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Design and Optimization of Molecular Beacon Real-Time Polymerase Chain Reaction Assays

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Summary

During the last few years, several innovative technologies have become available for performing sensitive and accurate genetic analyses. These techniques use fluorescent detection strategies in combination with nucleic acid amplification protocols. Most commonly used is the real-time polymerase chain reaction (PCR). To achieve the maximum potential of a real-time PCR assay, several parameters must be evaluated and optimized independently. This chapter describes the different steps necessary for establishing a molecular beacon real-time PCR assay: (1) target design, (2) primer design, (3) optimization of the amplification reaction conditions using SYBR Green, (4) molecular beacon design, and (5) molecular beacon synthesis and characterization. The last section provides an example of a multiplex quantitative real-time PCR.

Key Words: Real-time PCR; molecular beacon; SYBR Green; fluorescence; quantification of nucleic acids; SNP detection.

1. Introduction

The polymerase chain reaction (PCR), first described by Mullis and Saiki in 1985 ***(1)***, has made it possible to detect rare target nucleic acid sequences isolated from cell, tissue, or blood samples. Real-time PCR is a powerful improvement on the basic PCR technique ***(2)***. The use of fluorescent detection strategies in combination with appropriate instrumentation enables accurate quantification of nucleic acids. Quantification of nucleic acids is achieved by measuring the increase in fluorescence during the exponential phase of PCR. The point at which the fluorescence rises significantly above background is called the threshold cycle (Ct) (**Fig. 1A**). There is an inverse linear relationship between the log of the starting amount of template and the corresponding

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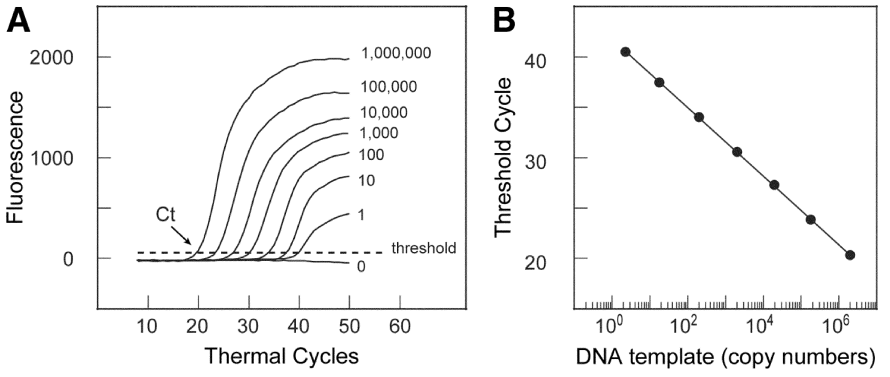

Fig. 1. **(A)** The threshold cycle (Ct) is the cycle at which the fluorescence rises significantly above the background. The fluorescence increases as the molecular beacons bind to the amplification products that accumulate during each successive cycle. In the early cycles of amplification, the change in fluorescence is usually undetectable, but at some point during amplification, the accumulation of amplified DNA results in a detectable change in the fluorescence of the reaction mixture. The Ct number decreases as the number of target molecules initially present in a reaction increases. **(B)** The standard curve can be used to determine the starting amount of an unknown template, based on its Ct. Given known starting amounts of the target, a standard curve can be constructed by plotting the log of the starting amount vs the Ct. The Ct is inversely proportional to the logarithm of the number of target molecules initially present. In real-time PCR quantitative results can be obtained over a wide dynamic range of target concentrations.

Ct-value during real-time PCR (**Fig. 1B**). Compared with end point quantification methods, real-time amplification assays offer reproducible results and have a much wider dynamic range. In addition, the use of fluorescent agents and probes that only generate a fluorescence signal on binding to their target enables real-time amplification assays to be carried out in sealed tubes, eliminating the risk of carryover contamination. Different techniques are available to monitor real-time amplification. The amplification process can be monitored using nonspecific double-stranded deoxyribonucleic acid (DNA) binding dyes or specific fluorescent hybridization probes. Dyes, such as SYBR Green, produce enhanced fluorescence signals on intercalating with double-stranded DNA complexes ***(3)***. Although double-stranded DNA binding dyes are a simple, fast, and inexpensive method to monitor the amplification of a template, their major disadvantage is that the dye binds nonspecifically to all double-stranded DNA complexes, such that primer–dimers and nonspecific amplification products cannot be distinguished from the intended amplification product. The use of fluorescent hybridization probes enhances the overall

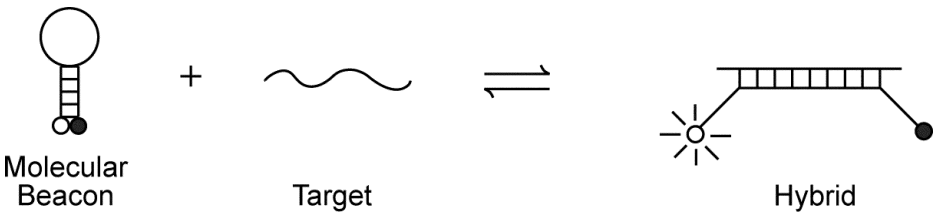

Fig. 2. Principle of operation of molecular beacons. Molecular beacons are single-stranded oligonucleotides that possess a hairpin structure. Free molecular beacons are nonfluorescent because the hairpin stem keeps the fluorophore in close proximity to the quencher. When the probe sequence in the hairpin loop hybridizes to its target, forming a rigid double helix, a conformational change occurs that removes the quencher from the vicinity of the fluorophore, thereby restoring fluorescence.

assay sensitivity by eliminating background signals owing to the synthesis of nonspecific amplification products. The hybridization probe is designed to be complementary to a target sequence within the expected amplification product, so that the probes do not bind to nonspecific amplification products, thus enhancing the specificity of the assay. The different fluorescent probe-based techniques include adjacent probes ***(4)***, 5' nuclease probes ***(5)***, molecular beacons ***(6),*** and duplex scorpion primers ***(7)***. This chapter describes the use of molecular beacons in conjunction with PCR. Molecular beacons (**Fig. 2**) hybridize at the annealing temperature to the amplification products and do not interfere with primer annealing and extension. Molecular beacon real-time PCR assays are simple, fast, sensitive, accurate, allow a high-throughput format, and enable the detection of a series of different agents in the same assay tube ***(8)***. Molecular beacon real-time PCR assays have been used in numerous studies to detect genomic DNA sequences, single nucleotide polymorphisms (SNPs), messenger ribonucleic acid (mRNA) expression levels, and pathogens ***(9–18)***. The development and application of molecular beacons was recently reviewed by Vet et al. ***(19)***, and more information is available at www.molecular-beacons.org.

To achieve the maximum potential of real-time PCR, several parameters must be evaluated and optimized independently. The amplification conditions, the molecular beacon design, and synthesis affect the efficiency and accuracy of real-time PCR. When determining which conditions to optimize, the ultimate assay goal must be considered. When the goal is to screen for a specific sequence, for example, in SNP detection assays or in assays to identify bacterial species with conserved DNA regions, both the design of the primers and molecular beacon is limited to the region that contains the specific sequence. In those cases, the selection of primers depends on the molecular beacon target

sequence, that is, the primers should not overlap with the molecular beacon sequence. As a result, the choice of primers is limited. When the choice of primers and molecular beacon is not limited to a restricted sequence region, for example, in assays that determine the mRNA expression of a gene, the primers should be designed and tested prior to developing the molecular beacon. After optimization of the PCR assay, a molecular beacon can be designed that will detect the amplified template. We have outlined the different steps necessary for establishing a reliable real-time PCR assay. These steps include: (1) target design, (2) primer design, (3) optimization of the amplification reaction conditions using SYBR Green, (4) molecular beacon design, and (5) molecular beacon synthesis and characterization. The last section provides an example of a multiplex quantitative real-time PCR.

2. Materials

1. 5 U/µL AmpliTaq Gold DNA polymerase (Applied Biosystems, CA) (*see* **Note 1**).
2. 10X concentrated PCR buffer (supplied with DNA polymerase, containing 100 m*M* Tris-HCl, pH 8.3, 500 m*M* KCl, 0.01% (w/v) gelatin, without $MgCl_2$).
3. 25 m*M* $MgCl_2$ (Applied Biosystems, CA).
4 Deoxynucleotide-triphosphate (dNTP) mixture: 25 m*M* dATP, 25 m*M* dCTP, 25 m*M* dGTP, 25 m*M* dTTP (Promega, WI).
5. TE buffer: 1 m*M* EDTA, 10 m*M* Tris-HCl, pH 8.0.
6. PCR primers (dissolved in TE). Stock solutions of 100 µ*M* should be kept for longtime storage at –20°C. Work solutions (diluted in Milli-Q water) should be kept at –20°C.
7. 50X SYBR Green for DNA in Milli-Q water. SYBR Green is available as a 10,000X stock in dimethyl sulfoxide (DMSO) (Molecular Probes, OR); 200X SYBR Green solutions in DMSO can be kept for longtime storage protected from light at –20°C. Work solutions of 50X SYBR Green in Milli-Q water are prepared fresh weekly and should be kept protected from light at –20°C prior to use.
8. Molecular beacon (dissolved in TE). Stock solutions of 100 µ*M* should be kept for longtime storage protected from light at –20°C. Work solutions (diluted in TE at, for example, 5 µ*M*) should be kept protected from light at –20°C for 1 mo.
9. Oligonucleotide target that is perfectly complementary to the probe sequence of the molecular beacon (but not complementary to the arm sequences). Store work solutions (diluted in Milli-Q water at 25 µ*M*) at –20°C.
10. Spectrofluorometric thermal cycler. There are several platforms available for performing real-time PCR analyses (**Table 1**). These platforms range from ultrarapid, air-heated thermal cyclers, where PCR is performed in glass capillaries, to tube-based and microtiter plate-based systems for high-throughput assays.

Table 1
Specification of the Different Available Spectrofluorometric Thermal Cyclers

Company	Model	Fluorophore choices	Multiplex capabilities	Sample capacity	Remarks
Applied Biosystems	PRISM® 7000 Sequence Detection System	FAM, TET, TMR and Texas Red	Up to four targets	96 wells	High throughput
Applied Biosystems	PRISM® 7700 and 7900HT Sequence Detection System	FAM, TET, Rhodamine-6G, TMR and Texas Red	Up to five targets (*see* **Note 9**)	96 wells or 384 wells	High throughput
Bio-Rad	iCycleriQ™ real-time PCR Detection System	FAM, HEX, Texas Red and Cy5	Up to four targets	96 wells	High throughput, temperature gradient block (*see* **Note 2**)
Cepheid	SmartCycler	FAM, Cy3, Texas Red and Cy5	Up to four targets	16 units	Rapid thermal cycles, each unit is independently programmable (*see* **Note 2**)
MJ Research	Opticon	FAM and TMR	Up to two targets	96 wells	High throughput, temperature gradient block (*see* **Note 2**)
Roche Applied Science	LightCycler System	FAM, ROX and Cy5.5	Up to three targets (*see* **Note 9**)	32 wells	Rapid thermal cycles
Stratagene	Mx3000P and Mx4000™ Multiplex Q-PCR System	FAM, TMR, Texas Red and Cy5	Up to four targets	96 wells	High throughput

3. Methods

3.1. Target Design

A successful real-time PCR requires efficient amplification of the template. Both the source of the template ***(20)*** and the sequence of the primers and template affect this efficiency. Significant secondary structures of the template may hinder the primers or molecular beacon from annealing and prevent complete product extension by the DNA polymerase. It is therefore recommended to design primer pairs that amplify a target region of 75 to 250 basepairs. Besides a more efficient amplification of the target sequences, these shorter amplification products produce higher fluorescent signals because molecular beacons are better able to compete with the complementary strands for binding to the target strands. To avoid selecting a molecular beacon target sequence within a region with strong secondary structures, the target sequence can be analyzed using a DNA folding program, such as the DNA mfold server ***(21)*** (available at http://www.bioinfo.rpi.edu/applications/mfold).

3.2. Primer Design

The goal is to design primers with a melting temperature (T*m*) higher than the T*m* of any of the predicted template secondary structures. This ensures that the majority of possible secondary structures have been unfolded before the primer-annealing step. The following parameters are important when designing primers:

1. Design primers with a 50–60% GC content.
2. Maintain a T*m* between 50–65°C.
3. Eliminate strong secondary structures (more than two basepairs).
4. Avoid repeats of Gs or Cs longer than three bases.
5. Check the sequence of the primers against each other to ensure that there are no 3' complementarities (avoids primer–dimers).
6. Place Gs and Cs on ends of the primers.
7. Avoid false priming by verifying the primers specificity using sites such as the Basic Local Alignment Search Tool (available at http://www.ncbi.nml.nih.gov/blast/).

We recommend designing a few primer sets for each individual PCR and testing these sets using SYBR Green as a reporter molecule.

3.3. Optimization Using SYBR Green Real-Time PCR

For optimizing the PCR and monitoring the amplification of nonspecific amplification products, different concentrations of $MgCl_2$, different PCR primer sets in varying concentrations, and different annealing temperatures should be tested.

1. Prepare 50-µL reactions that contain 1X PCR buffer, 2.5 U AmpliTaq Gold DNA polymerase, 250 µ*M* of each dNTP, 1X SYBR Green, and variable concentrations of $MgCl_2$ (3–6 m*M*, in 0.5 m*M* steps), PCR primers (100 n*M*, 250 n*M*, 500 n*M*, and 1000 n*M*).
2. For each amplification condition, prepare two reactions. To one reaction, add approx 100,000 copies of template molecules to test the target amplification efficiency. To monitor the formation of nonspecific amplification products, Milli-Q water (instead of template) is added to the other reaction.
3. Program the thermal cycler to heat the reaction mixture at 95°C for 10 min to activate the AmpliTaq Gold DNA polymerase, followed by 40 amplification cycles. Each cycle consists of denaturation at 95°C for 30 s, primer annealing at the temperature determined by the primer selection software for 30 s (*see* **Note 2**), and primer extension at 72°C for 30 s.
4. Monitor the fluorescence of SYBR Green during the annealing step of the amplification reaction. Refer to the instrument's manual to enable the appropriate channel or emission filter for monitoring the fluorescence of SYBR Green. If no specific SYBR Green option is available on the instrument, monitor the fluorescence using the same channel or emission filter chosen to monitor the fluorescence of fluorescein (FAM). FAM and SYBR Green have roughly the same emission spectrum.

3.3.1. Data Interpretation

1. Analyze the acquired fluorescence data with the appropriate settings for background and baseline subtraction as indicated in the instrument's manual.
2. Determine the Ct for each reaction condition for both the template and no target control.
3. A reaction initiated with 100,000 copies of the template will give a Ct in the range of 21 to 25. The different conditions can be compared to determine the reaction with earliest Ct and strongest fluorescence signal. The reaction with no template added will indicate the presence of nonspecific amplification products. If the Ct for nonspecific amplification products is 30 or lower, a new primer pair should be chosen. The formation of the nonspecific amplification products will most likely affect the amplification of the template. If the threshold of nonspecific amplification products is between 30 and 40, a new primer pair should be chosen if the assay is designed to amplify template molecules as low as 100 copies. If the assay is designed for the amplification of template molecules as low as one copy, then it is preferable to have Cts of nonspecific amplification products to be higher than 40, or, even better, have no nonspecific amplification products.
4. The efficiency of the PCR can be determined by performing a serial dilution experiment, using 100,000, 10,000, 1000, 100, and 10 copies of template to initiate the PCR, and constructing a standard curve. Refer to the thermal cycler manual to automatically determine the Ct for the standards and to calculate a standard curve. A slope of –3.322 represents a PCR efficiency of 100% (*see* **Note 3**).

The correlation coefficient should be at least 0.995. (For multiplex quantitative PCR guidelines, *see* **Note 4**.)
After careful selection of the best primer pair and other optimized reaction conditions, a suitable molecular beacon can be designed.

3.4. Molecular Beacon Design

To successfully monitor PCR reactions, molecular beacons should be designed that are able to hybridize to their targets at the annealing temperature of the PCR, whereas the free molecular beacons should stay closed and be nonfluorescent at this temperature. This can be ensured by appropriately choosing the length of the probe sequence and the length of the arm sequences. The length of the probe sequence should be such that the molecular beacon will dissociate from its target at temperatures above the annealing temperature of the PCR. The T*m* of the probe target-hybrid can be predicted using the "percent-GC" rule, which is available in most primer and hybridization probe design programs. The prediction should be made for the probe sequence alone before adding the stem sequences. In practice, the length of the probe sequence is usually between 15 and 30 nucleotides. The T*m* of the probe-target hybrid should not be above 72°C to avoid interference of the DNA polymerase. Molecular beacons are very versatile. The general design, just outlined, can be altered to fit specific applications.

3.4.1. Molecular Beacons for Standard Target Detection

For standard target detection, in which the molecular beacon target sequence is not limited to a specific sequence region of the amplification product, a probe length of 22–30 nucleotides can be used, and the T*m* of the probe-target hybrid should be 7–10°C above the annealing temperature of the PCR. Thermal denaturation profiles can be performed to confirm the theoretical prediction of the T*m* (*see* **Subheading 3.6.2.**). Longer loop sequences ensure that probe-target hybrids that contain mismatches are stable at the annealing temperature of the PCR. This can be of importance to detect, for example, a retroviral subtype that contains one or two nucleotide substitutions ***(18)*** (*see* **Note 5**).

3.4.2. Allele-Discriminating Molecular Beacons

To ensure that a molecular beacon can discriminate single-nucleotide variations at the detection temperature, the following criteria should be met: In the presence of perfectly complementary targets, the molecular beacons must form a stable probe-target hybrid; and in the presence of mismatched targets, the molecular beacons must remain closed. Therefore:

1. Select a probe sequence that will dissociate from its target at temperatures 5–8°C higher that the annealing temperature of the PCR. The length of the probe sequence usually falls in the range between 15 and 21 nucleotides.

2. Measure the fluorescence of solutions of molecular beacons in the presence of each kind of target as a function of temperature, to determine the window of discrimination, which is the range of temperature in which perfectly complementary probe targets can form and in which mismatched probe-target hybrids cannot form (*see* **Subheading 3.6.2.**).

3.4.3. Selecting the Stem Sequence

After selecting a probe sequence, two complementary arm sequences should be added on either side of the probe sequence. To ensure that the molecular beacon remains closed in the absence of a target sequence, the stem should be stable at the annealing temperature of the PCR. Because the stem is formed by an intramolecular hybridization event, its T*m* cannot be predicted by the percent-GC rule or nearest neighbor method used in the primer and hybridization probe software. Instead, a DNA folding program (such as the DNA mfold server ***[21],*** available at http://www.bioinfo.rpi.edu/applications/mfold) should be used to predict its T*m*. Usually the stem is 5–7 nucleotides long (*see* **Note 6**). In general, GC-rich stems that are five basepairs in length will melt between 55 and 60°C, GC-rich stems six basepairs in length will melt between 60 and 65°C, and GC-rich stems seven basepairs in length will melt between 65 and 70°C. Longer stems enhance the specificity of molecular beacons ***(22)***. The folding program also predicts if the chosen molecular beacon sequence will form an unwanted secondary structure (*see* **Note 7**). Molecular beacons can also be designed with the help of a software package called Beacon Builder, which is available from Premier Biosoft International (*see* **Note 8**).

3.4.4. Choice of Fluorophores

Molecular beacons can be labeled with a wide range of fluorophores, and all fluorophores can be efficiently quenched by the same quencher, such as DABCYL, BHQ-1, and BHQ-2 ***(8,23)***. The choice of the fluorophore is therefore mainly dependent on the apparatus used to perform the real-time PCR assay. **Table 1** provides information on what fluorophores can be used with the different spectrofluorometric thermal cyclers. For each spectrofluorometric thermal cycler, the fluorophores are listed that have minimum emission overlap and allow reliable multiplex detection assays to be carried out. However, this list only provides a small subset of the fluorophores that can be used. In the manual provided with each thermal cycler, a detailed list is available of fluorophores that are suitable for the selected light and emission filter installed on the thermal cycler (*see* **Note 9**).

3.5. Molecular Beacon Synthesis

Molecular beacons can be obtained from a large number of oligonucleotide synthesis companies (a list is available at www.molecular-beacons.org). These

companies specialize in the synthesis, purification, and characterization of molecular beacons. It is recommended to order simultaneously an oligonucleotide target that is complementary to the probe (loop) sequence of the molecular beacon, which is used for additional characterization experiments of the molecular beacon (*see* **Subheadings 3.6.1.** and **3.6.2.**).

If a DNA synthesizer and high-pressure (performance) liquid chromatography (HPCL) apparatus are available, molecular beacon probes can be prepared in a one- or two-step synthesis process, followed by one purification step. A rising number of fluorophores and quenchers have become available as phosphoramidite derivatives or linked to controlled pore glass (CPG) columns (Glen Research, VA; Amersham, NJ; Biosearch Technologies, CA). This allows the synthesis of a complete molecular beacon in a single synthesis step on a DNA synthesizer. In most cases, no changes in the DNA synthesis protocol have to be made to incorporate the fluorophores and quenchers; however, we suggest referring to the instruction manuals that are supplied with the fluorophores and quenchers for special coupling and postsynthesis protocols.

In case a fluorophore or quencher is not available for direct incorporation during the DNA synthesis, chemically reactive fluorophore derivatives can be introduced in a post-DNA-synthesis step. In a first DNA synthesis step, the quencher DABCYL, BHQ-1, or BHQ-2, linked to a CPG column (Glen Research, VA; Biosearch Technologies, CA) is introduced at the 3'-terminal position and an amino or sulfhydryl phosphoramidite (Glen Research, VA) is incorporated at the 5'-terminal position of the oligonucleotides. In the second post-DNA-synthesis step, a succinimidyl ester or an iodoacetamide (or maleimide) derivative of a fluorophore (Molecular Probes, OR) is coupled to the 5'-amino or sulfhydryl moiety, respectively. Protocols describing the coupling of fluorophore derivatives to oligonucleotides can be downloaded at www.molecular-beacons.org.

All molecular beacons are purified by HPCL through a C-18 reverse-phase column, using a linear elution gradient of 20 to 70% buffer B (0.1*M* triethylammonium acetate [TEAA] in 75% acetonitrile, pH 6.5) in buffer A (0.1*M* TEAA, pH 6.5) that forms over 25 min at a flow rate of 1 mL/min (refer to www.molecular-beacons.org for typical chromatograms.) After the purification, the molecular beacons are precipitated with 1/10 vol 3*M* sodium acetate, pH 5.2, and 2.5 vol 100% ethanol. The molecular beacons are dissolved at a concentration of 100 µ*M* in TE buffer and stored at –20°C.

3.6. Characterization of Molecular Beacons

The molecular beacon synthesis and purification is very crucial for the production of high-quality probes and, subsequently, for accurate and reliable real-time PCR experiments. Weak fluorescence signals during the real-time PCR

assay are often caused by poor signal-to-background ratios of the molecular beacons. These poor signal-to-background ratios are caused by the presence of uncoupled fluorophores in the preparation or by the presence of oligonucleotides containing a fluorophore but lacking a quencher. To determine the purity of a molecular beacon preparation, one can measure the extent to which its fluorescence increases on binding to its target. Preferably, the molecular beacons should have signal-to-background ratios above 20.

3.6.1. Signal-to-Background Ratio

The signal-to-background ratio of a molecular beacon can be determined using the same spectrofluorometric thermal cycler used for the real-time PCR assay. All measurements are taken at the same temperature as optimized for the annealing of the primers during the PCR, and the appropriate excitation and emission source settings are selected for the fluorophore that is used as a label for the molecular beacon.

1. Prepare one 25-μL reaction that contains 1X PCR buffer and the concentration of $MgCl_2$ that will be used in the PCR assay.
2. Determine the fluorescence signal of this solution (F_{buffer}).
3. Add 1 μL of a 5 μ*M* molecular beacon solution to the 25 μL solution, and measure the new level of fluorescence (F_{closed}). Ensure that the fluorescence level exceeds that of the F_{buffer}. In case the fluorescence level is the same as F_{buffer}, add an additional 1 μL of a 5 μ*M* molecular beacon solution to the solution and repeat the measurement.
4. Add 1 μL of 25 μ*M* of an oligonucleotide target whose sequence is perfectly complementary to the probe sequence of the molecular beacon. Allow the molecular beacon to hybridize to the oligonucleotide target (about 1 min), and measure the fluorescence of the solution (F_{open}).
5. Calculate the signal-to-background ratio as $(F_{open}–F_{buffer})/(F_{closed}–F_{buffer})$.

3.6.2. Thermal Denaturation Profiles

To determine the window of discrimination, the fluorescence of the molecular beacon as a function of temperature is measured in the absence of target, in the presence of perfectly complementary target oligonucleotides, and in the presence of the mismatch target oligonucleotides:

1. For each molecular beacon, prepare three tubes containing 200 n*M* molecular beacon in 1X PCR buffer and the concentration of $MgCl_2$ that will be used in the PCR assay.
2. Add to one of the tubes a fivefold molar excess of an oligonucleotide that is perfectly complementary to the molecular beacon probe sequence, add a fivefold excess of an oligonucleotide that contains the mismatched target sequence to the other tube, and add only buffer to the third tube.

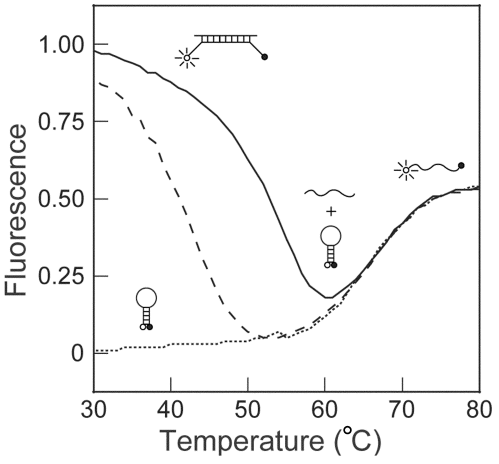

Fig. 3. Thermal denaturation profiles of molecular beacons in the presence of either wild-type target (continuous line), mutant target (dashed line), or no target (dotted line). A diagram indicates the state of the molecular beacon over the thermal denaturation profiles. Mismatched hybrids denature 10 to 12°C below the T*m* of perfectly matched hybrids. In this example, an annealing temperature of 50°C will be optimal to ensure that the molecular beacon generates a high fluorescence signal in the presence of its perfect complementary target and that no fluorescence signals are generated in the presence of a mutant target.

3. Determine the fluorescence of each solution as a function of temperature, using a 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.

Figure 3 shows an example of a thermal denaturation profile. Select the appropriate annealing temperature, that is, the temperature at which only the perfectly complementary probe-target hybrids are formed.

3.7. Real-Time PCR

After characterization of the molecular beacon, the last step in the optimization of the real-time PCR is to determine the optimal concentration of the molecular beacon. In general, the amount of molecular beacon to be used is about the same as the amount of primers, so that both will be in excess to the expected amount of amplification products. It is suggested to test three molecular beacon concentrations: half the amount of the primers, the same amount as the primers, and twice as much. Use the reaction conditions as determined with the SYBR Green PCR, and test the performance of each molecular beacon concentration with different target concentrations (e.g., 100,000, 10,000, 1000, 100,

and 10 molecules). Compare the Ct-values of the different amounts of template molecules for the three molecular beacon concentrations. If the Ct values are equal at all template amounts for the three molecular beacon concentrations, choose the lowest concentration of the molecular beacon used. Otherwise, choose the molecular beacon concentration that results in the lowest Ct value for the different amount of target molecules.

3.8. Multiplex Detection of Four Pathogenic Retroviruses Using Molecular Beacons

Four primer sets and four molecular beacons were designed according to the procedures described above. To detect HIV-1, HIV-2, HTLV-I, and HTLV-II equally well in the same assay, primer sets and molecular beacons were chosen to be compatible. The primers generated short amplification products (100–130 basepairs) and were chosen so that their target sequences would be unique to each retrovirus, highly conserved, present in most clinical subtypes, and not found in the human genome. The assay described here is adapted from Vet et al., 1999 ***(18)***. The concentration of each primer set was adjusted so that the efficiency of amplification of each of the four types of amplification products was approximately equal during the exponential phase of the reaction. The length of each of the probe sequences (25–33 nucleotides) was selected so that probe-target hybrids were likely to form even if the retroviral target sequence was from a subtype that contained one or two nucleotide substitutions. The arm sequences were 6 nucleotides long, to ensure that the T*m* of the stem would be above 65°C. The molecular beacons designed to detect HIV-1, HIV-2, and HTLV-I were labeled with, respectively, FAM, HEX, and Texas Red, and used DABCYL as a quencher. The molecular beacon to detect HTLV-II was labeled with Cy5 and used BHQ-2 as a quencher. These fluorophores were chosen to minimize crosstalk of the emission fluorescence between the filters available on the Bio-Rad iCycler IQ system that was used for this assay.

3.8.1. PCR Assays

Each 50-µL reaction contained the relevant template DNA, 1.00 µ*M* of each HIV-1 primer, 0.25 µ*M* of each HIV-2 primer, 0.50 µ*M* of each HTLV-I primer, 0.25 µ*M* of each HTLV-II primer, 0.10 µ*M* of HIV-1–FAM, 0.20 µ*M* HIV-2–HEX, 0.10 µ*M* HTLV-I–Texas red, 0.10 µ*M* HTLV-II–Cy5, 250 µ*M* of each dNTP, 2.5 U AmpliTaq Gold DNA polymerase, 3 m*M* $MgCl_2$ in 1X PCR buffer. After activating the DNA polymerase by incubation for 10 min at 95°C, 40 cycles of amplification (94°C for 30 s, 55°C for 30 s, and 72°C for 30 s) were carried out in a 96-well spectrofluorometric thermal cycler (Bio-Rad iCycler IQ) and fluorescence was monitored during every thermal cycle at the 55°C annealing step.

To determine how well individual retroviruses can be distinguished from one another in this multiplex format, four assays were carried out in parallel, each initiated with 100,000 molecules of a different retroviral DNA. The results are shown in **Fig. 4**. The intensity of the fluorescence of each of the four molecular beacons (normalized on a scale from 0 to 1 to aid in their comparison) is plotted as a function of the number of thermal cycles completed. The only significant fluorescence that appeared in the course of the amplification reactions carried out in each assay tube was fluorescence from the molecular beacon that was complementary to the sequence of the expected amplification product. The color of the fluorescence identified the retroviral DNA that was originally added to the reaction mixture. No significant fluorescence developed in a control assay that did not contain any template DNA. These results demonstrate that each molecular beacon binds only to its intended target amplification product and that there is no crosstalk between the emission filters.

4. Notes

1. The use of hot-start *Taq* DNA polymerase is recommended because it often results in higher quality real-time PCR assays compared to the use of conventional *Taq* DNA polymerase. The major advantage of hot-start *Taq* DNA polymerases is that primer extension cannot occur during the preparation of the master mix. This greatly minimizes the extension of nonspecific primer annealing events that might occur prior to starting the PCR reaction. As a result, they increase the amplification yield of low copy numbers of template and result in more efficient multiplex amplification assays.
2. Some spectrofluorometric thermal cyclers have the possibility to program a temperature gradient that enables the user to optimize the annealing temperature at the same reaction plate with the different $MgCl_2$ and primer concentrations. The temperature gradient is also a very convenient option for optimizing SNP detection assays.
3. PCR efficiency can be calculated using the following formula:

$$E = e^{\ln 10/-s} - 1$$

where E = efficiency, s = slope of the standard curve,
e = 2.718 (approx) and ln10 = 2.303 (approx)

4. Multiplex quantitative assays require both precise and accurate quantification of multiple gene targets in one assay tube and should therefore be carefully optimized. PCR efficiencies of the different amplification reactions should be maximized and equalized. Maximizing the efficiency of a reaction allows accurate quantification over a wider range of starting template concentrations and improves the reproducibility of replicate samples. It is equally important that the individual reactions have similar efficiencies because any difference in individual efficiencies will be amplified when the two reactions are combined. Always test for crossreactivity of the individual reaction components by comparing a set of wells containing one reaction alone to a set of wells containing all ingredients for

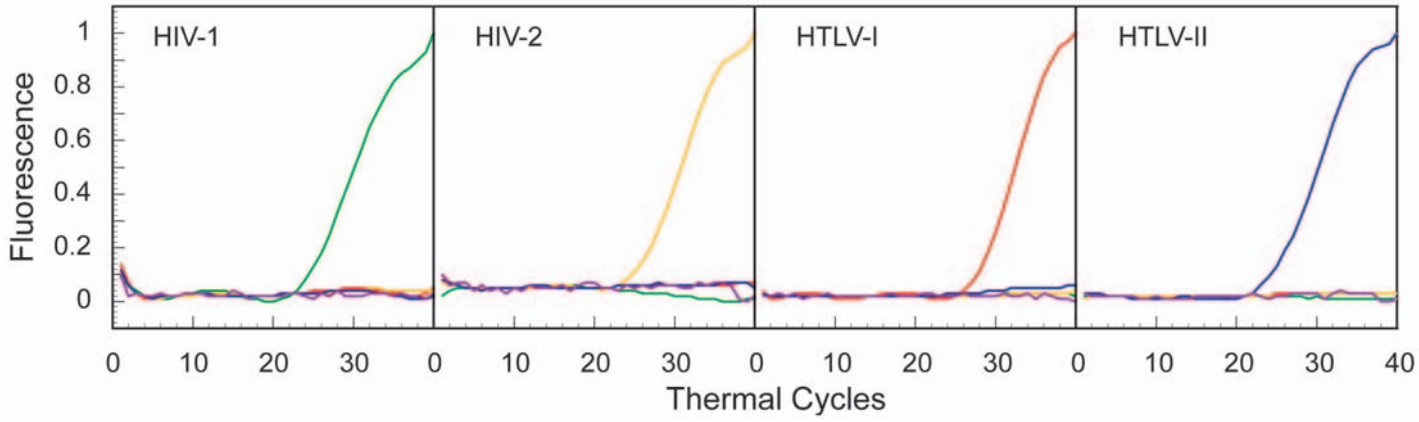

Fig. 4. Real-time detection of four retroviral DNAs in a multiplex format. Four assays were carried out in sealed tubes, each initiated with 100,000 molecules of different retroviral DNA. Each reaction contained four sets of PCR primers specific for unique HIV-1, HIV-2, HTLV-I, and HTLV-II nucleotide sequences and four molecular beacons, each specific for one of the four amplification products and labeled with a differently colored fluorophore. Fluorescent signals from the FAM-labeled molecular beacon (HIV-1 specific), from the HEX-labeled molecular beacon (HIV-2 specific), from the Texas Red-labeled molecular beacon (HTLV-I specific), and from the Cy5-labeled molecular beacon (HTLV-II specific), are plotted in green, yellow, red, and blue, respectively.

the multiplex PCR. If there is no crossreactivity, the Ct value of template X when amplified alone will be identical to the Ct value of template X in the presence of the other components.

5. Use a complementary oligonucleotide target that possesses the relevant nucleotide substitution(s) to perform a thermal denaturation profile as described in **Subheading 3.6.2.** Determine the temperature at which the mismatch hybrids are stable and use this as the assay temperature.
6. It has been observed that nucleotides can quench the fluorescence of fluorophores ***(23)***. Guanosine exhibits the highest degree of quenching. Therefore, it is recommended not to use the guanosine nucleotide at the 5'-end next to the fluorophore.
7. It is important that the conformation of the free molecular beacons is 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 the latter will make the molecular beacon sluggish in binding to its target. If the alternative structure results from the choice of the stem sequence, the identity of 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 two to three nucleotides long do not adversely affect the performance of molecular beacons.
8. This software for designing primers and molecular beacons is convenient to use when the DNA fragment to work with is rather large. With small fragments often no suitable primers or probe sequences are found.
9. In spectrofluorometric thermal cyclers possessing a monochromatic light source, such as a laser or light emitting diode, wavelength-shifting molecular beacons can expand the number of targets that can be detected in one assay tube ***(24)***. Wavelength-shifting molecular beacons contain a "harvester" fluorophore that, when hybridized to an amplification product, transfers the absorbed energy from the light source to a second "emitter" fluorophore that fluoresces in the desired color. However, when not hybridized to an amplification product, the energy from the harvester fluorophore is directly transferred to a quencher, and the wavelength-shifting molecular beacon remains dark.

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