

Multiplex PCR using Q5[®] High-Fidelity DNA Polymerase

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Introduction

Multiplex PCR is a type of polymerase chain reaction (PCR) in which numerous pairs of primers are used to amplify multiple targets in a single experiment. This technique is routinely used in genotyping, pathogen detection and enrichment techniques and *Taq*-based products are commonly used in many of these applications. Although primer design is arguably the most significant contributing factor to multiplex PCR success, reactions employing *Taq*-based enzymes can also require significant optimization. Among those components that most often require optimization are Mg⁺⁺, dNTPs, primer and enzyme concentrations. Specific mixes that provide optimized compositions for *Taq*-based multiplexing reactions are commercially available, but for some users, maintaining a mix just for multiplex needs is not convenient. In addition, for PCR-based DNA enrichment upstream of next generation sequencing, the lower fidelity of *Taq*-based mixes can be problematic. For this study, we sought to determine how our typical, off-the-shelf, ultra-high fidelity Q5 DNA Polymerase products performed in multiplex PCR.

Q5 High-Fidelity DNA Polymerase is composed of a novel polymerase that is fused to the processivity-enhancing Sso7d DNA binding domain, improving speed, fidelity and reliability of performance. In work described elsewhere (www.neb.com/what-is-polymerase-fidelity), Q5's fidelity has been determined by both traditional blue-white assay methods and Sanger sequencing to be at least 100X higher than that of *Taq* DNA Polymerase.

Materials & Methods

Unless otherwise noted, materials were obtained from, and manufactured by, New England Biolabs[®] (NEB[®]), Ipswich, MA.

Experiments shown in this application note employed Q5 High-Fidelity 2X Master Mix and primer sets designed to amplify 4 targets from human genomic DNA (Xp21.2, 19p13.2, and 2 from Xp21.1). These targets ranged from 36-59% GC content (Table 1) and could easily be resolved using traditional agarose-based gel

methods or a higher-throughput microfluidic-based system. Primer sets were designed according to recommendations (see Appendix) and were previously demonstrated to be appropriate for multiplex experiments using *Taq* DNA Polymerase. Except where otherwise noted, Q5 High-Fidelity DNA Polymerase products were used according to the manufacturer's recommendations.

 TABLE 1:
Target Details

AMPLICON	SIZE (bp)	GC CONTENT	Ta*
A	723	59%	72°C
B	547	40%	68°C
C	331	36%	66°C
D	139	52%	72°C

* Annealing temperature (Ta) as calculated by the NEB Tm Calculator for experiments using Q5; www.neb.com/TmCalculator.

Reaction Setup

Because Q5 typically requires higher annealing temperatures than *Taq*-based products, the NEB Tm Calculator (www.neb.com/tmcalculator) was used to determine appropriate annealing temperatures for each primer set.

Amplification reactions were either set up on ice and added to a pre-heated thermocycler or, for hot start enzymes, set up at room temperature and added to a room-temperature thermocycler. Amplification results were visualized either by traditional ethidium bromide staining and agarose gel electrophoresis or via a microfluidic-based LabChip[®] GXII system using a 5K DNA/RNA chip. For analysis on the LabChip, samples were first diluted 1:1 with water to reduce mobility differences that can arise from buffer effects. Primary data (electropherograms) are converted to virtual gels by the machine software. Settings for all experiments used either default software values (v. 4.0.1418.0) or, when modified, used the same modifications across all lanes to permit comparisons within and between experiments.

Compatible Products

Q5 High-Fidelity DNA Polymerase (NEB #M0491)

Q5 High-Fidelity 2X Master Mix (NEB #M0492)

Q5 Hot Start High-Fidelity DNA Polymerase (NEB #M0493)

Q5 Hot Start High-Fidelity 2X Master Mix (NEB #M0494)

NEBNext[®] High-Fidelity 2X PCR Master Mix (NEB #M0541)

Deoxynucleotide (dNTP) Solution Mix (NEB #N0447)

Jurkat genomic DNA (NEB #N4100)

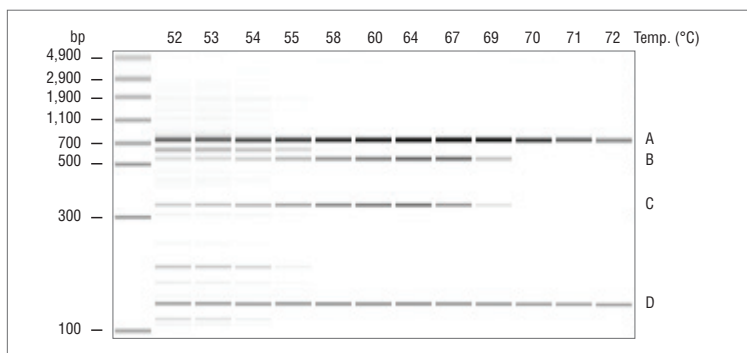
Results

To determine an annealing temperature for subsequent experiments, a gradient PCR with annealing temperatures from 52–72°C was conducted [98°C/30s, 30 x (98°C/10s, 52–72°C/30s, 72°C/30s), 72°C/5m] using Q5 High-Fidelity 2X Master Mix. As seen in Figure 1, annealing temperatures from 60–67°C supported specific and robust amplification of all four targets, without any additional optimization of reaction components. For remaining experiments, an annealing temperature of 65°C was used.



FIGURE 1:
Effect of annealing temperature (T_a) on Q5 multiplex PCR.

Q5 High-Fidelity 2X Master Mix was used in a gradient PCR (T_a from 52–72°C) to determine conditions that would support robust and specific amplification of four human gDNA targets (A–D). Reactions were diluted 1:1 with water before being visualized on a LabChip® GX II system.

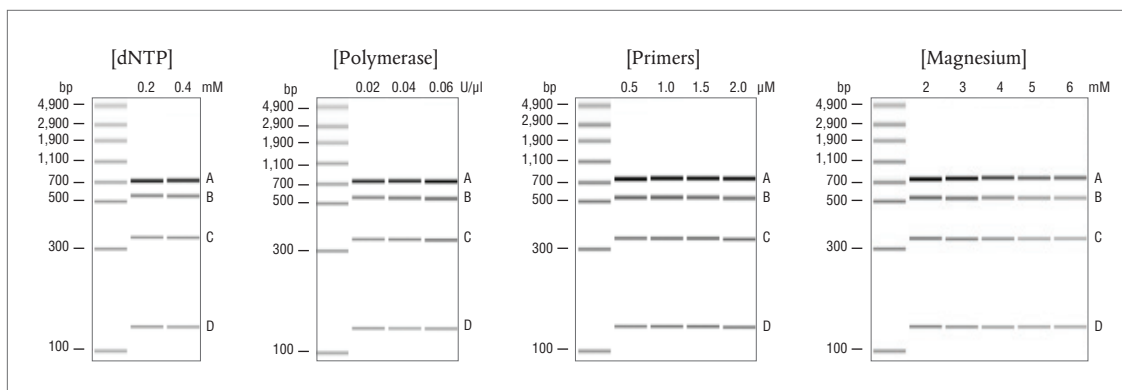


Reaction components that often require optimization for multiplex experiments using *Taq* DNA Polymerase were then modified to determine whether Q5 would also benefit from similar optimizations. As seen in Figure 2, none of the methods for optimization investigated (increasing concentrations of dNTPs, enzyme, primers or Mg^{++}) offered significant improvement over standard Q5 reaction conditions. In addition, for these 4 targets, increasing the Mg^{++} concentration over 3 mM had a deleterious effect on yield, though specificity remained high. Experiments conducted to examine the lower limits of primer concentration showed robust yields and high specificity using concentrations as low as 0.13 μM for each primer (data not shown).



FIGURE 2:
Q5 multiplex PCR optimization.

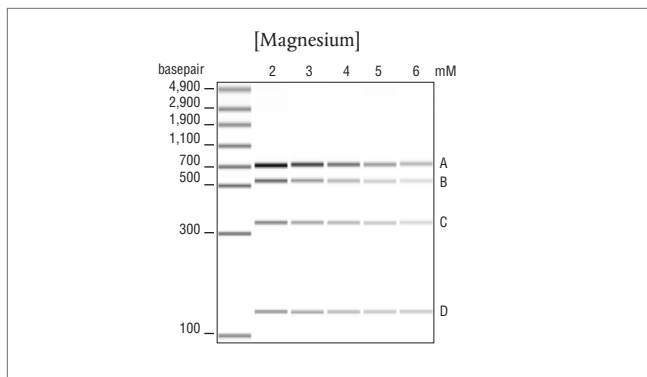
Using an annealing temperature of 65°C, reaction components that are commonly optimized to ensure robust multiplex PCR results were varied. The final concentration of the component varied in each experiment is indicated above each panel. For each experiment, the first non-ladder lane represents the typical recommended conditions for Q5. Few modifications were able to improve amplification of the four human gDNA targets examined (A–D) compared to standard Q5 conditions, which supported specific and robust amplification. Mg^{++} concentrations greater than 3 mM were inhibitory.



Further investigation of the Mg^{++} -related reduction in yield using 2X enzyme (0.04 U/ μ l in the final reaction, achieved by the addition of stand-alone Q5 into the master mix) demonstrated that Mg^{++} -related inhibition was not relieved by increasing the polymerase concentration (Figure 3). Interestingly, specificity was maintained over all reaction conditions investigated.

FIGURE 3: Q5 multiplex PCR optimization.

The effect of additional Mg^{++} was investigated in the presence of 2X enzyme (0.04 U/ μ l in the final reaction). Although specificity of the 4 targets (A-D) was maintained, excess Mg^{++} still resulted in a decrease in yield, suggesting that typical Q5 recommendations are already optimized to support successful multiplex PCR.



Summary

Using Q5 High Fidelity DNA Polymerase 2X Master Mix, we demonstrated successful multiplex amplification of human targets using a wide range of annealing temperatures, from 0.13 to 2 μ M primers, from 0.02–0.06 U/ μ l and up to 4 mM Mg^{++} . These studies and additional work investigating larger primer sets ($n \geq 12$) and other commercially available forms of Q5 (data not shown) suggest that Q5 DNA Polymerase is capable of robust and highly specific multiplex PCR results with little-to-no optimization, other than typical primer design criteria. Q5 offers not only ultra high-fidelity amplification critical for downstream workflows, but also a convenient option for multiplex PCR.

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Appendix

• Primer Design Recommendations

Primer design is critical to successful multiplex PCR. Primers are generally 24–35 nucleotides in length and ideally have a GC content of 40–60% (preferably 50–60%). Complementary sequences at the 3' end of all primers, runs of three or more G/C at the 3' end, and secondary structures within primers should all be avoided if possible. Computer programs such as PrimerSelect™ and Primer3 (primer3.ut.ee) can be used to design or analyze single primer pairs. As a starting point for *Taq*-based protocols, the melting temperature of all primers for mPCR should be more than 60°C according to the formula $T_m (^{\circ}C) = 2 \times (nA + nT) + 4 \times (nG + nC)$. For Q5, use of the NEB *Tm* Calculator (www.neb.com/TmCalculator) is highly recommended. Primers with *Tm* higher than 68°C are preferred. For best results, each primer pair should be tested individually before conducting multiplex experiments. When using sets that have been previously optimized for *Taq* DNA Polymerase, a gradient PCR that spans all the recommended Q5 annealing temperatures should be conducted.

• Primer Quality and Molar Concentration

Primers should be purchased desalted or HPLC-purified, as the primer quality is a critical factor for good multiplex PCR. Primers should be dissolved in 0.5X TE buffer (5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0) and the concentration should be accurately measured by a spectrophotometer. The molar concentration should be calculated using the molar extinction coefficient (ϵ_{260}) and absorbance at 260 nm. $Molar\ conc.\ of\ primers\ (M) = A_{260} / \epsilon_{260}$
 $\epsilon_{260} = 0.89 \times (nA \times 15480 + nC \times 7340 + nG \times 11760 + nT \times 8850)$ where n is the number of respective bases. For example, if a primer is composed of 6A, 7C, 8G, and 9T, then its ϵ_{260} is 283,011 $[0.89 \times (6 \times 15480 + 7 \times 7340 + 8 \times 11760 + 9 \times 8850)]$. Adjust the concentration of the primer stock to 50 μ M (store at $-20^{\circ}C$ to $-80^{\circ}C$). Mix all primers at equimolar concentration to 1 μ M in 0.5X TE buffer and store in small aliquots at $-20^{\circ}C$ or $-80^{\circ}C$. Repeated freeze-thaw cycles should be avoided, since they may lead to primer degradation. The final concentration of each primer in a typical multiplex PCR is between 0.05–0.5 μ M. In most cases, a final concentration of 0.15 μ M gives satisfactory results. Increasing the primer concentration up to 0.5 μ M may increase the yield.