Optimizing probe hybridization in real-time PCR for quantification and SNP genotyping

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Real-time PCR is used for quantitative gene expression analysis as well as single nucleotide polymorphism (SNP) detection. A prerequisite for successful quantification and genotyping is high PCR specificity. Adapting PCR to increase specificity often also increases sensitivity of the assay, due to the lack of non-specific PCR products competing for limiting PCR reagents in the course of the reaction (1). Therefore, highly sensitive quantitative and genotyping assays that employ minimal amounts of DNA are more likely to work efficiently.

High PCR specificity is not only required for specific primer annealing and efficient detection of the PCR product but is also a critical factor in SNP analysis using real-time PCR probes, which are used to detect single nucleotide differences in the template. The optimized formulation of QuantiTect™ Probe PCR Master Mix, which selectively destabilizes nonspecific base pairing, provides highly specific and efficient probe hybridization conditions, enabling accurate allele calling in SNP analysis. Real-time PCR-based quantitative gene expression analysis and SNP genotyping are now possible without lengthy assay optimization, even when only minute amounts of template are available.

Materials and methods

Quantitative real-time PCR gene expression assays were performed on the DNA Engine Opticon™ Instrument (MJ Research), which enables users to program a temperature gradient. SNP analysis was performed using an ABI PRISM® 7700 Sequence Detection System. The performance of QuantiTect Probe PCR Master Mix was compared with reagents from supplier A_{II} in reactions containing Molecular Beacons or TaqMan® dual-labeled probes. Setup and cycling conditions followed provided standard protocols. The final volume of SNP analysis reactions was 25 µl. Reactions were used to detect the cytochrome P450 SNP, CYP2D6*4 G1934A, using TaqMan MGB probes.

LightCycler® Software Version 3.5 was used for analysis on the LightCycler System with LightCycler Hybridization (FRET) probes. ▶

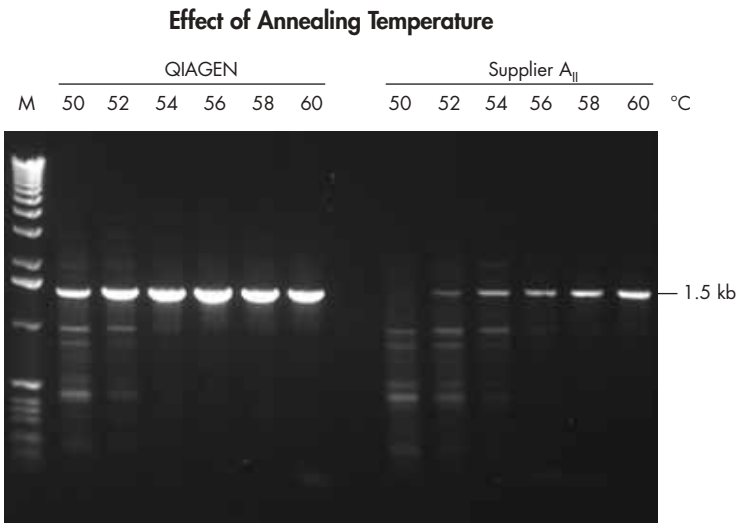

Figure 1 *Amplification of the human single-copy cystic fibrosis gene at the indicated annealing temperatures using QIAGEN® PCR Buffer and QIAGEN Taq DNA Polymerase. The same PCR was performed in parallel using PCR buffer and Taq DNA polymerase from another supplier (**Supplier A_{II}**). **M:** markers.*

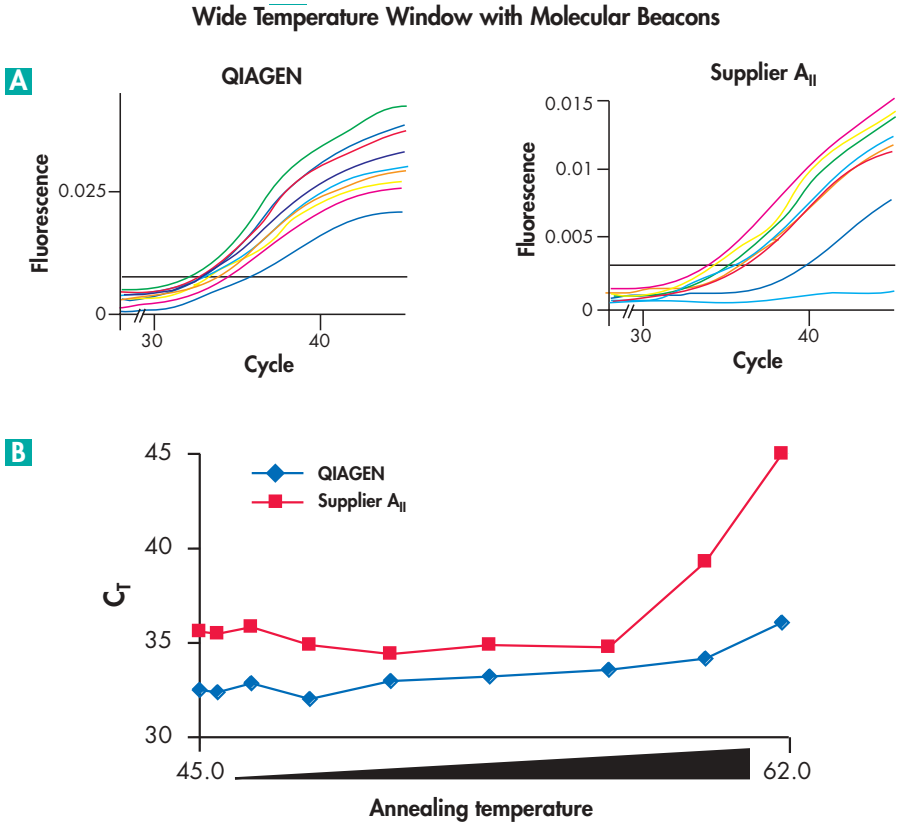

Figure 2 *One-step, quantitative RT-PCR was carried out using the QuantiTect Probe RT-PCR Kit* ***(QIAGEN)*** *or a one-step, quantitative RT-PCR kit from Supplier A, as indicated, using 10 pg HeLa total RNA. Reactions were performed on the DNA Engine Opticon Instrument according to suppliers' instructions, except that a temperature gradient from 45 to 62°C was used during the annealing/detection step of the 3-step cycling program. Primers and probe were specific for a fragment of the human GAPDH transcript.* **A** *Amplification plots. The horizontal line indicates the threshold limit.* **B** *Threshold-cycle (C_T) values with different annealing temperatures.*

Effect of buffer formulation on specificity and temperature of hybridization

PCR specificity depends on maintaining a high ratio of specific to nonspecific primer annealing. This ratio can be modulated by PCR buffer formulation. Cations such as K^+ or Mg^{2+} neutralize negatively charged phosphate groups on the DNA backbone and consequently weaken electrorepulsive forces between the two DNA strands. Reduction of these repulsive forces facilitates hybridization of primer or probe to template DNA or cDNA. NH_4^+, which is also contained in the buffer, can interact with hydrogen bonds between bases to destabilize weak hydrogen bonds at mismatched bases (2). The combined effect of K^+ and NH_4^+ maintains a high ratio of specific to nonspecific primer–template binding. This results in a wider temperature window for efficient and specific primer binding, providing more robust PCR conditions without lengthy PCR optimization (Figure 1).

The same principle holds true for fluorescent probe binding in quantitative or SNP real-time PCR analysis. To demonstrate the effect of a broader temperature tolerance for specific and efficient probe hybridization, real-time, quantitative, one-step RT-PCR was performed using the temperature gradient function of the DNA Engine Opticon Instrument. Assays were performed using either TaqMan dual-labeled probes or Molecular Beacons. Figure 2 shows that QuantiTect Probe RT-PCR Master Mix provides a wide temperature window for binding and detection of the Molecular Beacon. Binding of the probe becomes inefficient only at temperatures above 62°C.

The assay performed using the QuantiTect Probe RT-PCR Kit also shows high sensitivity, as indicated by the low C_T value. In contrast, when using reagents from Supplier A, hybridization of the probe is significantly weakened at temperatures as low as 57°C, limiting the range of temperatures suitable for analysis.

A similar phenomenon is observed when a TaqMan probe is used (not shown). Using QuantiTect reaction chemistry, probe binding and hydrolysis are uniform over a broad temperature range, eliminating the need for optimization, such as by adjusting the concentration of magnesium. In contrast, when using reagents from Supplier A, which do not contain the optimized ion composition used in QuantiTect buffers, probe hybridization and generation of fluorescent signal vary with temperature.

Reliable SNP genotyping, even from small amounts of DNA

In SNP genotyping, a single mismatch between probe and target sequences needs to be discriminated during PCR. Therefore, a PCR environment needs to be created that supports specific and strong oligonucleotide binding, as long as the probe perfectly matches its target sequence. In contrast,

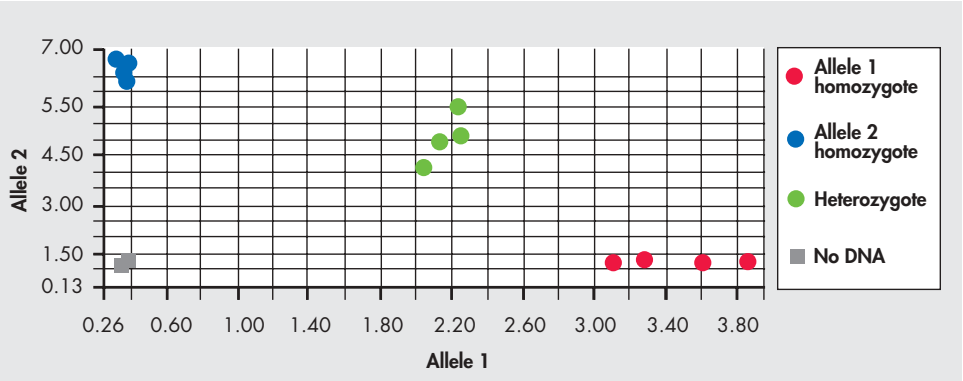

Figure 3 *PCR was carried out using the QuantiTect Probe PCR Kit and dual-labeled (TaqMan) probes for the CPY2D6*4 SNP, with 1 ng and 5 ng DNA template. End-point reads were performed on the ABI PRISM Sequence Detection System using SDS software version 1.7 (Applied Biosystems) for SNP genotyping. The allelic discrimination plots clearly indicate the homozygotes for each allele and the heterozygotes.*

probes that are mismatched in a single nucleotide position should not bind to the target. Binding of mismatched primers is prevented by a buffer composition that destabilizes weak hydrogen bonds at mismatched bases (see above). For genotyping, it is important that buffer composition offers such discrimination in a robust PCR environment using small amounts of DNA, since samples are often precious and cannot be routinely collected from an individual. We have tested the robustness of QuantiTect Probe PCR chemistry in SNP genotyping by reducing the amount of input DNA. Usually, use of 10 to 100 ng genomic DNA is recommended for real-time PCR SNP genotyping assays. Figure 3 shows results of assays where as little as 1 ng genomic DNA was used per reaction. DNA from individuals who were homozygous wildtype (allele 1), heterozygous (allele 1/2), or homozygous mutant (allele 2) for the CYP2D6*4, G1934A SNP was examined. Figure 3 shows that as little as 1 ng genomic DNA used in a 25 µl PCR provides accurate SNP genotyping with alleles clearly clustered in distinct regions of the scatter plot.

Probe hybridization on the LightCycler System

Stabilizing the probe–template complex is particularly important for rapid cycling using the LightCycler system. In this instrument, the detection of fluorescence with LightCycler Hybridization (FRET) probes takes place during the annealing step of the PCR cycle. ▶

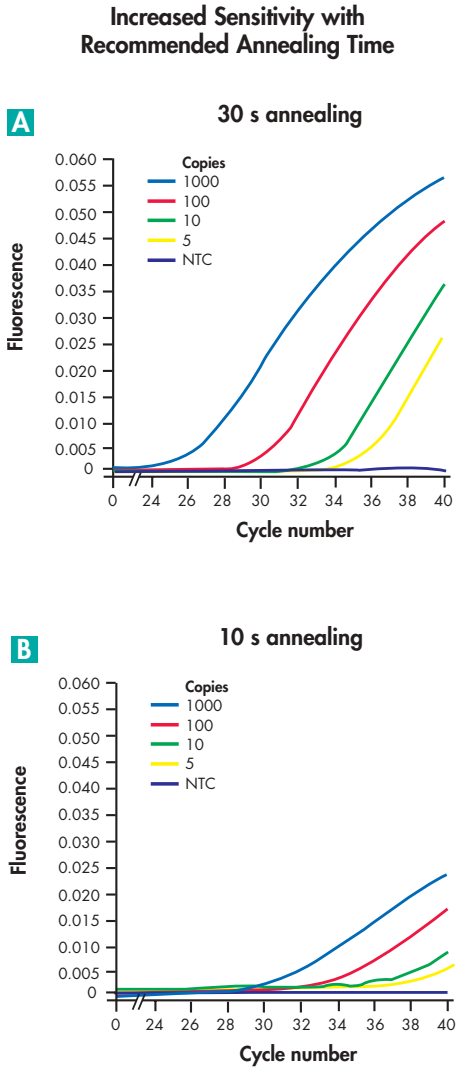

Figure 4 *Real-time, quantitative PCR was carried out using the QuantiTect Probe PCR Kit using primers and LightCycler Hybridization Probes specific for a 125 bp fragment of the human insulin-like growth factor 1. Reactions were performed on the LightCycler System with human genomic DNA corresponding to the indicated number of gene copies.* **A** *PCR was carried out using the 30-second annealing time recommended in the QuantiTect Probe protocol.* **B** *PCR using a shorter, 10-second annealing time.* ***NTC****: no template control.*

References

1. *QuantiTect Probe PCR and RT-PCR Kits — minimize your PCR optimization for highly sensitive results. QIAGEN News 2002 No.1, 1.*
2. *Missel, A., Decker, K., Arentzen, S., Kobsch, S., and Löffert, D. (2002) Reducing PCR optimization and costs with QuantiTect SYBR® Green PCR Kits on the LightCycler system. QIAGEN News 2002 No. 2, 3.*

Buffers used for this instrument often require optimization of salt concentration (e.g., Mg^{2+}) to sufficiently stabilize the probe–template complex. Salt concentrations (including Mg^{2+}) in QuantiTect Probe PCR Master Mix (or QuantiTect Probe RT-PCR Master Mix for one-step RT-PCR) are optimized for specific annealing of primers and probe. NH_4^+ ions in the Master Mix destabilize nonspecific primer and probe binding, and hybridization kinetics are slightly slowed down. Therefore, a moderate increase of the annealing time to 30 seconds is recommended for optimal sensitivity (Figure 4). Using pre-optimized QuantiTect Probe Master Mix allows more stringent primer and probe hybridization in real-time PCR, eliminating the need for lengthy optimization procedures.

Conclusions

Kits were developed for optimal performance with all types of fluorescent probes on all available real-time PCR instruments. The optimized formulation of QuantiTect Probe PCR and RT-PCR reagents increases PCR specificity, sensitivity, and efficiency. Using QuantiTect Probe Master Mix, probes bind to their target sequence with high specificity over a wider range of PCR conditions, such as temperature or Mg^{2+} concentrations, eliminating extensive assay optimization. QuantiTect Probe PCR and RT-PCR Kits enable highly sensitive and precise quantitative real-time analysis and accurate allele calling in SNP genotyping, even when minute amounts of genomic DNA are used. ■

Ordering Information

Product	Contents	Cat. No.
QuantiTect Probe Kits — for quantitative, real-time PCR and RT-PCR using sequence-specific probes		
QuantiTect Probe PCR Kit (200)	For 200 x 50 µl reactions: 3 x 1.7 ml QuantiTect Probe PCR Master Mix,* 2 x 2.0 ml RNase-free water	204343
QuantiTect Probe RT-PCR Kit (200)	For 200 x 50 µl reactions: 3 x 1.7 ml QuantiTect Probe RT-PCR Master Mix,* 100 µl QuantiTect RT Mix, 2 x 2.0 ml RNase-free water	204443
QIAGEN Operon® Oligonucleotide Synthesis Service — high-quality oligos, modified oligos, and longmers		
Oligonucleotide Synthesis Service	Custom-made oligonucleotides and a wide range of modified oligos, including Molecular Beacons, dual-labeled probes, and many more	Inquire

** Contains 8 mM $MgCl_2$*