# The Gradient Feature: Use in Optimization of Allelic Discrimination Assays

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## Introduction

### Real-Time PCR

PCR has been greatly refined since its advent 16 years ago. Several improvements in the technology and associated equipment have significantly changed the way PCR is performed today relative to earlier protocols. One of the most remarkable PCR advancements has been the development of real-time PCR (Higuchi et al. 1993), a system that permits the detection of nucleic acids while they accumulate during amplification. Various applications of real-time PCR have been published recently that show this technique to be a powerful tool for the quantitation of RNA and DNA targets as well as for the detection of single nucleotide polymorphisms (SNPs) (Foy and Parkes 2001, Bustin 2003). The advantages of using real-time PCR over the basic PCR technology are numerous. For example, the chances for generating carryover contamination are reduced because no post-PCR handling is required. In addition, the real-time detection of PCR products eliminates post-PCR detection as a potential error source. For quantitative experiments, the dynamic range of real-time PCR assays is several orders of magnitude greater than the range generated by end-point measurements.

### PCR Optimization

Under optimal reaction conditions, a high PCR efficiency allows the synthesis of micrograms of DNA from a few starting molecules of template DNA or RNA. However, reaction optimization requires careful design of primers as well as the adjustment of the cycling conditions and buffer composition. For the development of PCR cycling conditions, one of the most important variables to be optimized is the annealing temperature ($T_a$). A low $T_a$ may cause the synthesis of nonspecific PCR products while the use of a high $T_a$ may reduce the yield of a desired PCR product. Careful selection of the $T_a$ is therefore critical for the success of PCR.

### Allele-Specific Discrimination

The advent of real-time PCR allowed the development of homogeneous methods for SNP detection such as TaqMan (Holland et al. 1991), molecular beacon (Tyagi and Kramer 1996), and Scorpions (Whitcombe et al. 1999) primer/probe-based assays. Most of these homogeneous assays for mutation detection combine amplification of the DNA sequence that spans the target polymorphic nucleotide with the use of allele-specific oligonucleotide (ASO) probes (Wallace et al. 1979). In addition to appropriate probe design and PCR buffer optimization, proper choice of $T_a$ is essential to achieve good allele-specific discrimination. The use of optimal $T_a$ minimizes the cross-hybridization of target DNA to an ASO probe even under the presence of a mismatch.

### The Gradient

The gradient feature was developed to be used with the Bio-Rad iCycler iQ™ system. The gradient allows evaluation of up to eight annealing, polymerization, or denaturation temperatures in a single experiment. Temperature optimization can be accomplished using SYBR Green I or fluorescently labeled specific probes as the detection reagent. In addition, the gradient has proven to be a valuable tool for the optimization of reaction conditions for SNP detection using homogeneous assays. This report describes applications of the gradient feature that show its performance characteristics.

## Methods

### Optimization of $T_a$

$T_a$ optimization was performed for the amplification of a fragment of the coagulation factor V gene (van den Bergh et al. 2000). Duplicate PCRs were carried out in a volume of 50 µl containing 20 mM Tris, pH 8.4, 50 mM KCl, 200 µM each dNTP, 3 mM $MgCl_2$, 0.2 µM each PCR primer (M3= 5'-CTTGAAGGAAATGCCCCATTA, and M5= 5'-TGCCCAGTGCTTAACAAGACCA), a 1:100,000 dilution of SYBR Green I (Molecular Probes, Eugene, OR), 1.25 U Platinum *Taq* polymerase (Invitrogen), and 10 ng human genomic DNA. Reactions were incubated in the iCycler iQ real-time PCR detection system for 3.5 min at 95°C and then subjected to 50 two-step cycles of heating at 95°C for 10 sec and annealing for 50 sec at 54, 55.2, 57.1, 59.8, 64, 66.9, 68.9, or 70°C (temperature gradient). Fluorescent data were collected during the annealing step. A melt-curve protocol immediately followed the amplification. Reactions were incubated first at 95°C for 60 sec and subsequently at 55°C for 60 sec, followed by 80 repeats of heating for 10 sec starting at 55°C with 0.5°C increments.

**Optimization of Annealing Temperature for SNP Detection Using a TaqMan Assay**

For the optimization of a TaqMan assay for SNP detection, an experiment was designed to amplify and detect the factor V Leiden mutation (Bertina et al. 1994). The sequences of the primers and probes used for this experiment are shown in Table 1. Duplicate PCR reactions were carried out in a 50 µl volume containing 20 mM Tris pH 8.4, 50 mM KCl, 200 µM each dNTP, 3 mM $MgCl_2$, 0.2 µM each PCR primer, 0.15 µM each ASO TaqMan probe, 1.25 U Platinum *Taq* DNA polymerase, and 10 ng human genomic DNA. PCR conditions consisted of a 3.5 min incubation at 95°C followed by 50 cycles of 15 sec at 95°C and 60 sec at 55, 56.1, 57.9, 60.5, 64.3, 67.1, 68.9, or 70°C (temperature gradient).

**Table 1. Sequences of primers and probes of a TaqMan assay for SNP detection.**

| Name | Sequence |
|---|---|
| FV-Rev primer | 5'-CATCGCCTCTGGGCTAATAGG-3' |
| FV-Fwd primer | 5'-TTCTGAAAGGTTACTTCAAGGACAAA-3' |
| FV-WT probe | 5'-6-FAM-CCTGGACAGGCGAGGAATA-CAGG-BHQ*-1-3' |
| FV-mutant probe | 5'-HEX-CCCTGGACAGGCAAGGAATAC-AGG-BHQ*-1-3' |

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## Results and Discussion

The iCycler iQ gradient feature was used to optimize the $T_a$ of PCR experiments for the amplification of a 220 bp fragment of the coagulation factor V gene. The iCycler iQ system was programmed to perform a two-step (denaturation-annealing) PCR protocol to test a range of annealing temperatures between 54 and 70°C. To identify the number of amplified PCR products by the incorporation of SYBR Green I, a melt-curve protocol followed the amplification reactions. Melt-curve analysis, which is used to determine the melt curve of double-stranded DNA fragments, is a useful tool to check for the specificity of PCR amplifications. The presence of primer-dimers as well as nonspecific PCR fragments is clearly identified by the presence of peaks with melting temperatures ($T_m$) that differ from that of the expected PCR fragment. Results from the $T_a$ optimization experiments are shown in Table 2 and Figure 1. Data analysis showed the presence of the expected PCR products, as confirmed by a 1.5% agarose gel (Figure 2), within the temperature range of 54–68.9°C. As a result of reduced oligonucleotide priming efficiency, no amplification products were observed when using 70°C as $T_a$. Maximal yield of PCR products, measured by relative fluorescence and threshold cycle ($C_T$), was obtained within

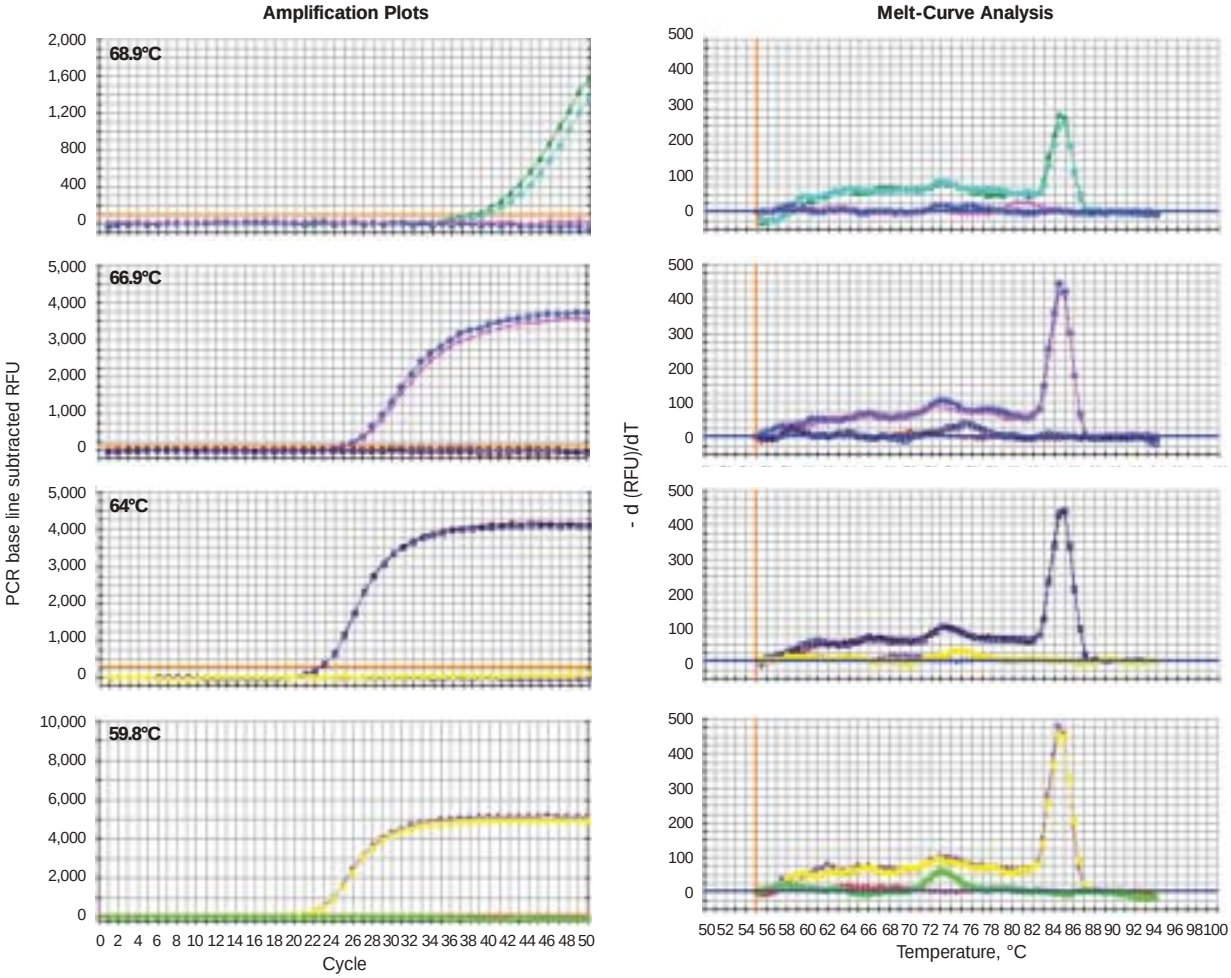


**Fig. 1. Optimization of annealing temperature for primer sets with SYBR Green I.** Amplification of a 220 bp fragment of the factor V coagulation gene; left panels, amplification plots of human genomic DNA at the specified $T_a$; right panels, melt-curve analysis of the same reactions. Representative real-time amplification plots for four out of eight $T_a$ tested with the gradient are shown. Melt curves indicate the specificity of the reaction (specific product $T_m$ = 84.5°C).

**Table 2. Melt-curve analysis.**

| $T_a$ | RFU | $C_T$ | $T_m$ |
|---|---|---|---|
| 70°C | – | – | – |
| 68.9°C | 1,500 | 36.0 | 84.5°C |
| 66.9°C | 3,750 | 23.5 | 84.5°C |
| 64°C | 4,250 | 20.4 | 84.5°C |
| 59.8°C | 5,000 | 20.2 | 84.5°C |
| 57.1°C | 5,000 | 20.2 | 84.5°C |
| 55.2°C | 5,000 | 20.2 | 84.5°C |
| 54°C | 5,000 | 20.2 | 84.5°C |

the range of 54–59.8°C. Additional information regarding PCR specificity is derived from the analysis of the melt curves (Figure 1 and Table 2). Melt-curve analysis showed neither primer-dimer formation nor the presence of nonspecific PCR fragments resulting from hybridization of the primers to sequences on the human genomic DNA template other than the specific priming sites. This information was confirmed by loading an aliquot of the PCR reactions in a 1.5% agarose gel (Figure 2).

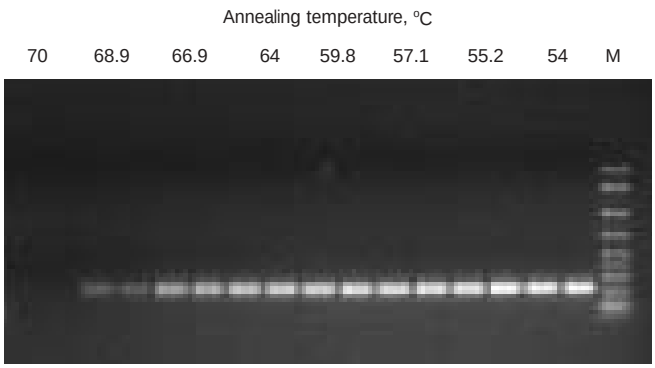


**Fig. 2. Effect of annealing temperature on amplification efficiency.** Products of amplification of a 220 bp fragment from the coagulation factor V gene were subjected to electrophoresis in a 1.5% agarose gel. Lane M, AmpliSize® 50–2,000 bp molecular ruler.

The detection of mutations and polymorphisms by TaqMan and molecular beacon assays is based on the principle of ASO hybridization (Wallace et al. 1979). SNP detection with TaqMan or molecular beacons requires the synthesis of ASO probes that are labeled at the 5' end with a different reporter dye such as FAM, HEX, TET, Cy5, Texas Red, etc. for each ASO probe. For the analysis of a biallelic genetic locus, two allele-specific probes are synthesized: a probe specific for the detection of allele 1 labeled with one dye, and a second probe specific for the other allelic variant labeled with a different dye. Proper choice of the PCR annealing temperature along with probe design and buffer composition is essential for the development of highly specific assays for allelic discrimination. A specific and robust genotypic assay based on the use of ASO probes will favor hybridization of an exact-match probe over a mismatched probe.

To demonstrate the utility of the gradient tool for the optimization of a genotypic DNA probe assay, a TaqMan approach was developed for factor V Leiden genotyping. PCR primers were designed to synthesize a 99 bp fragment of the coagulation factor V gene. The differentially labeled wild-type (WT) and mutant TaqMan ASO probes were mixed in the same reaction along with the PCR primers and subjected to a $T_a$ gradient from 55 to 70°C. Results from the optimization experiment are shown in Figure 3. Duplicate PCRs containing genomic DNA from a WT individual, genomic DNA from a factor V Leiden homozygous mutant individual, or distilled water (no-template control) were performed at each tested temperature. For both the WT and mutant probes, reactions worked well within the range of 55–67.1°C. The analysis of fluorescence in Figure 3 shows a reduced PCR efficiency at 67.1 and 64.3°C. However, when the goal of the experiment is to find specific reaction conditions to develop a robust assay for allelic discrimination, specificity should be favored over maximum yield of PCR products. For example, Figure 3 shows that 64.3°C is not the best $T_a$ to generate maximal product yield, but it is an optimal temperature to achieve good allele-specific discrimination. The amplification plot obtained at 64.3°C (Figure 3, left panel) shows that the WT probe hybridized specifically to its complementary WT PCR products (red plot) but not to the homozygous mutant DNA (blue plot). Similarly, the right panel shows that when 64.3°C was used as the $T_a$, the mutant TaqMan ASO probe hybridized only to homozygous mutant PCR products (pink plot) but not to their WT counterparts (teal plot). Results obtained using annealing temperatures lower than 64.3°C (60.5, 57.9, 56.1, and 55°C) showed lack of specificity for both the WT and mutant TaqMan probes. For example, the amplification plot generated at 60.5°C shows that the WT probe hybridized to the WT PCR products (red plot) but also cross-hybridized to the homozygous mutant PCR products (blue plot). A similar cross-hybridization pattern was observed at 60.5°C for the mutant probe. It is clear from the right panel that the mutant probe not only hybridized to its complementary mutant PCR products (pink plot) but also to PCR products with the WT genotype (teal plot).

In summary, we have demonstrated that the gradient feature is a powerful tool for the optimization of PCR protocols. Useful technical information such as the optimal $T_a$ for obtaining maximal PCR product yield (Rychlik et al. 1990) or the maximal temperature at which PCR works (Wu et al. 1991) can be generated using the gradient in a single experiment. The gradient not only dramatically accelerates the PCR $T_a$ optimization process but also the optimization of SNP detection protocols. In addition to TaqMan and molecular beacons, the iCycler iQ gradient feature can be used to optimize genotypic assays that involve allele-specific PCR (Whitcombe et al. 1999) and fluorescence resonance energy transfer (FRET) using dual-labeled probes (van den Bergh et al. 2000).

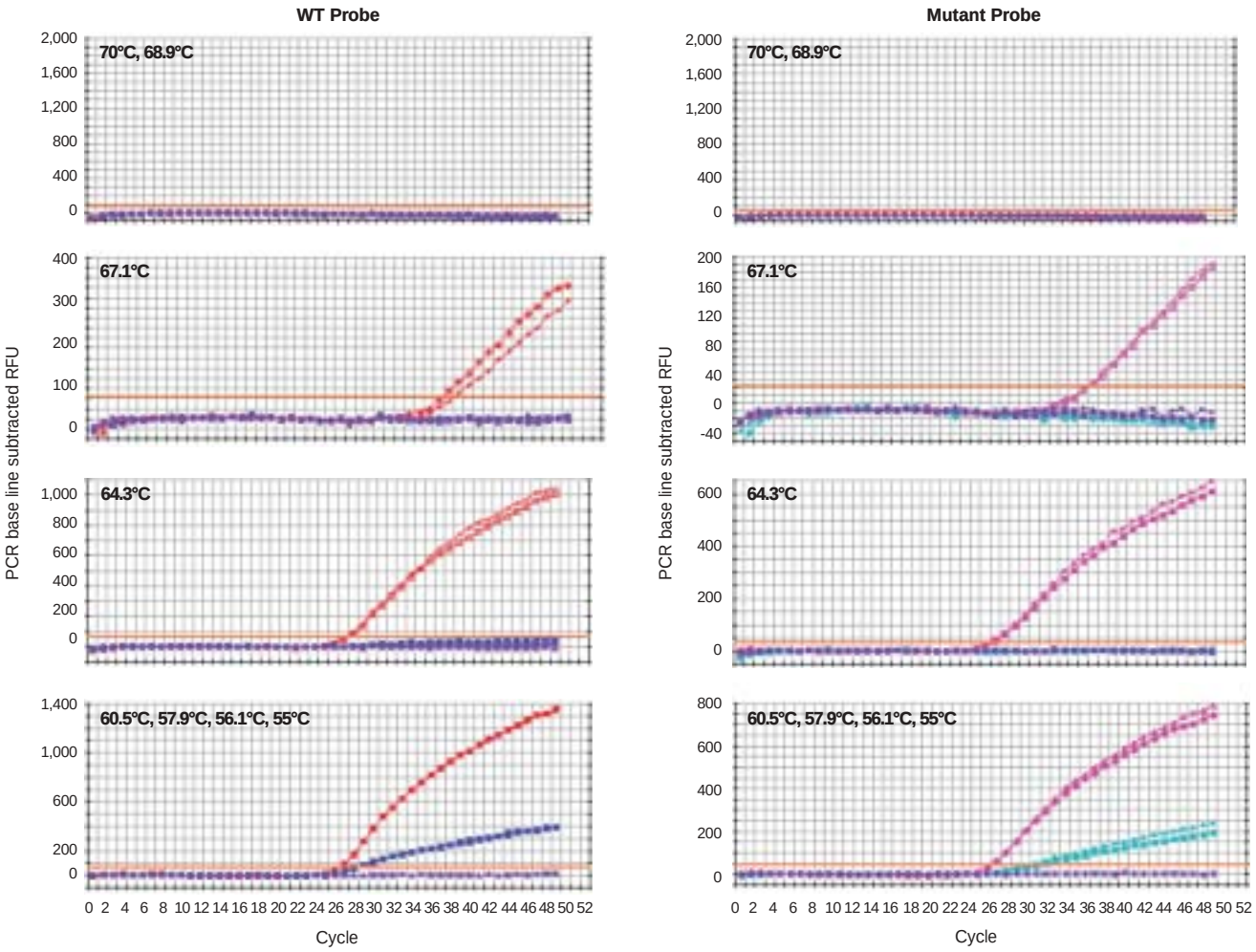


**Fig. 3. Optimization of annealing temperature for TaqMan probes.** Left panels, WT probe; right panels, mutant probe. The factor V Leiden mutation was detected using a TaqMan genotypic assay. Shown are the amplification plots generated by hybridizing the amplified WT and homozygous mutant PCR products with factor V Leiden probes. The $T_a$ gradient used was from 55 to 70°C. Representative amplification plots are shown.

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