PCR Optimization (E0555)

Overview

The following guidelines are provided to ensure successful PCR using Q5 High-Fidelity DNA Polymerase. These guidelines cover routine PCR. Amplification of templates with high GC content, high secondary structure, low template concentrations or longer amplicons may require further optimization.

Protocol

1. Template: Use of high quality, purified DNA templates greatly enhances the success of PCR. Recommended amounts of DNA template for a 50 µl reaction are as follows:

DNA	AMOUNT
Genomic	1 ng - 1 µg
Plasmid or Viral	1 pg - 10 ng

2. Primers:
   Oligonucleotide primers are generally 20–40 nucleotides in length and ideally have a GC content of 40–60%. Computer programs such as Primer3 can be used to design or analyze primers. The best results are typically seen when using each primer at a final concentration of 0.5 µM in the reaction.

3. Mg^{++} and additives:
   The Q5 High-Fidelity Master Mix contains 2.0 µM Mg^{++} when used at a 1X concentration. This is optimal for most PCR products generated with this mix.

4. Deoxynucleotides:
   The final concentration of dNTPs is 200 µM of each deoxynucleotide in the 1X Q5 High-Fidelity Master Mix. Q5 High-Fidelity DNA Polymerase cannot incorporate dUTP and is not recommended for use with uracil-containing primers or template.

5. Q5 High-Fidelity DNA Polymerase Concentration:
   The concentration of Q5 High-Fidelity DNA Polymerase in the Q5 High-Fidelity 2X Master Mix has been optimized for best results under a wide range of conditions.

6. Denaturation:
   An initial denaturation of 30 seconds at 98°C is sufficient for most amplicons from pure DNA templates. Longer denaturation times can be used (up to 3 minutes) for templates that require it.

   During thermocycling, the denaturation step should be kept to a minimum. Typically, a 5–10 second denaturation at 98°C is recommended for most templates.

7. Annealing:
   Optimal annealing temperatures for Q5 High-Fidelity DNA Polymerase tend to be higher than for other PCR polymerases. **The NEB Tm Calculator should be used to determine the annealing temperature when this enzyme.** Typically, use a 10–30 second annealing step at 3°C above the Tm of the lower Tm primer. A temperature gradient can also be used to

optimize the annealing temperature for each primer pair.

For high Tm primer pairs, two-step cycling without a separate annealing step can be used (see note 10).

8. Extension:
   The recommended extension temperature is 72°C. Extension times are generally 20–30 seconds per kb for complex, genomic samples, but can be reduced to 10 seconds per kb for simple templates (plasmid, *E. coli*, etc.) or complex templates < 1 kb. Extension time can be increased to 40 seconds per kb for cDNA templates, if necessary. A final extension of 2 minutes at 72°C is recommended.

9. Cycle number:
   Generally, 25–35 cycles yields sufficient product. For genomic amplicons, 30–35 cycles are recommended.

10. 2-step PCR:
    When primers with annealing temperatures ≥ 72°C are used, a 2-step thermocycling protocol (combining annealing and extension into one step) is possible.

    Thermocycling conditions for a routine 2-step PCR:

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25–35 Cycles	98°C	5–10 seconds
	72°C	15–30 seconds/kb
Final Extension	72°C	2 minutes
Hold	4°C	∞

11. Amplification of long products:
    When amplifying products > 6 kb, it is often helpful to increase the extension time to 40–50 seconds/kb.

12. PCR Product:
    The PCR products generated using Q5 High-Fidelity DNA Polymerase have blunt ends. If cloning is the next step, then blunt-end cloning is recommended. If T/A-cloning is preferred, the DNA should be purified prior to A-addition, as Q5 High-Fidelity DNA Polymerase will degrade any overhangs generated.

    Addition of an untemplated -dA can be achieved with *Taq* DNA Polymerase (NEB #M0267) or Klenow Fragment (3' → 5' exo$^-$) (NEB #M0212).