

PCR Using Phusion[®] High-Fidelity PCR Master Mix with HF Buffer (NEB #M0531)

Materials Required but not Supplied

Phusion[™] High-Fidelity PCR Master Mix with HF Buffer

- Nuclease-free Water (NEB #B1500)

Overview

The following guidelines are provided to ensure successful PCR using Phusion[®] Master Mixes. These guidelines cover routine PCR. Amplification of templates with high GC content, high secondary structure, low template concentrations, or long amplicons may require further optimization. The NEB [T_m calculator](#) should be used to determine the annealing temperature when using Phusion. Please note that protocols with Phusion DNA Polymerase may differ from protocols with other standard polymerases. As such, the conditions recommended below should be used for optimal performance.

Protocol

Reaction Setup:

1. Assemble all reaction components on ice. All components should be mixed and centrifuged before use. It is important to add Phusion Master Mix last to prevent any primer degradation caused by the 3' → 5' exonuclease activity. Gently mix the reaction. Collect all liquid to the bottom of the tube with a quick spin if necessary. Overlay the sample with mineral oil if using a PCR machine without a heated lid.

Component	25 µl Reaction	50 µl Reaction	Final Concentration
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
DMSO (optional)	(0.75 µl)	(1.5 µl)	(3%)
2X Phusion Master Mix	12.5 µl	25 µl	1X
Template DNA	variable	variable	< 250 ng
Nuclease-free water	to 25 µl	to 50 µl	

2. Quickly transfer the reactions in PCR tubes to a thermocycler preheated to the denaturation temperature (98°C).

Thermocycling conditions for a routine PCR:

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds

25-35 Cycles	98°C 45-72°C 72°C	5-10 seconds 10-30 seconds 15-30 seconds/kb
Final Extension	72°C	5-10 minutes
Hold	4-10°C	

Thermocycling conditions for a routine 2-step PCR:

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25-35 Cycles	98°C 72°C	5-10 seconds 15-30 seconds per kb
Final Extension	72°C	5-10 minutes
Hold	4°C	

Note: When primers with annealing temperatures $\geq 72^\circ\text{C}$ are used, a 2-step thermocycling protocol is recommended.

General Guidelines

1. Template:

Use high-quality, purified DNA templates to greatly enhance the success of PCR. If the template DNA is obtained from a cDNA synthesis reaction, the volume added should be less than 10% of the total reaction volume. Recommended amounts of DNA template for a 50 μl reaction are as follows:

DNA	Amount
Genomic	50 ng–250 ng
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^{++} , deoxynucleotides, and additives:

At 1X concentration, Phusion Master Mix provides 1.5 mM MgCl_2 and 200 μM of each dNTP in the final reaction. Phusion cannot incorporate dUTP and is not recommended for use with uracil-containing primers or template.

Amplification of difficult targets, such as those with GC-rich sequences or secondary structure, may be improved by the use of the Phusion[®] High-Fidelity PCR Master Mix with GC Buffer ([NEB #M0532](#)) or presence of additives such as DMSO (included). A final concentration of 3% DMSO is recommended, although concentration can be optimized in 2% increments. It is important to note that if a high concentration of DMSO is used, the annealing temperature must be lowered as it decreases the primer T_m (1). Phusion DNA polymerase is also compatible with other additives such as formamide or glycerol.

4. Phusion DNA Polymerase Concentration:

The concentration of Phusion DNA Polymerase in the Phusion PCR Master Mix has been optimized for best results under a wide range of conditions. If reactions are set up according to recommendations listed, the final concentration of Phusion DNA Polymerase in the reaction is 1 unit/50 μl or 0.4 units/20 μl .

5. Buffers:

This master mix contains Phusion HF Buffer. HF buffer is recommended as the default buffer for high-fidelity amplification. For difficult templates, such as GC-rich templates or those with secondary structure, Phusion High-Fidelity PCR Master Mix with GC Buffer ([NEB #M0532](#)) can improve reaction performance. GC buffer should be used in experiments where HF buffer does not work.

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:

Annealing temperatures required for use with Phusion tend to be higher than with other PCR polymerases. The [NEB Tm calculator](#) should be used to determine the annealing temperature when using Phusion. Typically, primers greater than 20 nucleotides in length anneal for 10–30 seconds at 3°C above the T_m of the lower T_m primer. If the primer length is less than 20 nucleotides, an annealing temperature equivalent to the T_m of the lower primer should be used. A temperature gradient can also be used to optimize the annealing temperature for each primer pair. For two-step cycling, the gradient can be set as high as the extension temperature.

For high T_m primer pairs, two-step cycling without a separate annealing step can be used.

8. Extension:

The recommended extension temperature is 72°C. Extension times are dependent on amplicon length and complexity. Generally, an extension time of 15 seconds per kb can be used. For complex amplicons, such as genomic DNA, an extension time of 30 seconds per kb is recommended. Extension time can be increased to 40 seconds per kb for cDNA templates, if necessary.

9. Cycle number:

Generally, 25–35 cycles yield sufficient product.

10. 2-step PCR:

When primers with annealing temperatures ≥ 72°C are used, a 2-step thermocycling protocol is recommended.

11. PCR product:

The PCR products generated using Phusion High Fidelity PCR Master Mix with HF Buffer have blunt ends; if cloning is the next step, then blunt-end cloning is recommended. If TA-cloning is preferred, then DNA should be purified prior to A-addition, as Phusion High Fidelity PCR Master Mix with HF Buffer will degrade any overhangs generated.

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

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.

References:

1. Chester, N. and Marshak, D.R. (1993). *Analytical Biochemistry*. 209, 284-290.

Related Resources

- [Tm Calculator](#)