

# Phusion™ Hot Start

## High-Fidelity DNA Polymerase

**Product codes: F-540S, 100 U**  
**F-540L, 500 U**



Stable for one year from the assay date. Store at -20°C.







### 1. Introduction

Finnzymes' Phusion™ Hot Start High-Fidelity DNA Polymerase offers superior performance for all PCR applications. A unique processivity-enhancing domain makes this *Pyrococcus*-like proofreading enzyme extremely processive, accurate and rapid. The error rate of Phusion Hot Start DNA Polymerase is equal to Phusion DNA Polymerase,  $4.4 \times 10^{-7}$  in Phusion HF-buffer, determined with a modified *lacI*-based method<sup>1</sup>. It is approximately 50-fold lower than that of *Thermus aquaticus* DNA polymerase and 6-fold lower than that of *Pyrococcus furiosus* DNA polymerase. Phusion Hot Start DNA Polymerase is capable of amplifying long amplicons such as 7.5 kb genomic and 20 kb  $\lambda$  DNA, used in our quality control tests.

Phusion Hot Start DNA Polymerase combines the DNA polymerase and a reversibly bound, specific Affibody<sup>®</sup> protein<sup>2,3</sup>, which inhibits the DNA polymerase activity at ambient temperatures and thus prevents the amplification of non-specific products. In addition, the Affibody ligand inhibits the 3'→5' exonuclease activity of the polymerase, preventing degradation of primers and template DNA during reaction setup. At polymerization temperatures, the Affibody molecule is released, rendering the polymerase fully active. Phusion Hot Start DNA Polymerase does not require any separate activation step in the PCR protocol.

Phusion Hot Start DNA Polymerase possesses the following activities: 5'→3' DNA polymerase activity and 3'→5' exonuclease activity. It generates blunt ends in the amplification products.

**Phusion™ Hot Start DNA Polymerase is unlike other enzymes. Please read the Quick Guide to modify your protocol for optimal results!**

Quick Guide	
	Use Phusion Hot Start DNA Polymerase at 0.5-1.0 U per 50 $\mu$ l reaction volume. Do not exceed 2 U/50 $\mu$ l. (See 4.1)
	Use 15-30 sec/kb for extension. Do not exceed 1 min/kb. (See 6.4)
	Use 98°C for denaturation. (See 6.1 & 6.2)
	Anneal at $T_m+3^\circ\text{C}$ (> 20 nt) or use 2-step protocol. (See 6.3)
	Use 200 $\mu\text{M}$ of each dNTP. Do not use dUTP. (See 4.3)
	Note: Phusion Hot Start DNA Polymerase produces blunt end DNA products.

### 2. Package Information

<b>F-540S</b>	<b>100 U (2 U/<math>\mu</math>l)</b> Material provided: Phusion™ Hot Start DNA Polymerase 100 U (50 $\mu$ l), 5x Phusion™ HF Buffer (2 x 1.5 ml), 5x Phusion™ GC Buffer (1.5 ml), DMSO (500 $\mu$ l) and 50 mM MgCl <sub>2</sub> solution (1.5 ml).
<b>F-540L</b>	<b>500 U (2 U/<math>\mu</math>l)</b> Material provided: Phusion™ Hot Start DNA Polymerase 500 U (250 $\mu$ l), 5x Phusion™ HF Buffer (6 x 1.5 ml), 5x Phusion™ GC Buffer (2 x 1.5 ml), DMSO (500 $\mu$ l) and 50 mM MgCl <sub>2</sub> solution (2 x 1.5 ml).

**Reaction buffer:** 5x Phusion HF Buffer and 5x Phusion GC Buffer both contain 7.5 mM MgCl<sub>2</sub>.

### 3. Guidelines for Using Phusion™ Hot Start DNA Polymerase

Phusion Hot Start DNA Polymerase (2U/ $\mu$ l) is provided with 5x Phusion HF Buffer and 5x Phusion GC Buffer. Both buffers contain 1.5 mM MgCl<sub>2</sub> at final reaction concentrations. Separate tubes of DMSO and 50 mM MgCl<sub>2</sub> solutions are provided for further optimization.

#### 3.1 Basic reaction conditions for PCR amplifications

Carefully mix and centrifuge all tubes before opening to improve recovery. When using Phusion Hot Start DNA Polymerase, it is not necessary to perform the PCR setup on ice. Prepare a master mix for the appropriate number of samples to be amplified. The DNA polymerase should be pipetted carefully and gently as the high glycerol content (50 %) in the storage buffer may otherwise lead to pipetting errors.

**Table 1. Pipetting instructions (in order).**

Component	Volume / 50 $\mu$ l reaction	Volume / 20 $\mu$ l reaction	Final conc.
H <sub>2</sub> O	add to 50 $\mu$ l	add to 20 $\mu$ l	
5x Phusion HF Buffer*	10 $\mu$ l	4 $\mu$ l	1x
10 mM dNTPs	1 $\mu$ l	0.4 $\mu$ l	200 $\mu\text{M}$ each
primer A**	x $\mu$ l	x $\mu$ l	0.5 $\mu\text{M}$
primer B**	x $\mu$ l	x $\mu$ l	0.5 $\mu\text{M}$
template DNA	x $\mu$ l	x $\mu$ l	
(DMSO***, optional)	(1.5 $\mu$ l)	(0.6 $\mu$ l)	(3 %)
Phusion Hot Start DNA Polymerase (2 U/ $\mu$ l)	0.5 $\mu$ l	0.2 $\mu$ l	0.02 U/ $\mu$ l

\* Optionally 5x Phusion GC Buffer can be used, see section 4.2. for details.

\*\* The recommendation for final primer concentration is 0.5  $\mu\text{M}$ , but it can be varied in a range of 0.2-1.0  $\mu\text{M}$  if needed.

\*\*\* Addition of DMSO is recommended for GC-rich amplicons. DMSO is not recommended for amplicons with very low GC % or amplicons that are >20 kb.

## 4. Notes about Reaction Components

### 4.1 Enzyme

The optimal amount of enzyme depends on the amount of template and the length of the PCR product. Usually 1 unit of Phusion Hot Start DNA Polymerase per 50 µl reaction volume gives good results, but optimal amounts could range from 0.5–2 units per 50 µl reaction depending on amplicon length and difficulty. **Do not exceed 2 U/50 µl (0.04 U/µl), especially for amplicons that are > 5 kb.**

When cloning fragments amplified with Phusion Hot Start DNA Polymerase, blunt end cloning is recommended. If TA cloning is required, it can be performed by adding A overhangs to the blunt PCR product with e.g. DyNAzyme™ II DNA Polymerase (F-501S/L). However, before adding the overhangs it is very important to remove all the Phusion Hot Start DNA Polymerase by purifying the PCR product carefully, as the proofreading activity in Phusion Hot Start DNA Polymerase is very strong at 72°C. Any remaining Phusion Hot Start DNA Polymerase will degrade the A overhangs, thus creating the blunt ends again. A detailed protocol for TA cloning of Phusion PCR products can be found on Finnzymes' web site [www.finnzymes.com](http://www.finnzymes.com).

### 4.2 Buffers

Two buffers are provided with the enzyme: 5x Phusion HF Buffer (F-518) and 5x Phusion GC Buffer (F-519). The error rate of Phusion Hot Start DNA Polymerase in HF Buffer ( $4.4 \times 10^{-7}$ ) is lower than that in GC Buffer ( $9.5 \times 10^{-7}$ ). Therefore, HF Buffer should be used as the default buffer for high-fidelity amplification. However, GC Buffer can improve the performance of Phusion Hot Start DNA Polymerase on some difficult or long templates, *i.e.* GC-rich templates or those with complex secondary structures. Use of GC Buffer is recommended when amplification with HF Buffer has failed. Use of detergent-free buffers (F-520, F-521) is not recommended with Phusion Hot Start DNA Polymerase.

### 4.3 Mg<sup>2+</sup> concentration and dNTP concentration

The concentration of Mg<sup>2+</sup> is critical since Phusion Hot Start DNA Polymerase is a magnesium-dependent enzyme. Excessive Mg<sup>2+</sup> stabilizes the DNA double strand and prevents complete denaturation of DNA. Excess Mg<sup>2+</sup> can also stabilize spurious annealing of primers to incorrect template sites and decrease specificity. Conversely, inadequate Mg<sup>2+</sup> could lead to lower product yield. The optimal Mg<sup>2+</sup> concentration depends on the dNTP concentration, the specific template DNA and the sample buffer composition. In standard PCR, the optimal Mg<sup>2+</sup> concentration is generally 0.5-1 mM higher than the total dNTP concentration. If the primers and/or template contain chelators such as EDTA or EGTA, the optimal concentration of free Mg<sup>2+</sup> may be higher. If further optimization is needed, increase Mg<sup>2+</sup> concentration in 0.2 mM steps.

High quality dNTPs (e.g. F-560S/L) should be used for optimal performance with Phusion Hot Start DNA Polymerase. Use of dUTP and other dUTP derivatives or analogues is not recommended. Due to the high processivity of Phusion Hot Start DNA Polymerase there is no advantage of increasing dNTP concentrations. For optimal results always use 200 µM of each dNTP.

### 4.4 Template

General guidelines are: 1 pg - 10 ng / 50 µl reaction with low complexity DNA (e.g. plasmid, lambda or BAC DNA); 50-500 ng / 50 µl reaction with high complexity genomic DNA.

### 4.5 PCR additives

The recommended reaction conditions for GC-rich templates include 3 % DMSO as a PCR additive, which aids in the denaturing of templates with high GC contents. For further optimization DMSO should be increased in 2 % steps. In some cases DMSO may also be required for supercoiled plasmids to relax for denaturation. Other PCR additives such as formamide (up to 3 %), glycerol and betaine are also compatible with Phusion Hot Start DNA Polymerase.

If high DMSO concentration is used, the annealing temperature must be lowered, as DMSO decreases the melting point of the primers. It has been reported that 10 % DMSO decreases the annealing temperature by 5.5-6.0°C<sup>4</sup>.

## 5. Cycling Conditions

Protocols optimized for Phusion DNA Polymerase can be applied to Phusion Hot Start DNA Polymerase reactions. However, use annealing temperature 60°C or higher (see section 6.3). Due to the novel nature of Phusion Hot Start DNA Polymerase, optimal reaction conditions may differ from standard enzyme protocols. Phusion Hot Start DNA Polymerase tends to work better at elevated denaturation and annealing temperatures due to higher salt concentrations in its buffer. Please pay special attention to the conditions listed below when running your reactions. Following the guidelines will ensure optimal enzyme performance.

Table 2. Cycling instructions.

Cycle step	Temp.	Time	Number of cycles
Initial denaturation	98°C	30 s	1
Denaturation	98°C	5-10 s	25-35
Annealing	60-72°C*	10-30 s	
Extension	72°C	15-30 s **/1 kb	
Final extension	72°C 4°C	5-10 min hold	1

\* See section 6.3

\*\* See section 6.4

## 6. Notes about Cycling Conditions

### 6.1 Initial denaturation

Denaturation should be done at 98°C (calculated sample temperature). Due to the high thermostability of Phusion Hot Start DNA Polymerase even higher than 98°C denaturation temperatures can be used. We recommend 30 s initial denaturation at 98°C for most templates. Some templates may require longer initial denaturation and the length of the initial denaturation time can be extended up to 3 minutes.

### 6.2 Denaturation

Keep the denaturation as short as possible. Usually 5-10 seconds at 98°C is enough for most templates. **Note:** the denaturation time and temperature may vary depending on the ramp rate and temperature control mode of the cyclor.

### 6.3 Primer annealing

With Phusion Hot Start DNA Polymerase, use primers with **T<sub>m</sub> 60°C or higher**. Typically the length of such primers is **20 nt or more**. The T<sub>m</sub> values should be calculated with the nearest-neighbor method<sup>5</sup>, because results from primer T<sub>m</sub> calculations can vary significantly depending on the method used. Instructions for T<sub>m</sub> calculation and a link to a calculator using the nearest-neighbor method can be found on Finnzymes' web site ([www.finnzymes.com](http://www.finnzymes.com)). As a basic rule, for primers >20 nt, anneal for 10 - 30 seconds at a T<sub>m</sub> +3°C of the lower T<sub>m</sub> primer. For primers ≤ 20 nt, use an annealing temperature equal to the T<sub>m</sub> of the lower T<sub>m</sub> primer. If necessary, use a temperature gradient to find the optimal annealing temperature for each template-primer pair combination. The annealing gradient should extend up to the extension temperature (two-step PCR). Two-step cycling without an annealing step is recommended for high T<sub>m</sub> primer pairs.

### 6.4 Extension

The extension should be performed at 72°C. The extension time depends on the length and complexity of the amplicon. For low complexity DNA (e.g. plasmid, lambda or BAC DNA) use extension time 15 s per 1 kb. For high complexity genomic DNA, 30 s per 1 kb is recommended.

## 7. Troubleshooting

#### No product at all or low yield

- Repeat the PCR and make sure that there are no pipetting errors.
- Use primers with T<sub>m</sub> 60°C or higher. T<sub>m</sub> values can be increased by lengthening the primers. Preferable primer length is 20 nt or longer (see section 6.3).
- Use Finnzymes' T<sub>m</sub> calculator ([www.finnzymes.com](http://www.finnzymes.com)).
- Use fresh high-quality dNTPs. Do not use dNTP mix that contains dUTP.
- Sample concentration may be too low. Use more template.
- Template DNA may be damaged. Use carefully purified template.
- Lengthen extension time.
- Increase the number of cycles.
- Lower annealing temperature.
- Optimize enzyme concentration.
- Titrate DMSO (2-8 %) in the reaction (see section 4.5).
- Denaturation temperature may be too low. Optimal denaturation temperature for most templates is 98°C or higher.
- Denaturation time may be too long or too short. Optimize denaturation time.
- Check the purity and concentration of the primers.
- Check primer design.
- Try using alternative GC Buffer (see section 4.2).

#### Non-specific products - High molecular weight smears

- Reduce enzyme concentration (see section 4.1).
- Shorten extension time (see section 6.4).
- Reduce the total number of cycles.
- Increase annealing temperature or try 2-step protocol (see section 6.3).
- Vary denaturation temperature (see section 6.2).
- Optimize Mg<sup>2+</sup> concentration (see section 4.3).
- Lower primer concentration.

#### Non-specific products - Low molecular weight discrete bands

- Raise annealing temperature (see section 6.3).
- Shorten extension time (see section 6.4).
- Lower enzyme concentration (see section 4.1).
- Optimize Mg<sup>2+</sup> concentration (see section 4.3).
- Titrate template amount.
- Lower primer concentration.
- Design new primers.

## 8. Component Specifications

### 8.1 Phusion™ Hot Start High-Fidelity DNA Polymerase (F-540)

Thermostable Phusion DNA Polymerase is isolated and purified from an *E. coli* strain carrying a plasmid into which the gene encoding Phusion DNA Polymerase is cloned. Phusion DNA Polymerase possesses the following activities: 5'→3' DNA polymerase activity and 3'→5' exonuclease activity. The Affibody ligand is isolated and purified from an *E. coli* strain carrying a plasmid with the cloned Affibody-encoding gene. Phusion Hot Start DNA Polymerase is purified free of contaminating endo- and exonucleases.

**Storage buffer:** 20 mM Tris-HCl (pH 7.4 at 25°C), 0.1 mM EDTA, 1 mM DTT, 100 mM KCl, 200 µg/ml BSA and 50 % glycerol.

**Storage and shipping:** Phusion Hot Start DNA Polymerase is shipped in gel ice. Upon arrival, store the components at -20°C. The Phusion Hot Start DNA Polymerase is stable for one year from the assay date when stored and handled properly.

**Unit definition:** One unit is defined as the amount of enzyme that will incorporate 10 nmoles of dNTPs into acid-insoluble form at 74°C in 30 minutes under the stated assay conditions.

**Unit assay conditions:** Incubation buffer: 25 mM TAPS-HCl, pH 9.3 (at 25°C), 50 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM β-mercaptoethanol, 100 µM dCTP, 200 µM each dATP, dGTP, dTTP.

Incubation procedure: 20 µg activated calf thymus DNA and 0.5 µCi [ $\alpha$ -<sup>32</sup>P] dCTP are incubated with 0.1 units of DNA polymerase in 50 µl incubation buffer at 74°C for 10 minutes. The amount of incorporated dNTPs is determined by trichloroacetic acid precipitation.

**DNA amplification test:** Performance in PCR is tested in amplification of 2.3 and 7.5 kb genomic DNA and 20 kb λ DNA.

**Exonuclease activity:** Incubation of 10 U for 4 hours at 72°C in 50 µl assay buffer with 1 µg sonicated <sup>3</sup>H DNA (2 x 10<sup>5</sup> cpm/µg) released < 1 % of radioactivity.

**Endonuclease assay:** No endonuclease activity is observed after incubation of 10 U of DNA polymerase with 1 µg of λ DNA in assay buffer at 72°C for 4 hours.

### 8.2 5x Phusion™ HF Buffer (F-518)

The 5x Phusion HF Buffer contains 7.5 mM MgCl<sub>2</sub>, which provides 1.5 mM MgCl<sub>2</sub> in final reaction conditions.

### 8.3 5x Phusion™ GC Buffer (F-519)

The 5x Phusion GC Buffer contains 7.5 mM MgCl<sub>2</sub>, which provides 1.5 mM MgCl<sub>2</sub> in final reaction conditions.

**Caution:** Repeated freezing and thawing of the buffer can result in the precipitation or accumulation of MgCl<sub>2</sub> in insoluble form. For consistent results heat the buffer to 90°C for 10 min and vortex prior to use if needed or store refrigerated.

## 8.4 50 mM MgCl<sub>2</sub> Solution (F-510MG)

Both Phusion Buffers supply 1.5 mM MgCl<sub>2</sub> at final reaction conditions. If higher MgCl<sub>2</sub> concentrations are desired, use a 50 mM MgCl<sub>2</sub> solution to increase the MgCl<sub>2</sub> titer. Using the following equation, you can calculate the volume of 50 mM MgCl<sub>2</sub> needed to attain the final MgCl<sub>2</sub> concentration: [desired mM Mg] – [1.5 mM] =  $\mu$ l to add to a 50  $\mu$ l reaction.

For example to increase the MgCl<sub>2</sub> concentration to 2.0 mM, add 0.5  $\mu$ l of the 50 mM MgCl<sub>2</sub> solution. Because the PCR reactions can be quite sensitive to changes in the MgCl<sub>2</sub> concentration, it is recommended that the 50 mM MgCl<sub>2</sub> stock solution is diluted 1:5 (to 10 mM) to minimize pipetting errors.

## 9. References

1. Frey & Suppmann (1995) *Biochemica* 2, 34-35.
2. Nord *et al.* (1997) *Nature Biotechnol.* 15, 772-777.
3. Wikman *et al.* (2004) *Protein Eng., Des. Sel.* 17, 455-462.
4. Chester & Marshak (1993) *Anal. Biochem.* 209, 284-290.
5. Breslauer *et al.* (1986) *PNAS* 83, 3746-3750.

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