General Guidelines for PCR Optimization

New England Biolabs offers a diverse group of DNA Polymerases for PCR-based applications. Specific recommendations for PCR optimization can be found in the product literature or on the individual product webpages. However, these general guidelines will help to ensure success using New England Biolabs' PCR enzymes.

Setup Guidelines

DNA Template

- Use high quality, purified DNA templates whenever possible. Please refer to specific product information for amplification from unpurified DNA (e.g., colony PCR or direct PCR).
- For low complexity templates (e.g., plasmid, lambda, BAC DNA), use 1 pg–10 ng of DNA per 50 µl reaction
- For higher complexity templates (e.g., genomic DNA), use 1 ng–1 µg of DNA per 50 µl reaction
- Higher DNA concentrations tend to decrease amplicon specificity, particularly when a high number of cycles are run

Primers

- Primers should typically be 20–40 nucleotides in length
- Ideal primer content is 40–60% GC
- Primer Tm calculation should be determined with NEB's Tm Calculator (www.neb.com/TmCalculator)
- Annealing temperatures should be determined according to specific enzyme recommendations. Please note that Q5 and Phusion annealing temperature recommendations are unique.
- Primer pairs should have Tm values that are within 5°C
- Avoid secondary structure (e.g., hairpins) within each primer and potential dimerization between the primers
- Final concentration of each primer should be 0.05–1 µM in the reaction. Please refer to the more detailed recommendations for each specific enzyme.
- Higher primer concentrations may increase secondary priming and create spurious amplification products
- When amplifying products > 20 kb in size, primers should be ≥ 24 nucleotides in length with a GC content above 50% and matched Tm values above 60°C
- When engineering restriction sites onto the end of primers, 6 nucleotides should be added 5′ to the site
- To help eliminate primer degradation and subsequent non-specific product formation, use a hot-start enzyme (e.g., Q5 and One*Taq* Hot Start DNA Polymerases)

Magnesium Concentration

- Optimal Mg^{++} concentration is usually 1.5–2.0 mM for most PCR polymerases
- Most PCR buffers provided by NEB already contain sufficient levels of Mg^{++} at 1X concentrations. Please refer to the specific product information for Mg^{++} content.
- NEB offers a variety of Mg-free reaction buffers to which supplemental Mg^{++} can be added for applications that require complete control over Mg^{++} concentration
- Further optimization of Mg^{++} concentration can be done in 0.2–1 µM increments, if necessary. For some specific applications, the enzyme may require as much as 6 mM Mg^{++} in the reaction
- Insufficient Mg^{++} concentrations may cause reaction failure but excess Mg^{++} may lead to spurious amplification

Deoxynucleotides

- **Ideal dNTP concentration is typically 200 µM of each, however, some enzymes may require as much as 400 µM each.** Please refer to specific product literature for more detailed recommendations.
- **Excess dNTPs can chelate Mg^{++} and inhibit the polymerase**
- Lower dNTP concentration can increase fidelity, however, yield is often reduced
- The presence of uracil in the primer, template, or deoxynucleotide mix will cause reaction failure when using archaeal-based PCR polymerases. Use One*Taq* or *Taq* DNA Polymerases for these applications.

Enzyme Concentration

- Optimal enzyme concentration in the reaction is specific to each polymerase. Please see the product literature for specific recommendations.
- In general, excess enzyme can lead to amplification failure, particularly when amplifying longer fragments

Starting Reactions

- Unless using a hot start enzyme (e.g., Q5 or One*Taq* Hot Start DNA Polymerase), assemble all reaction components on ice
- Add the polymerase last, whenever possible
- Transfer reactions to a thermocycler that has been pre-heated to the denaturation temperature. Please note that pre-heating the thermocycler is not necessary when using a hot start enzyme (e.g., Q5 or One*Taq* Hot Start DNA Polymerase).

PCR Troubleshooting Guide

The following guide can be used to troubleshoot PCR reactions. Additional tips for optimizing reactions can be found in the technical reference section of our website, www.neb.com.

PROBLEM	POSSIBLE CAUSE	SOLUTION
SEQUENCE ERRORS	Low fidelity polymerase	• Choose a higher fidelity polymerase such as Q5 (NEB #M0491), or Phusion* (NEB #M0530)
	Suboptimal reaction conditions	• Reduce number of cycles • Decrease extension time
	Unbalanced nucleotide concentrations	• Prepare fresh deoxynucleotide mixes
	Template DNA has been damaged	• Start with a fresh template • Try repairing DNA template with the PreCR Repair Mix (NEB #M0309) • Limit UV exposure time when analyzing or excising PCR product from the gel
	Desired sequence may be toxic to host	• Clone into a non-expression vector • Use a low-copy number cloning vector
INCORRECT PRODUCT SIZE	Incorrect annealing temperature	• Recalculate primer Tm values using the NEB Tm calculator (www.neb.com/TmCalculator)
	Mispriming	• Verify that primers have no additional complementary regions within the template DNA
	Improper Mg^{++} concentration	• Adjust Mg^{++} concentration in 0.2–1 mM increments
	Nuclease contamination	• Repeat reactions using fresh solutions
NO PRODUCT	Incorrect annealing temperature	• Recalculate primer Tm values using the NEB Tm calculator (www.neb.com/TmCalculator) • Test an annealing temperature gradient, starting at 5°C below the lower Tm of the primer pair
	Poor primer design	• Check specific product literature for recommended primer design • Verify that primers are non-complementary, both internally and to each other • Increase length of primer
	Poor primer specificity	• Verify that oligos are complementary to proper target sequence
	Insufficient primer concentration	• Primer concentration can range from 0.05–1 μM in the reaction. Please see specific product literature for ideal conditions
	Missing reaction component	• Repeat reaction setup
	Suboptimal reaction conditions	• Optimize Mg^{++} concentration by testing 0.2–1 mM increments • Thoroughly mix Mg^{++} solution and buffer prior to adding to the reaction • Optimize annealing temperature by testing an annealing temperature gradient, starting at 5°C below the lower Tm of the primer pair
	Poor template quality	• Analyze DNA via gel electrophoresis before and after incubation with Mg^{++} • Check 260/280 ratio of DNA template
	Presence of inhibitor in reaction	• Further purify starting template by alcohol precipitation, drop dialysis or commercial clean-up kit • Decrease sample volume
	Insufficient number of cycles	• Rerun the reaction with more cycles
	Incorrect thermocycler programming	• Check program, verify times and temperatures
	Inconsistent thermocycler block temperature	• Test calibration of heating block
	Contamination of reaction tubes or solutions	• Autoclave empty reaction tubes prior to use to eliminate biological inhibitors • Prepare fresh solutions or use new reagents
	Complex template	• Use One*Taq* DNA Polymerase or Q5 High-Fidelity DNA Polymerase • For GC-rich templates, use One*Taq* DNA Polymerase (NEB #M0480) with One*Taq* GC Reaction Buffer (plus One*Taq* High GC Enhancer, if necessary) or Q5 High-Fidelity DNA Polymerase (NEB #M0491) with the High GC Enhancer • For longer templates, we recommend LongAmp *Taq* DNA Polymerase (NEB #M0323)
MULTIPLE OR NON-SPECIFIC PRODUCTS	Premature replication	• Use a hot start polymerase, such as One*Taq* Hot Start DNA Polymerase (NEB #M0481) or Q5 Hot Start High-Fidelity DNA Polymerase (NEB #M0493) • Set up reactions on ice using chilled components and add samples to thermocycler preheated to the denaturation temperature
	Primer annealing temperature too low	• Recalculate primer Tm values using the NEB Tm calculator (www.neb.com/TmCalculator) • Increase annealing temperature
	Incorrect Mg^{++} concentration	• Adjust Mg^{++} in 0.2–1 mM increments
	Poor primer design	• Check specific product literature for recommended primer design • Verify that primers are non-complementary, both internally and to each other • Increase length of primer • Avoid GC-rich 3´ ends
	Excess primer	• Primer concentration can range from 0.05–1 μM in the reaction. See specific product literature for ideal conditions.
	Contamination with exogenous DNA	• Use positive displacement pipettes or non-aerosol tips • Set up dedicated work area and pipettor for reaction setup • Wear gloves during reaction setup
	Incorrect template concentration	• For low complexity templates (i.e., plasmid, lambda, BAC DNA), use 1 pg–10 ng of DNA per 50 μl reaction • For higher complexity templates (i.e., genomic DNA), use 1 ng–1 μg of DNA per 50 μl reaction

* Phusion DNA Polymerase was developed by Finnzymes Oy, now a part of Thermo Fisher Scientific.