Information Panel

PCR Primer Design

Arun Apte and Saurabha Daniel

INTRODUCTION

The objective of a polymerase chain reaction (PCR) is to amplify a specific DNA segment without any nonspecific by-products. In principle, each physical and chemical component of PCR can be modified to produce a potential increase in yield, specificity, or sensitivity. Yet the most critical parameter for successful PCR is optimal primer design. A poorly designed primer can result in little or no product, due to nonspecific amplification and/or primer-dimer formation leading to reaction failure, even when all the other parameters are properly optimized. This article provides general guidelines for PCR primer design, tips for development of primer pairs for more complex applications, and advice on the development of probes for real-time PCR.

BASICS OF PRIMER DESIGN

Several parameters must be taken into account when designing primers for PCR. Each parameter is discussed in detail below.

Primer Length

Primer length critically affects PCR success by influencing specificity, melting temperature, and time of annealing. A primer length of 18-30 bases is optimal for most PCR applications. Shorter primers could lead to nonspecific PCR amplification. Longer primers are more specific but have a higher probability of containing secondary structures, such as hairpin loops.

Primer Melting Termperature

The specificity of PCR strongly depends on the melting temperature (T_m) of the primers. For most PCR applications, the optimum melting temperature of primers ranges from 55°C to 60°C. In the absence of destabilizing agents, the T_m of a primer depends on its length, sequence composition, and concentration. The impact of ionic strength is negligible because the salt concentration does not vary significantly under different PCR conditions.

It is also important that all of the primers used in a reaction have similar melting temperatures. For most PCR applications, the primer pair T_m mismatch should not be more than 2°C-3°C. If the difference is greater, amplification will be less efficient or may not work at all because the primer with the higher T_m will misprime at a lower than optimal annealing temperature, and the primer with the lower T_m will only bind in low concentrations at a higher than optimal annealing temperature.

The most accurate T_m can be estimated by formulas based on the nearest-neighbor thermodynamic theory, which takes into account thermodynamic analysis of the duplex melting process:

$$T_m = [\Delta H/\Delta S + R \ln (C)] - 273.15.$$

The changes in enthalpy (ΔH) and entropy (ΔS) of a duplex formation are calculated from nearest-neighbor thermodynamic parameters, R is the molar gas constant, and C is the molar concentration of the oligonucleotide. This analysis determines the specific enthalpy and entropy contribution to the free energy of the duplex, made by each "nearest neighbor" in the sequence. The nearest

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neighbor starts at the 5′ end, and the enthalpy and entropy contributions are additive. A second term is added to the calculation of T_m to account empirically for the stabilizing effect of salt on the duplex:

$$T_m = [\Delta H/\Delta S + R \ln (C)] - 273.15 + 12.0 \log [Na^+].$$

Most primer design software uses either the Breslauer or SantaLucia nearest-neighbor parameter set to estimate the T_m of oligonucleotide duplexes (Breslauer et al. 1986; SantaLucia 1998, respectively).

A first-order approximation for short sequences with 20 bases or less can be calculated by the Wallace rule. The equation assumes a salt concentration of 0.9 M, common for dot-blot and hybridization assays:

$$T_m = 2°C\ (A + T) + 4°C\ (G + C).$$

A general rule of thumb is to use a temperature ~5°C lower than the primer melting temperature. Often, the annealing temperature determined this way will not be optimal, and empirical experiments will have to be performed to determine the optimal temperature. This is most easily accomplished using a gradient thermal cycler. Alternatively, more accurate equations can be used to calculate the T_a (optimum annealing temperature) (Rychlik et al. 1990):

$$T_a\ \text{Opt} = 0.3\ (T_m \text{ of primer}) + 0.7\ (T_m \text{ of product}) - 25,$$

where T_m of primer is the melting temperature of the less stable primer-template pair and T_m of product is the melting temperature of the PCR product, both calculated as shown above.

Amplicon Melting Temperature

In addition to calculating the melting temperatures of the primers, care must be taken to ensure that the melting temperature of the product is low enough to ensure complete melting at 92°C. In general, products between 100 and 600 bp are efficiently amplified in PCR. This parameter will help ensure a more efficient PCR, but is not always necessary for successful PCR. The product T_m can be calculated using the formula

$$T_m = 81.5 + 16.6\ (\log_{10}[K^+]) + 0.41\ (\%G+C) - 675/\text{length}.$$

Secondary Structures

An important factor to consider when designing a primer is the presence of secondary structures. A primer sequence with regions of self-homology can snap back to form partially double-stranded hairpin structures. Similarly, interprimer homology can lead to the formation of primer-dimers. Because high concentrations of primers, relative to the template, are used in the PCR, the primers may anneal to each other much more readily than they anneal to the template. The presence of 3′ hairpins or 3′ dimers is especially detrimental to amplification, because these will lead to the nonspecific amplification of sharp background products. A simple way to avoid secondary structures is to select primers that are 50% G + C and deficient in one of the four bases.

Repeat and Runs

Primers with long runs of a single base or nucleotide repeats should generally be avoided. The effects of different types of repeats and runs are shown in Table 1.

GC Clamp

Including a G or C residue at the 3′ end of primers increases the priming efficiency. The so-called "GC clamp" helps to ensure correct binding at the 3′ end of the primer due to the stronger hydrogen bonding of GC residues, thus providing enhanced specificity. Primers that end with a thymidine residue tend to have reduced specificity.

Amplification of High GC Content Targets

For amplification of GC-rich DNA, primers with a higher T_m (preferably 75°C-80°C) are recommended. The strand separation temperature for GC-rich DNA is significantly higher than for normal DNA. At lower temperatures, the two strands of PCR amplicons have a tendency to re-anneal faster and, as a

Table 1. Effects of primer repeats and runs

Repeat motifs	Description	Effect
Simple repeats	A repeated sequence of four or more nucleotides, which is repeated: (…AATCGA…AATCGA…)	Simple repeats can generate secondary binding sites for primers. Stable hybridization to secondary binding sites results in nonspecific amplification. Repeats >3-4.
Inverse repeats	A self-complementary sequence motif of four or more nucleotides (stem-loop or hairpin motifs): (…AATGGC…GCCATT…)	Inverse repeats can cause inefficient priming because they lead to formation of stable hairpins in the binding region, or within the amplicon.
Homopolymeric runs	A sequence of four or more identical nucleotides: (…AAAAA…)	Homopolymeric runs can be considered a special case of direct repeats. These can cause ambiguous binding of primers to their target site ("slippage effect"). Poly (A) and poly (T) stretches should also be avoided because these will "breathe" and open up stretches of the primer-template complex. Additionally, runs of three or more G residues can cause problems due to intermolecular stacking.

result, compete with primer annealing. Higher annealing temperatures during PCR favor primer annealing and therefore increase amplification efficiency.

Unintended Homologies

Primer sequences should be searched using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and checked for cross-homology with repetitive sequences or with other loci elsewhere in the genome. Such homologies could lead to false priming and the production of nonspecific amplicons.

MULTIPLEX PCR PRIMER DESIGN

Multiplex PCR (MPCR) uses one template and several sets of primers for the simultaneous amplification of multiple target sequences in a single PCR. It is an extremely useful technique that increases the throughput of PCR and allows more efficient use of each DNA sample. A variant of MPCR is combinatorial PCR. Combinatorial PCR uses several templates and several primer sets, all in the same reaction. The terms, MPCR and combinatorial PCR, are often used synonymously.

Design Strategy

MPCR requires that all the primer pairs in a reaction amplify their unique targets under a defined set of reaction conditions. Most multiplex reactions are restricted to amplification of five to ten targets. One reason for this is that a degree of flexibility is lost with each additional primer set included in the reaction. Increased numbers of primers also increase the probability of primer-dimer formation and nonspecific amplification. The development of an efficient MPCR requires strategic planning and often multiple attempts to optimize reaction conditions.

Ideally, all the primers in a multiplex reaction should amplify their individual target sequences with equal efficiency. Most often it is difficult to predict the efficiency of a primer pair, but oligonucleotides with near-identical annealing temperatures work well under similar conditions.

General Rules for Multiplex Primer Design and Optimization

When designing primers for use in MPCR, the general rules of primer design apply, but additional considerations must be taken into account. Generally, all the primers in a multiplex reaction should be matched for T_m. Care should be taken to avoid primers with complementary 3′ nucleotides. Each primer pair should be tested separately to determine optimal conditions. Once the panel of primer pairs is assembled, they need to be mixed sequentially and optimized.

1. The length of individual primers should be 18-24 bases. Longer primers are more likely to result in formation of primer-dimers.

2. Annealing temperature and cycle number are critical to the success of MPCR. The annealing temperature should be kept as high as possible. Identify the annealing temperatures for each primer pair and use the lowest temperature in the multiplex reaction. Similarly, use the minimum number of cycles.

3. Since multiple templates are simultaneously amplified, the pool of enzyme and nucleotides in a MPCR can be a limiting factor, and more time is required for complete synthesis of all products. It is important to optimize reagent concentrations and extension times for each reaction. Longer extension times are needed than in single-target PCR.

NESTED PCR

Nested PCR is designed to increase the sensitivity of PCR using a second PCR to amplify the product directly from a primary PCR. The second reaction uses primers placed internal to the first primer pair. These internal primers are referred to as "inner" or "nested" primers. The amplification product from the first round acts as a template for the second round, significantly improving sensitivity without impairing specificity (Albert and Fenyo 1990). Because nested PCR uses two sets of primer pairs, a higher total number of cycles are possible with replenishment of reaction components such as *Taq* DNA polymerase. If a full nested PCR (using two internal primers) cannot be performed, sensitivity and specificity can be improved by designing a heminested PCR using just one inner primer in conjunction with one of the outer primers from the first reaction.

The biggest problem with nested PCR is that it is prone to contamination. Tubes from the first PCR have to be opened so that the primary product can be transferred to a new tube for the second reaction. It also involves the additional cost of two rounds of PCR and additional primer synthesis.

To reduce the risk of contamination, a single-tube approach, referred to as drop-in/drop-out nested PCR, can be used. In this protocol, the inner primers are designed with a significantly lower melting temperature than the outer primer pair. Both primer pairs are included in the first reaction. During the first round of PCR, the annealing temperature is chosen so that only the outer pair will anneal and extend. During the second round of amplification, the annealing temperature is lowered so that the inner pair can function. Although this method is attractive from the contamination control perspective, it does not provide all the advantages of the original nested PCR protocol.

Design Strategy

The same general rules of PCR primer design can be applied to the design of primers for nested PCR. Because multiple primer pairs are used, the increased probability of primer-primer interaction should be carefully considered. When designing primers for single-tube nested PCR, it is important that the melting temperature of the inner (nested) primer pair is significantly lower than that of the outer (first-round) primer pair. The easiest way of achieving this is to reduce the length of the nested primers as compared to the outer primers (e.g., 18-20 bases vs. 25-28 bases). The T_m of the outer primer pairs should be high enough to prevent the inner primers from annealing, ensuring that only the longer product is produced in the first-round PCR. Internal primers should be used in excess (typically 40 times more) compared to the outer primers. The use of shorter annealing and extension times in the second PCR favors the annealing of the shorter primers and production of the smaller amplicon.

cDNA/gDNA SELECTIVE PRIMER DESIGN

Design Strategy

The major problem in reverse transcriptase (RT)-PCR is the presence of contaminating genomic DNA (gDNA), which can lead to false-positive signals, reduced specificity, or overestimation of specific RNA. To prevent any interference by gDNA in RT-PCR applications, primers can be designed to anneal to splice junctions, unique to cDNA sequences, so that gDNA will not be amplified.

The cDNA-specific primers can be designed in three ways (as illustrated in Fig. 1):

1. Primers spanning the exon-exon junction. The primer is designed so that one-half of its sequence will hybridize to the 3′ end of one exon and the other half to the 5′ end of the adjacent exon. This

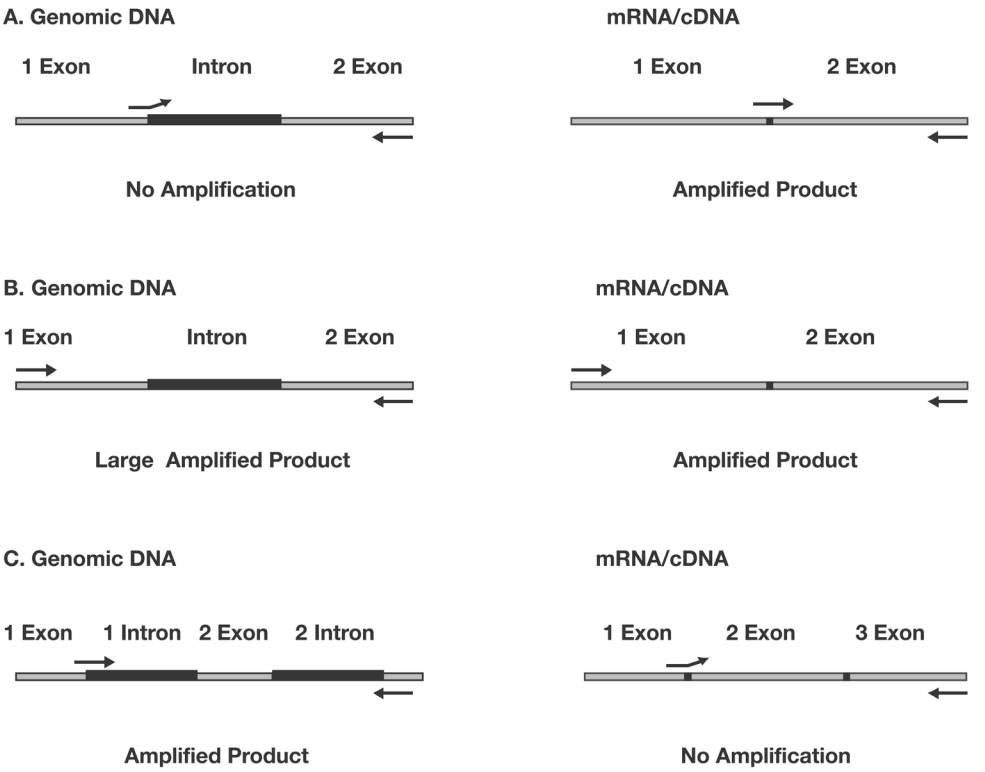

FIGURE 1. cDNA/gDNA selective primer design. (*A*) Primers spanning the exon-exon junction. (*B*) Primers flanking the exon-exon junction. (*C*) Exon-primed intron-crossing (EPIC) primers.

primer will anneal to mRNA, or to cDNA synthesized from spliced mRNAs, but not to gDNA, thus eliminating amplification of contaminating gDNA. Either the forward or the reverse primers can be designed to cross the exon junction; the second primer can be designed to bind at a second exon junction, or at a site completely within the exon.

2. Primers flanking the exon-exon junction. RT-PCR primers can be designed to flank a region that contains at least one intron. Products amplified from cDNA will be smaller than those amplified from gDNA. This size difference can be used to detect the presence of contaminating DNA.

3. Exon-primed intron-crossing (EPIC) primers. For selective amplification of the gDNA from a mixture of cDNA and gDNA, primers should be designed to anneal across the exon-intron junctions (see, e.g., Bierne et al. 2000). Because exon sequences are more highly conserved than intron sequences, EPIC primers can be used in phylogenetic studies to amplify homologous intron regions.

CROSS-SPECIES PRIMER DESIGN

In addition to the identification of genes and prediction of the encoded proteins, the current effort in genomics includes studies on genome organization to examine the interposition of genes with structural and regulatory elements. Cross-species analysis, which forms a major part of comparative genomics, is seen as an important method for the study of evolution, gene function, and human disease.

Cross-species analysis of microbial genomes, particularly bacteria, is beginning to identify genes conserved among bacteria, and virulence genes associated with subspecies (Fredricks and Relman 1996). These advances have facilitated the development of new, broad-based methods for the detection and discrimination of pathogenic microbes. Cross-species PCR can be used to study evolutionarily related species that share some common genomic properties; the conserved regions can be used

to identify species at the genomic level. The technique can also be used to amplify homologous genes in unsequenced genomes.

Design Strategy

Cross-species PCR primers are designed using multiple sequence alignment and basic primer design principles. The alignment of small numbers of large contiguous sequences identifies conserved regions, which constitute potential cross-species primer-binding sites. Primers annealing to the conserved regions can be used to amplify homologous loci in different species (see Fig. 2).

The following steps should be performed:

1. Align selected sequences using a multiple sequence alignment program such as ClustalW (available from http://www.ebi.ac.uk/Tools/clustalw/index.html).
2. Evaluate conserved regions of the alignment. Derive a consensus primer sequence by choosing the most commonly occurring nucleotide at each position of the conserved sequence.
3. The primer-binding site should lie entirely within the conserved region. For distantly related species, when sequences are not completely conserved, the minority consensus can also be used to design probes/primers. Degeneracy can be tolerated at the 5′ end of the primer, but mismatches at the 3′ end reduce both annealing specificity and PCR yield (Sommer and Tautz 1989).
4. Use general primer-design rules for PCR to avoid false priming and primer-dimer formation in cross-species PCR.

Real-Time PCR Probe Design

The introduction of real-time PCR has made it possible to accurately quantify the starting amounts of nucleic acid during the PCR, without the need for post-PCR analysis. In real-time PCR, a fluorescent reporter is used to monitor the PCR as it occurs (i.e., in real time). The fluorescence of the reporter molecule increases as products accumulate with each successive round of amplification. The reporter can be a nonspecific intercalating double-stranded DNA-binding dye or a sequence-specific fluorescent-labeled oligonucleotide probe. The oligonucleotide probe is labeled with both a reporter fluorescent dye and a quencher dye. While the probe is intact, the proximity of the quencher greatly reduces the fluorescence emitted by the reporter dye by Förster resonance energy transfer (FRET) through space. Adequate quenching is observed for probes with the reporter at the 5′ end and the quencher at the 3′ end. When a probe molecule is incorporated into an amplicon, quenching is disrupted and the probe fluoresces.

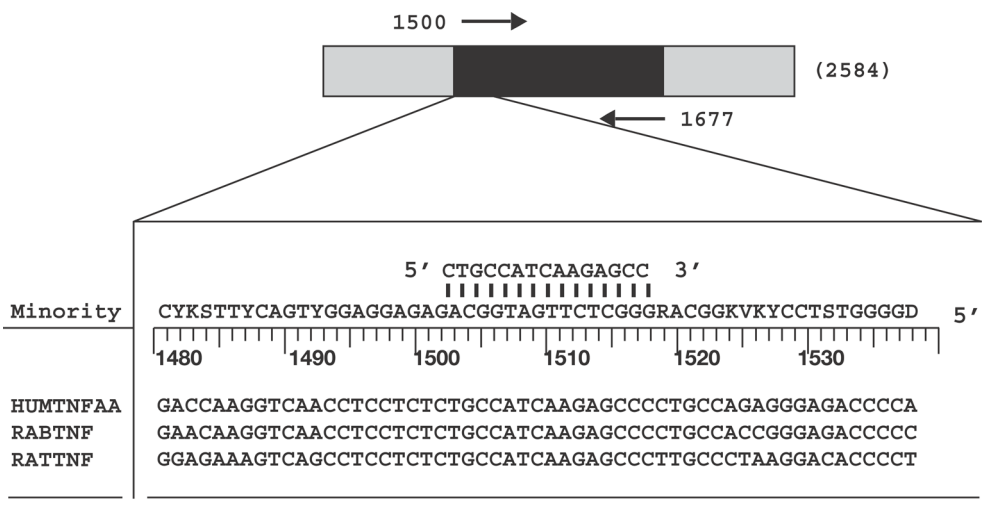

FIGURE 2. Cross-species primer design in conserved region of multiple sequence alignment for human tumor necrosis factor, rabbit tumor necrosis factor, and rat tumor necrosis factor gene sequence.

The ability to monitor the real-time progress of the PCR has completely revolutionized the approach to PCR-based quantification of DNA and RNA. It is now possible to quantify PCR products reliably by eliminating the variability associated with conventional quantitative techniques. Because reporter fluorescence is monitored externally, the reaction tubes do not need to be opened after the PCR is complete; this prevents aerosol contamination by PCR products and reduces the number of false-positive results.

TaqMan Assay

One popular real-time PCR probe strategy is the TaqMan assay (Applied Biosystems). This assay uses a hydrolysis probe, which exploits the 5′ exonuclease activity of *Taq* polymerase to cleave a labeled hybridization probe during the extension phase of PCR (Holland et al. 1991).

Strategy

The fluorogenic 5′ nuclease assay is a convenient, self-contained process. The probe is designed to anneal to the target sequence between the upstream and downstream primers. The probe is labeled with a reporter fluorochrome (usually 6-carboxyfluorescein [6-FAM]) at the 5′ end and a quencher fluorochrome (6-carboxy-tetramethyl-rhodamine [TAMRA]) at the 3′ end, or any T position.

During the PCR amplification, the probe anneals to its target on the template DNA. As the primer is extended, the newly synthesized DNA strand approaches the site of probe hybridization. On arrival, due to its intrinsic 5′ to 3′ nuclease activity, the polymerase cleaves the probe, releasing the reporter fluorochrome into the reaction buffer. When the reporter molecule parts company with the quencher, it starts to fluoresce, and synthesis of the DNA strand continues until the amplification cycle is complete. Because the reporter molecules are cleaved from the probes during every amplification cycle, the fluorescence intensity of the overall system increases proportionally to the amount of DNA amplified.

The following issues should be considered when designing TaqMan probes:

1. The 3′ end of the probe must be protected against chain elongation during PCR. To block the 3′-end phosphate, cordycepin, 2′,3′-dideoxynucleosides, inverse T, or the quencher dye itself can be used. Labeling the 3′ end with TAMRA or another quencher can be achieved by using a 3′ amino linker. It is also possible to label the 3′ end of oligonucleotides post-synthetically via an amino link. This method is available for all dyes, except Cy3, Cy5, Cy5.5, HEX, and TET.
2. The probe T_m should be 10°C higher than the amplification primer T_m and within 65°C-72°C for optimal *Taq* exonuclease activity.
3. Avoid incorporation of a G residue at the 5′ end of the probe; this will quench reporter fluorescence even after cleavage.
4. Probe must not have more Gs than Cs.
5. Avoid runs of single nucleotides, particularly G residues.
6. AT-rich sequences require longer primer and probe sequences to achieve the recommended T_m. Note that probes more than 40 bp long can exhibit inefficient quenching and produce lower yields.
7. Design the probe to anneal as close as possible to the primer without overlapping (at least one base away from primer 3′ end).
8. If a TaqMan probe is being designed for allelic discrimination, it is advisable to place the mismatching nucleotide (the polymorphic site) in the middle of the probe rather than at the ends. These probes should be as short as possible to provide the greatest discrimination by the mismatch.

Primer and Amplicon Properties

Amplicon size should range between 50 and 150 bp and should not exceed 400 bp. Smaller amplicons give more consistent results because PCR is more efficient and more tolerant of reaction conditions.

Commercially Available TaqMan Probes

Two types of TaqMan probes are commercially available, as shown in Table 2.

Only three possible fluorophores—FAM, HEX, and TET—can be used as reporter dyes when TAMRA is used as a quencher. This limits the use of the probes for multiplex PCR due to a spectral overlap between these fluorescent reporters. A larger range of fluorescent reporters is available for use with dabcyl (4-[4′-dimethylaminophenylazo]benzoic acid), a universal quencher for molecular beacons. Dabcyl can replace TAMRA at the 3′ end of the TaqMan probe, thus permitting the use of a large range of fluorescent reporters in the synthesis of probes, and simultaneous detection of different targets in a single reaction.

TaqMan Probes for Allelic Discrimination Assays

TaqMan MGB probes are most widely used for allelic discrimination assays. These probes have the following features:

1. A nonfluorescent quencher at the 3′ end. Because the quencher does not fluoresce, the fluorescence of the reporter dye can be measured more precisely.
2. A minor groove binder at the 3′ end. The minor groove binder increases the T_m of probes, allowing the use of shorter probes. Consequently, the TaqMan MGB probes exhibit greater differences in T_m values between matched and mismatched probes, which provides more accurate allelic discrimination.

Molecular Beacon Design

Molecular beacons (Tyagi and Kramer 1996) represent another example of specific fluorescent real-time probes (see Fig. 3). The molecular beacons enable dynamic, real-time detection of nucleic acid hybridization events both in vitro and in vivo (Tyagi and Kramer 1996; Kostrikis et al. 1998; Tyagi et al. 1998).

A molecular beacon is a dual-labeled oligonucleotide (25-40 nucleotides [nt]) that forms a hairpin structure with a loop (probe) and self-complementary stem. There is a fluorescent reporter molecule at the 5′ end and a quencher at the 3′ end. At room temperature, the molecular beacon assumes the hairpin formation, and the fluorescent reporter and quencher molecule are brought together by the probe's self-complementary stem structure, thus suppressing the fluorescent signal. During the annealing step of the PCR, when the probe encounters a target DNA sequence, thermodynamics favor the binding of the beacon to its target, rather than reformation of the hairpin structure. Disruption of the stem structure separates the fluorescent reporter from its quencher, thus allowing the reporter to fluoresce (see Fig. 3).

Unlike conventional oligonucleotide hybridization, in which unhybridized probe molecules must be removed to eliminate background, hybridization using molecular beacons has an inherently low background, due to the stability of the beacon-quencher association in the absence of target sequences. The low background eliminates the need for washing and probe degradation steps. Moreover, with each successive cycle of amplification, the proportion of molecular beacons that bind the target and emit light increases. Thus, the increase in fluorescence corresponds directly to the accumulation of product.

Due to the stability of the loop-stem structure, selectivity of molecular beacons is higher than that of a linear probe. Molecular beacons can discriminate between targets that have a single-nucleotide change, making them ideal tools for the investigation of single-nucleotide polymorphisms (SNPs). The perfectly matched probe-target hybrid is more stable than the single-stranded hairpin structure of the molecular beacon, whereas the mismatched probe-target hybrid is generally less stable, regardless of the base-pair combination of the mismatch. This thermodynamic feature is the key to the exquisite specificity of molecular beacons.

Table 2. TaqMan probes

TaqMan probe	5′ Label	3′ Label	Features
TaqMan	6-FAM, VIC, or TET	TAMRA	None
TaqMan MGB	6-FAM, VIC, TET, or NED	Nonfluorescent quencher	Minor groove binder

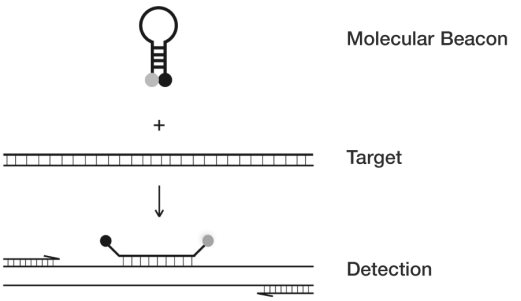

FIGURE 3. Schematic diagram of a molecular beacon and its principle for PCR detection.

Molecular beacons are finding a multitude of uses in many different quantitative and qualitative target detection assays. They are used for real-time monitoring of DNA amplification during PCR (Tyagi and Kramer 1996); they can be multiplexed using multiple dye labels and used for real-time fluorescent genotyping (Kostrikis et al. 1998; Tyagi et al. 1998) and in the simultaneous detection of different pathogens in clinical samples (Vet et al. 1999). Molecular beacons have also been used to detect RNA transcripts in living cells (Sokol et al. 1998) and to detect DNA-binding proteins (Heyduk and Heyduk 2002). Molecular beacons have been adapted to real-time and end-point PCR, and to RT-PCR assays.

Design Guidelines

To monitor PCRs successfully, molecular beacons must hybridize to their targets at PCR annealing temperatures, while the free molecular beacons remain closed and nonfluorescent. The annealing temperature and the buffer will influence probe specificity and must be carefully controlled. The loop or probe region of the beacon is target-specific and is flanked by the sequences that form the hairpin. The following general guidelines should be observed when designing beacons.

1. The probe region should be 15-33 nt long. Its sequence must be complementary to the target sequence of the assay.
2. The T_m of the probe region should be 7°C-10°C higher than the PCR annealing temperature (as predicted using the percent GC rule). The prediction should be made for the probe sequence alone, before adding the stem sequences.
3. To ensure preferential hybridization of the beacon to target sequences, the probe must be complementary to a region of minimal secondary structure. The template secondary structure can be evaluated by analyzing the sequence using folding software, such as mfold (Michael Zuker, Rensselaer Polytechnic Institute, http://frontend.bioinfo.rpi.edu/applications/mfold/).
4. The molecular beacon should bind at or near the center of the amplicon. The distance between the 3′ end of the upstream primer and the 5′ end of the molecular beacon (stem) should be greater than 6 nt.
5. The stem region of the molecular beacon should be 5-7 bp long, with a GC content of 70%-80%. Longer stems will make the molecular beacon sluggish in binding to its target.
6. The length, sequence, and GC content of the stem should be chosen such that the melting temperature is 7°C-10°C higher than the annealing temperature of the PCR primers.
7. The melting temperature of the stem is usually calculated using the mfold hairpin folding formula, which is part of the mfold software. The formula is used to estimate the free energy of the formation of the stem hybrid, from which its melting temperature can be predicted. Assuming a 100% GC content, a 5-bp stem will melt at 55°C-60°C, a 6-bp stem at 60°C-65°C, and a 7-bp stem at 65°C-70°C.
8. The more negative the free energy of a sequence, the more favorable and stable the structure will be. The stem-loop free-energy values obtained with mfold should range between -3 and +0.5 kcal/mol.

9. Because G residues may act as quencher molecules, avoid designing molecular beacons with a G directly adjacent to the fluorescent dye (typically at the 5′ end of the stem sequence). A cytosine at the 5′ end of the stem is preferred.

10. The beacons should be checked for the presence of alternate secondary structure (other than the intended hairpin stem), because this can change the position of the fluorophore relative to the quencher, causing an increase in background signals.

11. Avoid complementarity between molecular beacon and PCR primers; this may cause the molecular beacon to bind to primers and increase background.

12. The amplification primers used in molecular beacon PCRs should be designed to produce a relatively short amplicon, preferably less than 150 bp. The molecular beacon is an internal probe, which must compete with the opposite strand of the amplicon for its complementary target. A shorter amplicon is more likely to fully complete DNA synthesis, assuring that the target sequence is present and, therefore, gives more reproducible results.

Choosing Fluorophore and Quencher Molecules

Another consideration in molecular beacon design is the choice of fluorophore and quencher. Dabcyl is the optimal choice of the quenching fluorophore molecules available for molecular beacons. Dabcyl is a neutral, hydrophobic molecule, making it an ideal partner for a variety of fluorophores. Dabcyl has a short operational range for quenching as compared to the total length of a beacon probe. This means that it must be in close contact with the fluorophore, for energy-transfer quenching to be efficient. Consequently, a stem-loop beacon is quenched whereas a probe-target hybrid is not.

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