PCR Protocol for Phusion® High-Fidelity DNA Polymerase (M0530) Version 2

New England Biolabs

Abstract

This is the PCR protocol for Phusion® High-Fidelity DNA Polymerase (M0530)

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Guidelines

OVERVIEW

The following guidelines are provided to ensure successful PCR using Phusion® DNA Polymerase. 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.

PROTOCOL

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 and centrifuged prior to use. It is important to add Phusion DNA Polymerase last in order to prevent any primer degradation caused by the 3´→ 5´ exonuclease activity. Phusion DNA Polymerase may be diluted in 1X HF or GC Buffer just prior to use in order to reduce pipetting errors. Please note that protocols with Phusion DNA Polymerase may differ from protocols with other standard polymerases. As such, conditions recommended below should be used for optimal performance.

Component	**20 µl Reaction**	**50 µl Reaction**	**Final Concentration**
Nuclease-free water	to 20 µl	to 50 µl	
5X Phusion HF or GC Buffer	4 µl	10 µl	1X
10 mM dNTPs	0.4 µl	1 µl	200 µM
10 µM Forward Primer	1 µl	2.5 µl	0.5 µM
10 µM Reverse Primer	1 µl	2.5 µl	0.5 µM
Template DNA	variable	variable	< 250 ng
DMSO (optional)	(0.6 µl)	(1.5 µl)	3%
Phusion DNA Polymerase	0.2 µl	0.5 µl	1.0 units/50 µl PCR

Notes: Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Overlay the sample with mineral oil if using a PCR machine without a heated lid.

Transfer PCR tubes from ice to a PCR machine with the block preheated to 98°C and begin thermocycling:

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 per kb
Final Extension	72°C	5-10 minutes
Hold	4-10°C	

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
genomic	50 ng–250 ng
plasmid or viral	1 pg–10 ng

If the template DNA is obtained from a cDNA synthesis reaction, the volume added should be less than 10% of the total reaction volume.

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 final concentration of each primer in a reaction using Phusion DNA Polymerase may be 0.2–1 µM, while 0.5 µM is recommended.

3. Mg++ and additives:
Mg++ is critical to achieve optimal activity with Phusion DNA Polymerase. The final Mg++ concentration in 1X Phusion HF and GC Buffer is 1.5 mM. Excessive Mg++ can prevent full denaturation of DNA as well as cause non-specific binding of primers. The optimal Mg++ concentration is affected by dNTP concentration, the template being used and supplements that are added to the reaction. This can also be affected by the presence of chelators (e.g. EDTA). Mg++ can be optimized in 0.5 mM increments using the $MgCl_2$ provided.

Amplification of difficult targets, such as those with GC-rich sequences or secondary structure, may be improved by the 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 Tm (2). Phusion DNA polymerase is also compatible with other additives such as formamide or glycerol.

4. Deoxynucleotides:
The final concentration of dNTPs is typically 200 µM of each deoxynucleotide. Phusion cannot incorporate dUTP.

5. Phusion DNA Polymerase Concentration:
We generally recommend using Phusion DNA Polymerase at a concentration of 20 units/ml (1.0 units/50 µl reaction). However, the optimal concentration of Phusion DNA Polymerase may vary from 10–40 units/ml (0.5–2 units/50 µl reaction) depending on amplicon length and difficulty. Do not exceed 2 units/50 µl reaction, especially for amplicons longer than 5 kb.

6. Buffers:
5X Phusion HF Buffer and 5X Phusion GC Buffer are provided with the enzyme. 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, GC buffer can improve reaction performance. GC buffer should be used in experiments where HF buffer does not work. Detergent-free reaction buffers are also available for applications that do not tolerate detergents (e.g. microarray, DHPLC).

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:
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 Tm of the lower Tm primer. If the primer length is less than 20 nucleotides, an annealing temperature equivalent to the Tmof 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 Tm primer pairs, two-step cycling without a separate annealing step can be used.

9. 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.

10. Cycle number:
Generally, 25–35 cycles yields sufficient product.

11. 2-step PCR:
When primers with annealing temperatures ≥ 72°C are used, a 2-step thermocycling protocol is recommended.

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	4-10°C	

12. PCR product:
The PCR products generated using Phusion DNA Polymerase 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 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).

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

Before start

Annealing temperatures should be determined using the NEB Annealing Temp Calculator.

Protocol

Step 1.
Set up the following reaction on ice.

Component	20 µl Reaction	50 µl Reaction	Final Concentration
Nuclease-free water	to 20 µl	to 50 µl	
5X Phusion HF or GC Buffer	4 µl	10 µl	1X
10 mM dNTPs	0.4 µl	1 µl	200 µM
10 µM Forward Primer	1 µl	2.5 µl	0.5 µM
10 µM Reverse Primer	1 µl	2.5 µl	0.5 µM
Template DNA	variable	variable	< 250 ng
DMSO (optional)	(0.6 µl)	(1.5 µl)	3%
Phusion DNA Polymerase	0.2 µl	0.5 µl	1.0 units/50 µl PCR

PROTOCOL

. Mixture for M0530 Phusion PCR

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Step 1.1.

Nuclease-free water

Step 1.2.

5X Phusion HF or GC Buffer

NOTES

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GC buffer should be used in experiments where HF buffer does not work. Detergent-free reaction buffers are also available for applications that do not tolerate detergents (e.g. microarray, DHPLC).

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5X Phusion HF Buffer and 5X Phusion GC Buffer are provided with the enzyme. 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, GC buffer can improve reaction performance.

Step 1.3.

10 mM dNTPs

REAGENTS

Deoxynucleotide Solution Mix - 8 umol of each N0447S by New England Biolabs

NOTES

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Phusion cannot incorporate dUTP.

Step 1.4.

10 µM Forward Primer

Step 1.5.

10 µM Reverse Primer

Step 1.6.

Template DNA

Step 1.7.

DMSO (optional)

NOTES

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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 Tm (2).

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Amplification of difficult targets, such as those with GC-rich sequences or secondary structure, may be improved by the presence of additives such as DMSO (included). A final concentration of 3% DMSO is recommended, although concentration can be optimized in 2% increments.

Step 1.8.

Phusion DNA Polymerase

Step 2.
Gently mix the reaction.

Step 3.
Collect all liquid to the bottom of the tube by a quick spin if necessary and overlay the sample with mineral oil if using a PCR machine without a heated lid.

Step 4.
Quickly transfer PCR tubes from ice to a PCR machine with the block preheated to 98°C and begin thermocycling.

Warnings

Please note that protocols with Phusion DNA Polymerase may differ from protocols with other standard polymerases. As such, conditions recommended below should be used for optimal performance.