Rapid Genotyping and Quantification on the LightCycler™ with Hybridization Probes

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Abstract
Continuous monitoring of PCR using fluorescently labeled hybridization probes is used to demonstrate rapid PCR quantification and genotyping. Two hybridization probes are designed that recognize adjacent internal sequences within target DNA sequences. Both probes are fluorescently labeled such that, when annealed to the template, the fluorophores are separated by one base. Proximity of the fluor allows resonance energy transfer that is monitored by the LightCycler™. PCR product quantitation is demonstrated using a probe pair for β-globin. Quantification of the initial template copy number over a 10⁶-fold range is illustrated, with the ability to distinguish a single starting template copy from a no template control. A two-probe system is also used for rapid genotyping of the factor V Leiden mutation.

Introduction
Methods used for DNA analysis usually involve amplification of target regions by PCR followed by separate product analysis. Sequence-specific monitoring of PCR products is routinely performed by hybridization analysis using blots, gels, or microtiter plates. Hybridization of small oligonucleotide probes to template DNA can be visualized with radioactively labeled probes, fluorescently labeled probes, or chemiluminescent techniques. These techniques, however, are time-consuming and can involve several handling steps that increase the risk of end-product contamination and sample tracking errors. The LightCycler™ is a microvolume fluorometer integrated with a thermal cycler that combines rapid-cycle PCR with real-time fluorescence monitoring (1). Sequence-specific hybridization probes can be designed that allow detection and analysis of PCR products on the LightCycler without the need for any post-PCR sample manipulation (2), allowing high throughput genotyping and product quantification.

Fluorescence offers advantages over other techniques, notably its linear response over a large dynamic range. Fluorescence monitoring of amplification using hybridization probes is based on the concept that a fluorescence signal is generated if fluorescence resonance energy transfer (FRET) occurs between two adjacent fluorophores, illustrated in Figure 1 (3). Fluorescence-based, single step genotyping methods have been described for the factor V Leiden mutation (4) and the C677T point mutation in the methylenetetrahydrofolate reductase gene (5) using the LightCycler. Amplification and analysis take approximately 30 min and require no sample handling after loading on the LightCycler. Both these applications employed one labeled primer and one labeled probe with asym-
metric amplification of target sequences using a Cy5-labeled primer. Hybridization of a 3' fluorescein-labeled probe to the Cy5-labeled target strand results in the two fluorophores being brought into close proximity allowing FRET to occur. After amplification, a melting curve is generated that allows rapid genotyping. Mutant alleles are distinguished from wild type by the melting temperature ($T_m$) of the probe. Continuous fluorescence monitoring of the reaction as the temperature is raised from annealing to denaturation, results in a sharp decrease in fluorescence at the temperature at which the probe dissociates from the labeled template. A single base change (G1691A) caused by the Leiden mutation results in a decrease in probe $T_m$ of 7°C. A more stable substitution in the MTHFR gene (C677T) gives a 3°C difference in $T_m$ between wild type and mutant alleles. Both changes can easily be distinguished with the LightCycler, suggesting that this fluorescence method is suitable for all single base mismatches.

This paper describes experiments using two singly labeled probes for rapid, sequence-specific mutation detection and quantification. Hybridization probe pairs are designed that recognize adjacent internal sequences within target DNA sequences. Both probes are fluorescently labeled such that, when annealed to the template, the fluorophores are separated by one base, allowing a strong FRET signal. PCR product quantitation is demonstrated using a probe pair for β-globin. Fluorescence is monitored once each cycle at the end of the annealing step and increases above background at a cycle number that is dependent on the initial template concentration. A two probe system is also used for genotyping the factor V Leiden mutation, showing that both a primer-probe system and a probe-probe system are equally reliable for mutation detection.

Materials and Methods

Primer and probe design and synthesis

The factor V amplicon was derived from exon 10 and comprised a 220 base pair fragment. The sense and reverse amplification primers were a 22-mer and a 20-mer (antisense intron 10), respectively. The anchor probe was a 36-mer (antisense exon 10), labeled at the 5' end with LightCycler-Red 640 (LC-Red 640) and modified at the 3' end by phosphorylation. The
Fluorescence protocols

PCR was performed with 0.2 µM primers in a standard PCR reaction, supplemented with bovine serum albumin (BSA). For the factor V mutation detection experiments, 0.2 µM anchor probe and 0.2 µM hybridization probe and 0.4 U Taq DNA polymerase per 10 µl sample was included. For the quantitation experiments with β-globin, 0.2 µM fluorescein labeled probe and 0.4 µM Cy5-labeled probe was used. 0.8 U of exonuclease minus polymerase per 10 µl sample was used after preincubination with anti-Taq antibody.

Samples were spun into glass capillary cuvettes, capped and placed in the LightCycler. Fluorescein is used as the donor fluorophore and either Cy5™ or LC-Red 640 can be used as the acceptor fluorophore.

3’ fluorescein-labeled hybridization probe was a 23-mer (antisense exon 10/intron 10 junction).

The β-globin amplicon is a 110 base pair sequence that was amplified using a 20-mer forward primer and a 20-mer reverse primer. The first hybridization probe (35-mer) was 3’-labeled with fluorescein and the second probe (30-mer) was 5’-labeled with Cy5. Primers and probes were synthesized and purified as described previously (4). The binding of the fluorescently labeled probes is illustrated in Figure 2.

Sequence-specific quantification

Hybridization probes can be used for sequence-specific quantification of PCR products. Quantification of the initial template copy number over a 10⁷-fold range is possible (2). Figure 4 shows the amplification of purified β-globin serial dilutions, ranging from 10⁸ initial template copies to one copy with fluorescence plotted against cycle number. The higher the initial copy number, the earlier the fluorescence signal appears out of the background. As the initial template concentration decreases, the curves are shifted to the right. The sensitivity of the system is such that a single template copy can be distinguished from the absence of template. Fluorescence is acquired once per sample, just before extension when the probes are

Results and Discussion

FRET probes

Fluorescence monitoring of PCR amplification is based on the concept of fluorescence resonance energy transfer between two adjacent dyes. Excitation of a donor fluorophore with an emission spectrum that overlaps the excitation spectrum of an acceptor fluorophore results in non-radioactive energy transfer to the acceptor.

The emission spectra of three fluorophores used with the LightCycler are shown in Figure 3. Fluorescein is the donor fluorophore of choice for the LightCycler, Cy5® (Amersham), or LightCycler-Red 640 (Roche Molecular Biochemicals) can be used as the acceptor fluorophore.
hybridized, in order to obtain the strongest signal.

**Factor V genotyping**

A typical factor V genotyping experiment using two hybridization probes is illustrated below (Figure 5). A long anchor probe (a 36-mer) is labeled at the 5′ end with LightCycler Red 640 and a second shorter probe (a 23-mer) is labeled with fluorescein at the 3′ end. The probes recognize adjacent sequences with the shorter probe lying over the mutation site. The greater stability of the anchor probe means that loss of fluorescence occurs as the shorter probe melts off the template. A single base mismatch under the probe results in a Tm shift due to its poor stability and allows the mutant to be easily distinguished from the wild type. Other more stable mismatches, however, have also been detected using the same technique (5). One option for designing probes using this system is to determine which combination (wild type probe with mutant template or mutant probe with wild type template) is the least stable and design probes accordingly. If a mutant probe with a wild type template is selected, the probe will melt off the wild type allele at a lower Tm than the mutant allele for which it is a perfect match.

The probe design of these experiments places both probes on the same strand, as far from the extending primer as possible. This delays displacement of the probe by the polymerase and allows maximum hybridization time. The spacing between the fluorophores can be critical for a strong fluorescence signal since the efficiency of energy transfer between dyes decreases with distance. Ideally a spacing of one to four bases is optimal with the signal decreasing as the fluoros are separated by greater than ten bases. The fluorophores on the probe pairs used in these experiments were separated by one base to maximize the signal.

The Leiden mutation represents a G to A substitution within exon 10 of the factor V gene creating an A:C mismatch under the probe pair system. This mismatch results in a large Tm shift due to its poor stability and allows the mutant to be easily distinguished from the wild type. Other more stable mismatches, however, have also been detected using the same technique (5). One option for designing probes using this system is to determine which combination (wild type probe with mutant template or mutant probe with wild type template) is the least stable and design probes accordingly. If a mutant probe with a wild type template is selected, the probe will melt off the wild type allele at a lower Tm than the mutant allele for which it is a perfect match.

This paper has dealt with the usefulness of hybridization probe pairs in fluorescence monitoring of PCR products using the LightCycler. The ability to monitor PCR product formation in real-time is helpful no matter what fluorescence technique is employed. The advantage of hybridization probes is that real-time hybridization information is available, allowing mutation detection and quantification in the same reaction. This permits product identification, even down to a single base change, during amplification. Such a “dynamic dot blot” removes the requirements for processing after amplification and eliminates PCR contamination concerns. Rapid cycling combined with sequence-specific fluorescence monitoring on the LightCycler is a powerful tool.

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**References**