NEW ENGLAND Biolabs®

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

Overview

This protocol is intended for standard endpoint PCR (only) using the NEBNext® High-Fidelity 2X PCR Master Mix (NEB #M0541). Although this formulation of the Q5® High-Fidelity DNA Polymerase is also included as a component in the original NEBNext standard workflow library prep kits for Illumina® and in the NEBNext library prep kit for Ion Torrent™, this protocol is not intended for next-generation sequencing library amplification. Please contact Technical Support for questions about using this product for NGS PCR.

Reaction Setup:
We recommend assembling all reaction components on ice and quickly transferring the reactions to a thermocycler preheated to the denaturation temperature (98°C). All components should be mixed prior to use.

Note: If you are performing a cleanup step using AMPure XP Beads prior to PCR, be sure not to transfer any beads. Trace amounts of bead carried over may affect the optimal performance of the poymerase used in the NEBNext High-Fidelity 2X PCR Master Mix in the subsequent PCR step.

Component	Volume per 50 ul RXN	Final Concentration
NEBNext High Fidelity 2x PCR Master Mix	25 µl	1x
10 µM Forward Primer	2.5 µl	0.5 µM
10 µM Reverse Primer	2.5 µl	0.5 µM
Template DNA*	Variable	Variable
Nuclease-free water	To 50 µl	

*1 ng - 1 µg genomic DNA or 1 pg - 1 ng for plasmid or viral DNA

Gently mix the reaction. Collect all liquid at the bottom of the tube by a quick spin if necessary. Transfer PCR tubes to a PCR machine and begin thermocycling.

Recommended Thermocycling Conditions:

Standard PCR			
Step	**Temp**	**Time**	**Cycles**
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	5-10 seconds	25-35
Annealing	50-72°C	10-30 seconds	
Extension	72°C	20-30 seconds/kb	
Final Extension	72°C	2 minutes	1

Hold	4-10°C	∞	1

*Depending on primer design, annealing temperature may need to be optimized. Use of the NEB Tm Calculator is highly recommended. Further optimization may be required.

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 - 1 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. Q5 is a high fidelity polymerase and for NGS amplification we would recommend using a primer with a 3' phosphorothioate modification.

3. Mg++ and additives:

   The NEBNext High-Fidelity 2X Master Mix contains 2.0 mM Mg++ when used at a 1X concentration. This is optimal for most PCR products generated with this master mix.

4. Deoxynucleotides:

   The final concentration of dNTPs is optimized for robust amplification. NEBNext High-Fidelity 2X PCR Master Mix cannot incorporate dUTP and is not recommended for use with uracil-containing primers or templates.

5. Q5 High-Fidelity DNA Polymerase concentration:

   The concentration of DNA Polymerase in the NEBNext High-Fidelity 2X PCR 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 NEBNext High-Fidelity 2X PCR Master Mix tend to be higher than for other PCR polymerases. The NEB Tm Calculator should be used to determine the annealing temperature when suing 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 or long, complex templates, if necessary. A final extension of 2 minutes at 72°C is recommended.

9. Cycle number:

   Generally, 25-35 cycles yield 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.

11. PCR produce:

    The PCR products generated using Q5 High-Fidelity 2X Master Mix 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 done with Taq DNA Polymerase (NEB # M0267) or Klenow exo- (NEB #M0212).