

POLYMERASES & AMPLIFICATION

Phusion[®] High-Fidelity PCR Kit

Instruction Manual

NEB #E0553S/L
50/200 reactions

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Introduction:

The Polymerase Chain Reaction (PCR) is a powerful and sensitive technique for DNA amplification (1). PCR amplifies specific DNA sequences exponentially by using multiple cycles of a three-step process. First, the double-stranded DNA template is denatured at a high temperature. Sequence-specific primers are then annealed to sites flanking the target sequence. A thermostable DNA polymerase (2–6) extends the annealed primers, thereby doubling the amount of the original DNA sequence. This newly synthesized product then becomes an additional template for subsequent cycles of amplification. These three steps are repeated for 20 to 30 cycles, resulting in a 10^5 – 10^9 fold increase in target DNA concentration.

High Fidelity DNA Polymerases are important for applications in which the DNA sequence needs to be correct after amplification. Manufactured and quality controlled at New England Biolabs, Thermo Scientific® Phusion High-Fidelity DNA Polymerase offers both high fidelity and robust performance, and thus can be used for all PCR applications. Its unique structure, a novel *Pyrococcus*-like enzyme fused with a processivity-enhancing domain, increases fidelity and speed. Phusion DNA Polymerase is an ideal choice for cloning and can be used for long or difficult amplicons. With an error rate 50-fold lower than that of *Taq* DNA Polymerase and 6-fold lower than that of *Pyrococcus furiosus* DNA Polymerase (7), Phusion is one of the most accurate thermostable polymerases available. Phusion DNA Polymerase possesses 5'→3' polymerase activity, 3'→5' exonuclease activity and will generate blunt-ended products.

Kit Components:

This kit contains a sufficient supply of polymerase, buffers, deoxynucleotides, magnesium chloride, DMSO, and DNA size standard to perform 50 PCR reactions (small) or 200 PCR reactions (large). Control template and primers are provided for 20 control reactions.

Phusion DNA Polymerase (NEB #M0530)

50/200 units (2 units/ μ l)

Supplied in: 20 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM DTT, 100 mM KCl, stabilizers, 200 μ g/ml BSA and 50% glycerol.

Unit definition: One unit is defined as the amount of enzyme that will incorporate 10 nmol of dNTP into acid insoluble material in 30 minutes at 74°C.

Unit assay conditions: 25 mM TAPS-HCl (pH 9.3), 50 mM KCl, 2 mM $MgCl_2$, 1 mM β -mercaptoethanol, 200 μ M dNTPs including [3H]-dTTP and 400 μ g/ml activated Calf Thymus DNA.

Deoxynucleotide Solution Mix (NEB #N0447)

100/400 μ l (10 mM of each dATP, dCTP, dGTP and dTTP)

Deoxynucleotide Solution Mix is an equimolar solution of ultrapure dATP, dCTP, dGTP and dTTP. These deoxynucleotides are supplied at a 10 mM concentration in Milli-Q® water as a sodium salt at pH 7.5.

Phusion HF Buffer (NEB #B0518)

1.5/4.5 ml (5X)

5X Phusion HF Buffer contains 7.5 mM $MgCl_2$ (1.5 mM at 1X dilution). HF buffer is recommended as the default buffer for high-fidelity amplification.

Phusion GC Buffer (NEB #B0519)

1.5/1.5 ml (5X)

5X Phusion GC Buffer contains 7.5 mM $MgCl_2$ (1.5 mM at 1X dilution). For difficult templates, such as GC-rich templates or those with secondary structure, GC buffer can improve enzyme performance. GC buffer should be used in experiments where HF buffer does not work.

MgCl₂ Solution (NEB #B0510)

1.5/1.5 ml (50 mM)

When used at the recommended concentration, both Phusion buffers contain 1.5 mM MgCl₂. Additional MgCl₂ is provided to optimize polymerase activity if necessary. As Phusion DNA Polymerase is quite sensitive to changes in MgCl₂, the solution should be diluted with water to 10 mM to avoid pipetting errors.

Control Lambda Template (NEB #N3010)

40/40 µl (0.5 ng/µl)

Bacteriophage lambda DNA (GenBank #NC_001416, 48,502 bp) is provided as a control template. It is supplied in TE buffer at 0.5 ng/µl.

1.3 and 10 kb Primers (NEB #S0535/S0536)

50/50 µl (4 µM)

The 1.3 kb Control Primer Mix is a mix of primers in water for amplification of a 1.3 kb fragment using the Control Lambda Template. Primers are provided at a concentration of 4 µM each.

Sequences are as follows:

Primer 1 (27-mer): 5' GTC ACC AGT GCA GTG CTT GAT AAC AGG 3'

T_m 71.0°C, coordinates in lambda: 30,006–30,032

Primer 2 (28-mer): 5' GAT GAC GCA TCC TCA CGA TAA TAT CCG G 3'

T_m 73.2°C, coordinates in lambda: 31,325–31,352

The 10 kb Control Primer Mix is a mix of primers in water for amplification of a 10 kb fragment using the Control Lambda Template. Primers are provided at a concentration of 4 µM each.

Sequences are as follows:

Primer 1 (22-mer): 5' CAG TGC AGT GCT TGA TAA CAG G 3'

T_m 63.0°C, coordinates in lambda: 30,011–30,032

Primer 2 (20-mer): 5' GTA GTG CGC GTT TGA TTT CC 3'

T_m 62.7°C, coordinates in lambda: 40,024–40,043

DMSO (NEB #B0515)

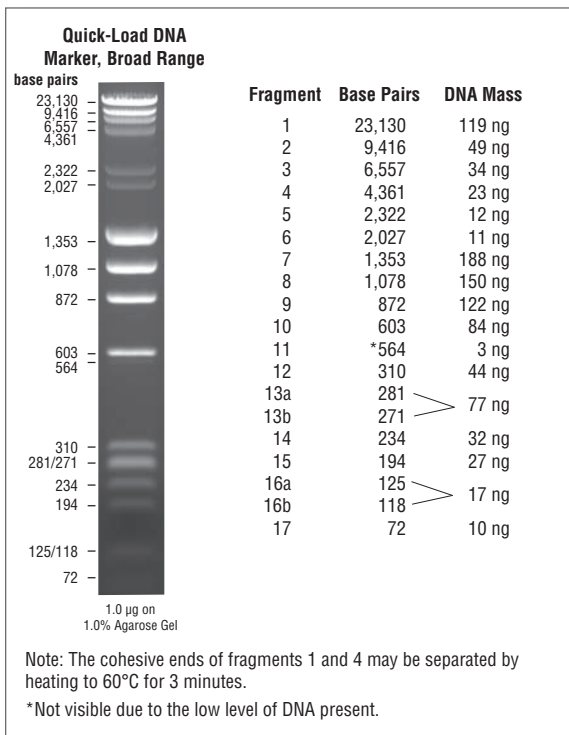
0.5/0.5 ml (100%)

DMSO can be used as an additive in the reaction when amplifying difficult targets. Recommended concentration is generally 3%, but can range from 1%–10%.

Quick-Load[®] DNA Marker, Broad Range (NEB #N0303)

200/400 μ l (100 μ g/ml)

The Quick-Load DNA Marker, Broad Range is a mix of HindIII digest of Lambda DNA and HaeIII digest of ϕ X174 DNA. It is supplied in 3.3 mM Tris-HCl (pH 8.0), 11 mM EDTA, 2.5% Ficoll 400, 0.017% SDS and 0.015% bromophenol blue. Recommended usage is 10 μ l (1 μ g) per gel lane.



Protocol for a Routine PCR Reaction:

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.**

1. Set up the appropriate reactions 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

2. Gently mix the reaction and spin down in microcentrifuge.

If the thermocycler does not have a heated lid, overlay the sample with mineral oil.

3. Cycling Conditions for a Routine PCR Reaction:

CYCLE STEP	CYCLES	TEMP	TIME
Initial Denaturation	1	98°C	30 seconds
Denaturation	30	98°C	5–10 seconds
Annealing		45–72°C	10–30 seconds
Extension		72°C	15–30 seconds per kb
Final Extension	1	72°C	5–10 minutes
Hold	1	4°C	∞

PCR Optimization:

The following guidelines are provided to ensure successful PCR using Phusion DNA Polymerase. These guidelines cover routine PCR reactions. Amplification of templates with high GC content, high secondary structure, low template concentrations or longer amplicons may require further optimization.

1. Template:

Use of high quality, purified DNA templates greatly enhances the success of PCR reactions. Recommended amounts of DNA template for a 50 μ l 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 (<http://frodo.wi.mit.edu/primer3>) can be used to design or analyze primers. The final concentration of each primer in a PCR reaction using Phusion DNA Polymerase may be 0.2–1 μ M, while 0.5 μ M 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. If the chelators (e.g. EDTA) are present, it may be necessary to increase the Mg⁺⁺ concentration. Mg⁺⁺ can be optimized in 0.5 mM increments using the MgCl₂ 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 T_m (8). Phusion DNA polymerase is also compatible with other additives such as formamide or glycerol.

4. Deoxynucleotides:

The final concentration of dNTPs is typically 200 μ M of each deoxynucleotide. Phusion cannot incorporate dUTP and is not recommended for use with uracil-containing primers or template.

5. **Phusion DNA Polymerase Concentration:**
We generally recommend using Phusion DNA Polymerase at a concentration of 20 units/ml (1.0 units/50 μ l reaction). However, the optimal concentration of Phusion DNA Polymerase may vary from 10–40 units/ml (0.5–2 units/50 μ l reaction) depending on amplicon length and difficulty. Do not exceed 2 units/50 μ l 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).
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 T_m Calculator (www.neb.com/TmCalculator) 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 yields sufficient product.

10. 2-step PCR:

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

Thermocycling conditions for a routine 2-step PCR:

CYCLE 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	∞

11. 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 T/A-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).

Amplification of the Control Template

1. Set up the appropriate reactions on ice:

COMPONENT	20 μl REACTION	50 μl REACTION	FINAL CONCENTRATION
Nuclease-free water	13.6 μl	34 μl	
5X Phusion HF Buffer	4 μl	10 μl	1X
10 mM dNTPs	0.4 μl	1 μl	200 μM
Primers*	1 μl	2.5 μl	0.2 μM
Control Template DNA	0.8 μl	2 μl	0.02 ng/ μl
Phusion DNA Polymerase	0.2 μl **	0.5 μl	0.02 U/ μl

*Reaction can be set up using either the 1.3 or 10 kb primer set

** Dilute polymerase with 1X reaction buffer to avoid pipetting errors

2. Recommended cycling conditions for the 1.3 kb fragment using a 2-step protocol:

CYCLE STEP	TEMP	TIME
Initial Denaturation	98°C	1 Minute
25 Cycles	98°C 72°C	5 Seconds 20 Seconds
Final Extension	72°C	10 Minutes
Hold	4°C	∞

2. Recommended cycling conditions for the 10 kb fragment using a 3-step protocol. Alternatively, this program can be used to amplify both fragments simultaneously.

CYCLE STEP	TEMP	TIME
Initial Denaturation	98°C	1 Minute
25 Cycles	98°C 60°C 72°C	5 Seconds 15 Seconds 2 Minutes 30 Seconds
Final Extension	72°C	10 Minutes
Hold	4°C	∞

Note: Controls have been shown to work in a variety of conditions.

Troubleshooting Guide:

Observation	Probable Cause(s)	Solution(s)
No Amplification Product	Incorrect annealing temperature	Verify annealing temperature using NEB T_m Calculator. Test temperature gradient.
	Poor 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	Increase primer concentration to 0.5 μ M.

Observation	Probable Cause(s)	Solution(s)
No Amplification Product (cont.)	Missing reaction component	Repeat reaction setup.
	Target sequence not present in template DNA	Try other sources of template DNA.
	Poor reaction conditions	Optimize [Mg ⁺⁺], annealing temperature and extension time. Check primer concentrations. Titrate enzyme concentration.
	Questionable template quality	Analyze DNA via gel electrophoresis after incubation with Mg ⁺⁺ .
	Inhibitory substance in reaction	Decrease sample volume. Try alcohol precipitation or drop dialysis to further purify DNA.
	Insufficient number of cycles	Rerun the reaction with more cycles.
	Incorrect thermocycler programming	Check program, verify times and temperatures.
	Inconsistent block temperature	Test calibration of heating block.
	Reaction tubes or solutions contaminated	Autoclave tubes prior to use to eliminate biological inhibitors.
	Complex template	Try Phusion GC buffer. Add DMSO to the reaction

Observation	Probable Cause(s)	Solution(s)	
Multiple or non-specific products	Premature replication	Set up reactions on ice with chilled components. Add samples to pre-heated (98°C) thermocycler.	
	Primer annealing temperature too low	Test temperature gradient. Verify annealing temperature using NEB T_m calculator.	
	Insufficient mixing of reaction buffer	Reaction buffer must be thoroughly mixed.	
	Improper Mg^{++} concentration	Adjust Mg^{++} concentration in 0.5 mM increments.	
	Poor primer design		Verify that primers have no complementary regions – either internally or to each other.
			Try longer primers.
			Avoid GC-rich 3' ends.
	Excess primer		Reduce primer concentration to 0.1–0.5 μM .
	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.
	Multiple target sequences in template DNA		Redesign primers with higher specificity to target sequence.
Poor reaction conditions		Reduce enzyme concentration.	
		Shorten extension time.	
		Titrate template. Reduce number of cycles.	

Quality Controls:

7.5 kb Genomic DNA PCR:

30 cycles of PCR amplification in a 50 µl reaction containing 50 ng genomic DNA with 1.0 unit of Phusion DNA Polymerase in the presence of 200 µM dNTPs and 1.0 µM primers in Phusion HF Buffer results in the expected 7.5 kb product.

20 kb Lambda DNA PCR:

22 cycles of PCR amplification in a 50 µl reaction containing 10 ng Lambda DNA with 1.0 unit of Phusion DNA Polymerase in the presence of 200 µM dNTPs and 1.0 µM primers in Phusion HF Buffer results in the expected 20 kb product.

Endonuclease Activity

Incubation of a 50 µl reaction in NEBuffer 2 containing a minimum of 10 units of Phusion DNA Polymerase with 200 µM dNTPs and 1 µg of supercoiled ϕX174 DNA for 4 hours at either 37°C or 72°C results in < 10% conversion to the nicked form as determined by agarose gel electrophoresis.

Reaction Buffers

The supplied Phusion reaction buffers and supplements are free of detectable nucleases.

Deoxynucleotide Solution

Deoxynucleotide solutions are certified free of detectable nucleases and phosphatases.

References:

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5. Longley, M.J., Bennett, S.E. and Mosbaugh D.W. (1990) *Nucleic Acids Res.*, 18, 7317-7322.
6. Lyamichev, V., Brow, M.A. and Dahlberg, J.E. (1993) *Science*, 260, 778-783.
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Ordering Information

PRODUCT	NEB #	SIZE
Phusion High-Fidelity PCR Kit	E0553S/L	50/200 rxns
KIT COMPONENTS SOLD SEPARATELY		
Phusion DNA Polymerase	M0530S/L	100/500 units
Deoxynucleotide Solution Mix	N0447S/L	8/40 μ mol of each
Phusion HF Buffer Pack	B0518S	6.0 ml
Phusion GC Buffer Pack	B0519S	6.0 ml
COMPANION PRODUCTS		
Detergent-free HF Buffer Pack	B0520S	6.0 ml
Detergent-free Phusion GC Buffer Pack	B0521S	6.0 ml
Deoxynucleotide Solution Set	N0446S	25 μ mol of each
Magnesium Chloride (MgCl_2) Solution	B9021S	6.0 ml
Phusion High-Fidelity PCR Master Mix with HF Buffer	M0531S/L	100/500 rxns
Phusion High-Fidelity PCR Master Mix with GC Buffer	M0532S/L	100/500 rxns
Lambda DNA	N3011S/L	250/1250 μ g



USA

New England Biolabs, Inc.
240 County Road
Ipswich, MA 01938-2723
Telephone: (978) 927-5054
Toll Free: (USA Orders) 1-800-632-5227
Toll Free: (USA Tech) 1-800-632-7799
Fax: (978) 921-1350
e-mail: info@neb.com
www.neb.com

Canada

New England Biolabs, Ltd.
Telephone: (905) 837-2234
Toll Free: 1-800-387-1095
Fax: (905) 837-2994
Fax Toll Free: 1-800-563-3789
e-mail: info@ca.neb.com

China, People's Republic

New England Biolabs (Beijing), Ltd.
Telephone: 010-82378265/82378266
Fax: 010-82378262
e-mail: info@neb-china.com

France

New England Biolabs France
Free Call: 0800/100 632
Free Fax: 0800/100 610
e-mail: info@fr.neb.com

Germany

New England Biolabs GmbH
Telephone: +49/(0)69/305 23140
Free Call: 0800/246 5227 (Germany)
Fax +49/(0)69/305 23149
Free Fax: 0800/246 5229 (Germany)
e-mail: info@de.neb.com

Japan

New England Biolabs Japan, Inc.
Telephone: +81 (0)3 5669 6191
Fax +81 (0)3 5669 6192
e-mail: info@neb-japan.com

United Kingdom

New England Biolabs (UK), Ltd.
Telephone: (01462) 420616
Call Free: 0800 318486
Fax: (01462) 421057
Fax Free: 0800 435682
e-mail: info@uk.neb.com

