PCR Using Q5® High-Fidelity DNA Polymerase (NEB #M0491)

Materials Required but not Supplied

Q5® High-Fidelity DNA Polymerase
- Deoxynucleotide (dNTP) Solution Mix (NEB #N0447)
- Nuclease-free Water (NEB #B1500)

Overview

This protocol describes methods for PCR using Q5® High-Fidelity DNA Polymerase, which offers high fidelity (~280X higher than Taq), resulting in ultra-low error rates. Please note that protocols with Q5 High-Fidelity DNA Polymerase may differ from protocols with other polymerases. The conditions recommended below should be used for optimal performance.

Protocol

1. Assemble all reaction components on ice. Each component should be gently mixed before adding to the reaction in a sterile thin-walled PCR tube. The Q5 High-Fidelity DNA Polymerase may be diluted in 1X Q5 Reaction Buffer just prior to use to reduce pipetting errors. The entire reaction should be mixed again to ensure homogeneous, consistent mixture. Collect all liquid to the bottom of the tube with a quick centrifuge spin if necessary. Overlay the sample with mineral oil if using a PCR machine without a heated lid.

2. Quickly transfer the reactions to a thermocycler preheated to the denaturation temperature (98°C) and begin thermocycling.

COMPONENT	25 µl REACTION	50 µl REACTION	FINAL CONCENTRATION
5X Q5 Reaction Buffer	5 µl	10 µl	1X
10 mM dNTPs	0.5 µl	1 µl	200 µM
10 µM Forward Primer	1.25 µl	2.5 µl	0.5 µM
10 µM Reverse Primer	1.25 µl	2.5 µl	0.5 µM
Template DNA	variable	variable	< 1,000 ng
Q5 High-Fidelity DNA Polymerase	0.25 µl	0.5 µl	0.02 U/µl
5X Q5 High GC Enhancer (optional)	(5 µl)	(10 µl)	(1X)
Nuclease-Free Water	to 25 µl	to 50 µl	

Thermocycling Conditions for a Routine PCR:

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25–35 Cycles	98°C 50–72°C* 72°C	5–10 seconds 10–30 seconds 20–30 seconds/kb
Final Extension	72°C	2 minutes
Hold	4–10°C	

**Use of the NEBTm Calculator is highly recommended.*

General Guidelines:

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
DNA 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. However, amplification of certain long, complex DNA targets (≥ 5 kb) may benefit from using a lower primer concentration (~ 0.2 to 0.3 µM).

3. **Mg^{++} and additives:**
   Mg^{++} concentration of 2.0 mM is optimal for most PCR products generated with Q5 High-Fidelity DNA Polymerase. When used at a final concentration of 1X, the Q5 Reaction Buffer provides the optimal Mg^{++} concentration.

   Amplification of some difficult targets, like GC-rich sequences, may be improved by the addition of 1X Q5 High GC Enhancer. The Q5 High GC Enhancer is not a buffer and should not be used alone. It should be added only to reactions with the Q5 Reaction Buffer when other conditions have failed.

4. **Deoxynucleotides:**
   The final concentration of dNTPs is typically 200 µM of each deoxynucleotide. Q5 High-Fidelity DNA Polymerase cannot incorporate dUTP and is not recommended for use with uracil-containing primers or templates. Should uracil-containing primers or templates be used, we recommend Q5U® Hot Start High-Fidelity DNA Polymerase (NEB #M0515).

5. **Q5 High-Fidelity DNA Polymerase concentration:**
   We generally recommend using Q5 High-Fidelity DNA Polymerase at a final concentration of 20 units/ml (1.0 unit/50 µl reaction). However, the optimal concentration of Q5 Hot Start High-Fidelity DNA Polymerase may vary from 10–60 units/ml (0.5–3 units/50 µl reaction) depending on amplicon length and difficulty.

6. **Buffers:**
   The 5X Q5 Reaction Buffer provided with the enzyme is recommended as the first-choice buffer for robust, high-fidelity amplification. For difficult amplicons, such as GC-rich templates or those with secondary structure, the addition of the Q5

High GC Enhancer can improve reaction performance. The 5X Q5 Reaction Buffer is detergent-free and contains 2.0 mM Mg^{++} at the final (1X) concentration.

7. **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.

8. **Annealing:**
   Optimal annealing temperatures for Q5 High-Fidelity DNA Polymerase tend to be higher than for other PCR polymerases. The NEB Tm Calculator sshould be used to determine the annealing temperature when using this enzyme. Typically, use a 10–30 second annealing step at the recommended T_a. A temperature gradient can also be used to optimize the annealing temperature for each primer pair.

   For high T_m primer pairs, two-step cycling without a separate annealing step can be used (see note 11).

9. **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 or long, complex templates, if necessary.

   A final extension of 2 minutes at 72°C is recommended.

10. **Cycle number:**
    Generally, 25–35 cycles yield sufficient product. For genomic amplicons, 30-35 cycles are recommended.

11. **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.

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

13. **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. The Monarch® Spin PCR & DNA Cleanup Kit (5 µg) (NEB #T1130) is recommended as an efficient method for purification and concentration up to 5 µg of high-quality, double-stranded and single-stranded DNA.

14. Addition of an untemplated -dA can be done with *Taq* DNA Polymerase (NEB #M0267) or Klenow exo$^-$ (NEB #M0212).

Related Resources

- Tm Calculator