of polymerase chain reactions (4,7) and nucleic acid sequence-based amplification reactions in sealed tubes (8,9). They can be used to construct self-reporting oligonucleotide arrays (10) and to detect specific mRNA sequences in living cells (11,12).

Molecular beacons are uniquely suited for the detection of single-nucleotide variations because they recognize their targets with significantly higher specificity than conventional oligonucleotide probes (4,7,13). Their high specificity is a consequence of their hairpin structure (13). When a molecular beacon binds to its target sequence, the formation of the probe-target hybrid occurs at the expense of the stem hybrid. Molecular beacons can be designed so that over a wide range of temperatures only perfectly complementary probe-target hybrids are sufficiently stable to force open the stem hybrid. Mismatched probe-target hybrids do not form, except at substantially lower temperatures (7,13). Therefore, a relatively wide range of temperatures exist in which perfectly complementary probe-target hybrids elicit a fluorogenic response, while mismatched molecular beacons remain dark. Consequently, assays using molecular beacons robustly discriminate targets that differ from one another by as little as a single nucleotide substitution.

A number of laboratories have confirmed the utility of using molecular beacons for the detection of single nucleotide polymorphisms (SNP). Molecular beacons have been used to genotype Factor V Leiden mutations (14), hereditary haemochromatosis gene mutations (14), cystic fibrosis and Tay-Sachs disease gene mutations (15), methylenetetrahydrofolate gene mutations (16), human chemokine receptor mutations (17,18), drug-resistance mutations in malarian parasites (19) and drug-resistance mutations in *Mycobacterium tuberculosis* (20). Usually the mutations are detected in real-time during amplification, but they can also be detected after amplification (21). In side-by-side comparisons, the specificity of molecular beacons has proven superior to probes that rely on the 5'-endonucleolytic cleavage activity of DNA polymerase (22). This high specificity allows detection of a small proportion of mutant DNA in the presence of an abundant wild-type DNA (23).

Materials

Synthesis of molecular beacons

Conventionally, molecular beacons were synthesized by the manual coupling of fluorophore and quencher moieties to oligonucleotides containing amino and sulfhydryl functionalities at each of their ends. Since the development of phosphoramidites linked to fluorophores and quenchers and controlled-pore glass (CPG) columns, containing covalently linked fluorophores and quenchers, molecular beacons can be made entirely by automated synthesis (24). However, phosphoramidites are still not available for most fluorophores. Mixed synthesis can be performed in order to utilize these fluorophores. In the mixed synthesis mode, the quencher moiety dabcyl is introduced during DNA synthesis and the fluorophore is coupled manually to an amino or a sulfhydryl group. Usually iodoacetamide

or maleimide derivatives of fluorophores are used for coupling with sulfhydryl groups and succinimidyl ester derivatives of fluorophores are used for coupling with amino groups. Although the non-fluorescent dye dabcyl has the ability to quench all fluorophores in molecular beacons, a number of other non-fluorescent quenchers, such as the "Black Hole" quenchers and QSY-7, have recently been introduced and found to be effective. DNA synthesis reagents for the incorporation of dabcyl and Black Hole quenchers can be obtained from Biosearch Technologies, and QSY-7 is available from Molecular Probes.

1. Start the DNA synthesis on a 3'-dabcyl CPG column of appropriate size. After incorporating all the nucleotides, introduce either a 5'-fluorophore, a 5'-sulfhydryl group, or a 5'-amino group. The 5'-modifiers should remain protected during synthesis to allow purification of the oligonucleotide in the presence of the protective trityl moiety. Perform the post synthetic steps according to the guidelines specific to each modifier. Dissolve the oligonucleotide in 600 µl buffer A (0.1 M triethyl-amonium acetate, pH 6.5).

2. Purify the tritylated dabcyl containing oligonucleotide by high-pressure liquid chromatography (HPLC) on a C-18 reverse-phase column, utilizing a linear elution gradient of 20 to 70 % buffer B (0.1 M triethylamonium acetate in 75 % acetonitrile, pH 6.5) in buffer A that forms over 25 minutes at a flow rate of 1 ml/min. Monitor the absorption of the elution stream at 260 nm (for DNA) and at 491 nm (for dabcyl). Collect the major peak that has a much higher absorption at 260 nm than at 491 nm and that appears near the end of the elution (due to the presence of its hydrophobic trityl moiety). Consult typical chromatograms that are available on the internet at http://www.molecular-beacons.org.

3. For oligonucleotides that are modified with sulfhydryl groups, precipitate them with ethanol and salt and dissolve the pellet in 250 µl buffer A. In order to remove the trityl moiety from the sulfhydryl group, add 10 µl of 0.15 M silver nitrate and incubate at room temperature for 30 min. Add 15 µl of 0.15 M dithiothreitol to this mixture and shake for 5 min. Centrifuge for 2 min at 10,000 rpm and transfer the supernatant to a new tube.

4. If an iodoacetamide derivative of the fluorophore will be coupled to the purified oligonucleotide, dissolve approximately 5 mg of the fluorophore in 250 µl of 0.2 M sodium bicarbonate, pH 9.0 and add it to the supernatant. Incubate the mixture for 90 min at room temperature. Remove the excess fluorophore by gel-exclusion chromatography through a Sephadex G-25 column (NAP-5, Pharmacia). Purify the dual-labeled oligonucleotide by HPLC on a C-18 reverse-phase column as described above, collecting the major peak that absorbs at 260 nm and at the maximal absorption wavelength of the fluorophore. The correct fraction will show substantial enhancement in fluorescence if you heat it with a hair dryer while holding it over a UV-transilluminator. Determine the absorbance at 260 nm and estimate the yield (1 OD_{260} = 33 µg/ml).

5. If a maleimide derivative of the fluorophores will be coupled to the purified oligonucleotide, remove excess dithiothreitol before coupling with the fluorophore by passing the supernatant from Step 3 through a Sephadex G-25 column that is equilibrated with 1 M Tris-HCl, pH 7.5. Dissolve approximately 5 mg of the fluorophore in 50 μl 1 M Tris-HCl, pH 7.5, mix with the deprotected oligonucleotide, and incubate for 90 min at room temperature. Remove the excess fluorophore by passing through a Sephadex G-25 column and purify the molecular beacon by HPLC as in the previous step.

6. For oligonucleotides that are modified with an amino group, precipitate them with ethanol and salt after the first purification (see Step 2) and dissolve the pellet in 50 μl buffer A. To remove the trityl moiety from the amino group, add 400 μl 80 % acetic acid and incubate for 1 hr at room temperature. Precipitate the oligonucleotide with ethanol and salt and wash the pellet with 70 % ethanol. Dissolve the pellet in 500 μl of 0.1 M sodium bicarbonate, pH 8.5. Dissolve approximately 5 mg of the fluorophore in 50 μl dimethylformamide and add the mixture to the oligonucleotide solution in several small aliquots. Incubate the mixture for 2 hr at room temperature. Remove the excess fluorophore by passing through a Sephadex G-25 column and purify and characterize the molecular beacon by HPLC as described in Step 4.

7. When a 5'-fluorophore is introduced via automated synthesis, a fully finished molecular beacon can be obtained in a single HPLC purification step, using the same procedure as in Step 4. Tetrachlorofluorescein (TET) increases the hydrophobicity of the oligonucleotides substantially, so that the dual-labeled oligonucleotide is clearly separated from the partially synthesized oligonucleotides. However, fluorescein (FAM) does not increase the hydrophobicity of the oligonucleotides substantially and the dual-labeled oligonucleotide may not clearly separate from the partially synthesized oligonucleotides. Therefore, a fluorescein phosphoramidite with a 5'-trityl moiety should be used, and the oligonucleotide should be synthesized and purified with the trityl moiety on. The trityl moiety should only be removed after purification (see Step 4).

A number of oligonucleotide synthesis companies specialize in the synthesis, purification, and characterization of molecular beacons. These companies are listed on the World Wide Web at http://www.molecular-beacons.org.

Sequences of molecular beacons and primers

The experimental example that is used to illustrate the principals of the method utilize a pair of molecular beacons that were designed to type a C to T transition at position 627 of the human chemokine receptor 2 gene (18). The sequence of the wild-type-specific molecular beacon is 5'-FAM-CGCCCTCTGGTCTGAA**G**GTTTATTGGTGCG-dabcyl-3' and the sequence of the mutant-specific molecular beacon is 5'-TET-CGCACCTCTGGTCTG-AA**A**GTTTATTGGTGCG-dabcyl-3', where the underlined nucleotides identify the arm sequences and the bold letters identify the site of the polymorphism. The sequences of the primers that were used are: 5'-AGATGAATGTAAATGTTCTTCTAG-3' and 5'-CTTTTT-AAGTTGAGCTTAAAATAAGC-3'.

Choice of fluorophores for different real-time instruments

A number of instruments that can monitor the progress of a polymerase chain reaction (PCR) by fluorescence have recently become available. Since the typing of each single nucleotide polymorphism (SNP) is performed using two molecular beacons, where one molecular beacon is specific for the wild-type sequence and the other is specific for the mutant sequence, it is important to carefully select two fluorophores that the available instrument is able to distinguish reliably. The Prism 7700 Sequence Detector spectrofluorometric thermal cycler (Applied Biosystem) uses a blue argon-ion laser (488 nm) as its light source, records the emission spectrum in the range of 500 to 600 nm, and then computes the fraction of each fluorophore that is present in each tube using a deconvolution algorithm. This instrument is able to excite and discriminate FAM and TET very well. The Smart Cycler (Cepheid), the iCycler (Bio-rad), and the Mx4000 (Stratagene) utilize either multicolored or white light sources in combination with specific filter sets that allow detection of up to four different fluorophores. These instruments rely on fluorophores that have widely separated spectra with minimum cross-talk, rather than relying on the deconvolution of the emission spectra of closely related fluorophores. With these instruments, either FAM or TET, which emit in the green range, can be used for one molecular beacon and either tetramethylrhodamine, Alexa 546, Cy 3, ROX, Texas red, or Cy 5, which emit in the red range, can be used for the other molecular beacon. The Light Cycler (Roche Diagnostics) utilizes a blue light-emitting diode and detects fluorescence in either the green or the red range. This instrument can therefore be used with a fluorescein labeled molecular beacon that emits in the green range and a Cy 5- or "Light Cycler"- red labeled wavelength-shifting molecular beacon (5) that emits in the red range (but is excited by the blue light-emitting diode).

Methods

Design of allele discriminating molecular beacons

In order to detect the synthesis of products during polymerase chain reactions, molecular beacons should be designed so that they are able to hybridize to their targets at the annealing temperature of the PCR, whereas the free molecular beacons should stay closed and be non-fluorescent at this temperature. This can be ensured by choosing the length of the probe sequence and arm sequences appropriately. In order to discriminate amplicons that differ from one another by a single nucleotide substitution, the length of the probe sequence should be such that it would dissociate from its target at temperatures 7-10 ºC higher than the annealing temperature of the PCR. If single-nucleotide allele discrimination is not desired, longer and more stable probes can be chosen. The melting temperature of the probe-target hybrid can be predicted using the "percent-GC" rule, which is available in most probe design software packages, such as Oligo 5.0. The prediction should be made for the probe sequence alone before adding the stem sequences. In practice, the length of the probe sequence usually falls in the range between 15 and 30 nucleotides.

After selecting the probe sequence, two complementary arm sequences should be added on either side of the probe sequence. In order to ensure that the molecular beacon remains closed in absence of target, three factors need to be considered in choosing the arm sequences: their length, their sequence and their G-C content of the resulting stem, these factors should be chosen so that the melting temperature of the stem is 7-10 ℃ higher than the detection temperature (usually the annealing temperature of the PCR). The melting temperature of the stem can not be predicted by the percent-GC rule, since the stem is formed by intramolecular hybridization. Instead, a DNA folding program, such as the Zuker DNA folding program, which is available on the internet, should be utilized to estimate its melting temperature. Usually the stems are 5-7 basepairs long. In general, GC-rich stems 5 basepairs in length will melt between 55 and 60 ℃, GC-rich stems 6 basepairs in length will melt between 60 and 65 ℃, and GC-rich stems 7 basepairs in length will melt between 65 and 70 ℃. Longer stems enhance the specificity of molecular beacons (13).

It is important that the conformation of the free molecular beacons be the intended hairpin structure, rather than other structures that either do not place the fluorophore in the immediate vicinity of the quencher, or that form longer stems than intended. The former will cause high background signals, and latter will make the molecular beacon sluggish in binding to its target. A folding of the selected sequence by the Zuker DNA folding program will reveal such problems. If the alternative structures result from the choice of the stem sequence, the stem sequence can be altered. If on the other hand, the alternative structures arise from the identity of the probe sequence, the frame of the probe can be moved along the target sequence to obtain a probe sequence that is not self-complementary. Small stems within the probe's hairpin loop that are 2 to 3 nucleotides long do not adversely effect the performance of molecular beacons.

As with PCR primers, the sequence of the molecular beacon should be compared with the sequences of the primers using a primer design software program to make ensure that there are no regions of substantial complementarity that may cause the molecular beacon to bind to one of the primers, permitting primer extension. The primers that are used should be designed to produce a relatively short amplicon, in general less than 150 basepairs. Molecular beacon are internal probes that must compete with the opposite strand of the amplicon for binding to its complementary target. Having a shorter amplicon allows the molecular beacon to compete more efficiently, and, therefore produces stronger fluorescence signals during real-time PCR.

Molecular beacons can also be designed with the help of a dedicated software package called "Beacon Builder", which is available from Premier Biosoft International.
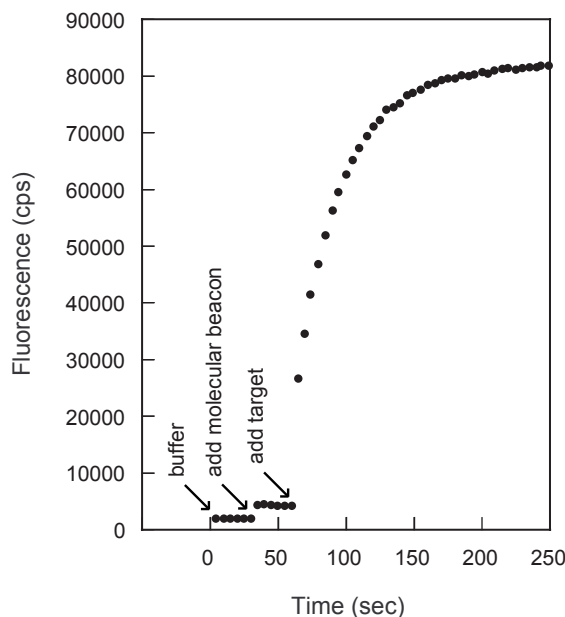
Figure 2. Functional characterization of a molecular beacon preparation by addition of a complementary oligonucleotide target. There was a 35-fold increase in the fluorescence of this preparation of molecular beacons upon the addition of the target.

Characterization of molecular beacons

In order to ensure that a molecular beacon will function as expected during real-time PCR, the extent to which its fluorescence increases upon binding to its target should first be measured (Figure 2). This "signal-to-background ratio" depends primarily on the purity of the molecular beacon preparation. Poor signal-to-background ratios are caused by the presence of uncoupled fluorophores in the preparation or by the presence of oligonucleotides in the preparation that have a fluorophore but do not have a quencher.

1. Determine the fluorescence (F_{buffer}) of 200 µl of a 4 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl (pH 8.0) solution with a QuantaMaster spectrofluorometer (Photon Technology International). Use the optimal excitation and emission wavelength of the fluorophore used to label the molecular beacon. The excitation and emission wavelengths for FAM and TET, the fluorophores used for the construction of molecular beacons used in the genotyping assay described in this protocol, were 491 and 515, and 522 and 535 nm, respectively.

2. Add 10 µl of a 0.1 µM molecular beacon solution to the 200 µl solution and record the new level of fluorescence (F_{closed}).

3. Add a two-fold molar excess of an oligonucleotide target (whose sequence is perfectly complementary to the probe sequence of the molecular beacon, but that does not possess sequences complementary to the arms of the molecular beacon), and monitor the rise in fluorescence until it reaches a stable level (F_{open}).

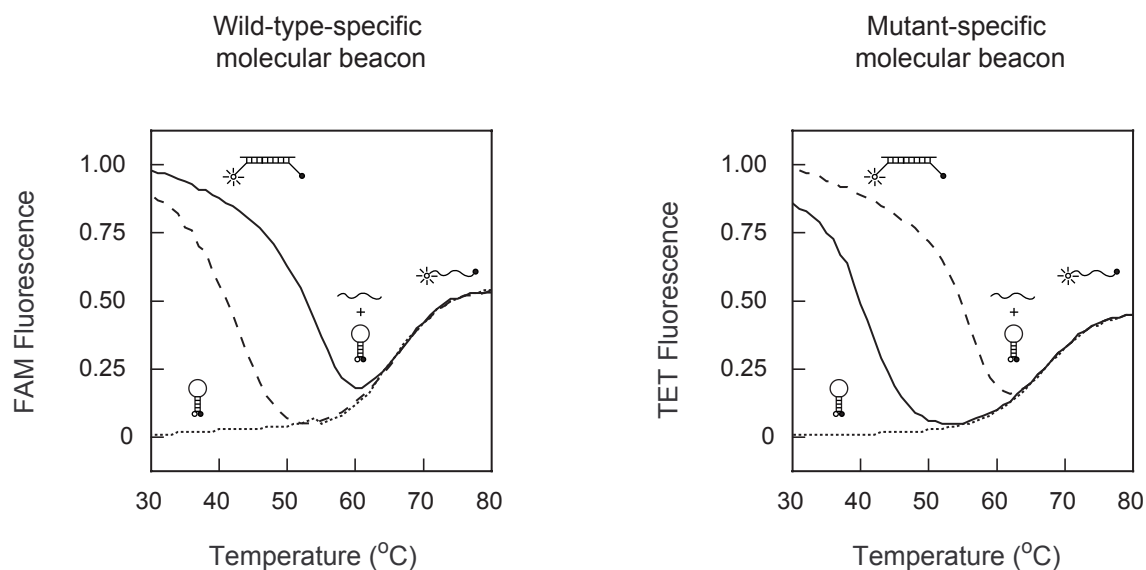4. Calculate the signal-to-background ratio as $(F_{open}-F_{buffer})/(F_{closed}-F_{buffer})$.

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Figure 3. Thermal denaturation profiles of molecular beacons in the presence of either wild-type target (continuous lines), mutant target (dashed lines), or no target (dotted lines). The state of the molecular beacons is indicated by a diagram over the thermal denaturation profiles. Mismatched hybrids denature 10 to 12 °C below the melting temperature of perfectly matched hybrids. Optimum discrimination is achieved at the center of this temperature range. Therefore, in this example 50 °C was chosen to be the assay temperature (the annealing temperature of the PCR).

Determination of thermal denaturation profiles

In order to determine the window of discrimination, which is the range of temperatures in which perfectly complementary probe-target hybrids can form and in which mismatched probe-target hybrids can not form, the fluorescence of solutions of molecular beacons in the presence of each kind of target is measured as a function of temperature (Figure 3). This experiment is also useful for confirming the theoretical predictions of different melting transitions.

1. For each molecular beacon, prepare three tubes containing 200 nM molecular beacon, 4 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl (pH 8.0), in a 50-µl volume.

2. Add a two-fold molar excess of an oligonucleotide that is perfectly complementary to the molecular beacon probe sequence (but not to the arm sequences arms) to one of the tubes, add a two-fold excess of an oligonucleotide that contains the mismatched target sequence to the other the tube, and add only buffer to the third tube.

3. Determine the fluorescence of each solution as a function of temperature, using a Prism 7700 Sequence Detector spectrofluorometric thermal cycler. Decrease the temperature of the solutions from 80 °C to 30 °C in 1 °C steps, with each step lasting 1 min, while monitoring fluorescence during each step.

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Real-time PCR

1. Prepare 50-µl reactions that contain 100 nM wild-type-specific molecular beacon, 100 nM mutant-specific molecular beacon, 500 nM of each primer, 2 units of Amplitaq Gold DNA polymerase (Applied Biosystems), 250 µM dATP, 250 µM dCTP, 250 µM dGTP, 250 µM dTTP, 4 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl (pH 8.0).

2. The thermal cycling program should consist of 10 min at 95 °C, followed by 40 cycles of 30 sec at 95 °C, 45 sec at 50 °C, and 30 sec at 72 °C. Monitor fluorescence during the 50 °C annealing steps in the Prism 7700 Sequence Detector spectrofluorometric thermal cycler.

Data analysis

1. Analyze the acquired PCR-run data by indicating in the software, that controls the spectrofluorometric thermal cycler, that "there is no quencher" (since dabcyl is non-fluorescent) and that there is no internal reference dye in the reactions. The default pure-dye spectra of FAM and TET fluorophores usually allow satisfactory determination of the fluorescence contributions from each of the two fluorophores. However, if low signals are obtained, it may be necessary to use the actual spectra of the molecular beacons that are being used in the PCR. These spectra, instead of the default pure-dye spectra, will then be used for the deconvolution of the emission spectra generated during the polymerase chain reactions. In that case, for each molecular beacon, dissolve 100 nM of the molecular beacon in 50 mM KCl, 10 mM Tris-HCl (pH 8.0), and 4 mM $MgCl_2$, add an excess of its complementary target oligonucleotide, and record its spectrum at the annealing temperature that will be used in the PCR.

2. For each sample, fluorescence is plotted as a function of the number of thermal cycles. Two curves are obtained, one for FAM and one for TET. Sample results for a homozygous wild-type or homozygous mutant and heterozygous sample are depicted in Figure 4. The homozygous wild-type sample exhibited a rise in fluorescence for the FAM molecular beacon and not for the TET molecular beacon. The homozygous mutant sample exhibited a rise in fluorescence for the TET molecular beacon and not for the FAM molecular beacon, and the heterozygous sample exhibited a rise in fluorescence for both the FAM molecular beacon and the TET molecular beacon. Samples with too little DNA exhibit no fluorescence change for both molecular beacons. The magnitude of the fluorescence in heterozygous samples in each color range is half of the value obtained for homozygous samples.

3. This analysis is sufficient for experiments in which a few samples are analyzed. However, for a large number of samples a more automated approach is necessary. In this method, the data showing fluorescence as a function of the number of thermal cycles performed for each color in every sample are exported into a spreadsheet. The fluorescence values at the final thermal cycle are then analyzed. For each sample, the ratio of the FAM fluorescence to the FAM plus TET fluorescence is determined. These ratios fall into three clearly segregated categories, high numbers (0.9-1.0) indicate homozygous wild-type individuals, low numbers (0-0.1) indicate homozygous mutant individuals and intermediate numbers (0.55-0.75) indicate heterozygous individuals. In order to illustrate the distribution of these ratios within these three ranges, we analyzed 793 DNA samples for the 627C to T polymorphism in the human chemokine receptor 2 gene. The number of samples that displayed particular ratios (width of the range = 0.01) is plotted in Figure 5. The results demonstrate that majority of the homozygous wild-type samples become fluorescent only for FAM and the majority of homozygous mutant samples become fluorescent only for TET. The three categories are clearly segregated and the boundary lines that define each of the categories can be selected easily. As a result, almost all of the samples can be assigned an unambiguous genotype. The error rate is so low that only two samples in this set of 793 samples were discordant with another method of genotyping (18).

Notes

1. Usually the 50 nmol, 200 nmol, and 1000 nmol scales of syntheses yield 10 nmol, 40 nmol, and 200 nmol molecular beacons respectively.

2. Store stock solutions of molecular beacons at –20 ºC or –70 ºC in TE buffer (10 mM Tris-HCl pH 8.0 and 0.1 mM EDTA) and prevent them from being exposed to light. Store the molecular beacons as a dried pellet for the long term.

3. Before performing the coupling reactions, prepare fresh solutions of both silver nitrate and dithiothreitol.

4. In order to remove unincorporated fluorophore derivatives from the coupling reactions, the reaction mixtures can also be precipitated with salt and ethanol, as the fluorophores remain dissolved in ethanol.

5. Refer to the data-sheets of the fluorophore derivatives for the information on their solubility. In case a fluorophore derivative is not soluble in water, as is the case for most succinimidyl ester derivatives, dissolve it in a small amount of dimethylformamide and then add this solution to the reaction mixture in small aliquots.

6. Although false amplicons and primer dimers are not detected by molecular beacons, when they do appear, the sensitivity of the PCR assay is reduced. Therefore, DNA polymerases that become active after a brief incubation at 95 ºC are recommended, as they minimize false priming.
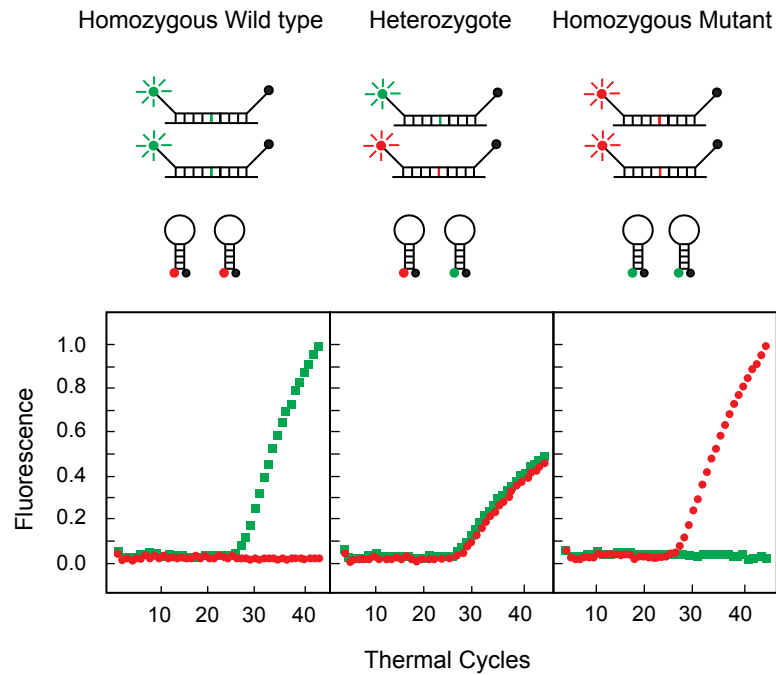
Figure 4. Principle of genotyping with molecular beacons. With DNA from homozygous wild-type individuals, only the FAM-labeled molecular beacons hybridize to the amplicons, generating green fluorescence, whereas the TET-labeled molecular beacons retain their stem-and-loop structure and do not produce a red fluorescent signal. With DNA from heterozygous individuals, both molecular beacons hybridize to the amplicons, generating both green and red fluorescence. With DNA from homozygous mutant individuals, only the TET-labeled molecular beacons hybridize to the amplicons, generating red fluorescence, whereas the FAM-labeled molecular beacons remain dark
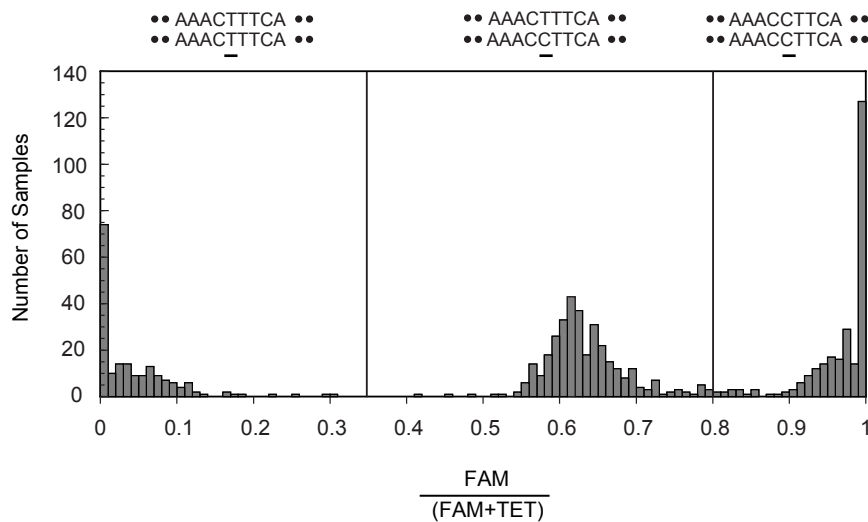

Figure 5. Genotyping a large number of individuals for the 627C to T polymorphism in the chemokine receptor 2 gene by the color of the fluorescence generated by the end of polymerase chain reactions. The ratio of FAM fluorescence to total fluorescence was calculated for each of the 793 samples. The plot shows the distribution of the ratios that were obtained. The ratios fall into three clearly segregated categories, high numbers (0.9-1.0) identify homozygous wild-type individuals, low numbers (0-0.15) identify homozygous mutant individuals and intermediate numbers (0.55-0.75) identify heterozygous individuals.

Notes (continued)

7. In case you obtain low signals in real-time PCR with molecular beacons, try to optimize the concentration of the molecular beacons, decrease the size of the amplicon, decrease the annealing temperature, and alter the relative concentrations of the two primers so that the PCR becomes asymmetric, favoring of the target strand.

8. In case you do detect a good signal but observe poor discrimination between the alleles, check if there is no bleed through of fluorescence from one color channel to the other. If the instrument is able to distinguish between the two fluorophores perfectly, increase the annealing temperature of the PCR. If you still observe poor discrimination, increase the length of the stems of the molecular beacons or decrease the length of the probe sequences.

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