FINNZYMES

Phusion™

High-Fidelity DNA Polymerase

Product codes: F-530S, 100 U
F-530L, 500 U

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

1. Introduction

Finnzymes' Phusion™ High-Fidelity DNA Polymerase offers extreme performance for all PCR applications. Incorporating an exciting new technology, Phusion DNA Polymerase brings together a novel *Pyrococcus*-like enzyme with a processivity-enhancing domain. The Phusion DNA Polymerase generates long templates with an accuracy and speed previously unattainable with a single enzyme, even on the most difficult templates. The extreme fidelity makes Phusion DNA Polymerase a superior choice for cloning. Using a *lac*I-based method modified from previous studies[1], the error rate of Phusion DNA Polymerase in Phusion HF Buffer is determined to be 4.4 x 10^{-7}, which is approximately 50-fold lower than that of *Thermus aquaticus* DNA polymerase, and 6-fold lower than that of *Pyrococcus furiosus* DNA polymerase.

Phusion DNA Polymerase possesses the following activities: 5´→3´ DNA polymerase activity and 3´→5´ exonuclease activity. It generates blunt ends in the amplification products. The polymerase is suitable also for amplification of long amplicons such as 7.5 kb genomic and 20 kb λ DNA used in Finnzymes' quality control assays.

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

Quick Guide:

- Use Phusion DNA Polymerase at 0.5-1.0 U per 50 µl reaction volume. Do not exceed 2 U/50 µl. (See 4.1)
- Use 15-30 s/kb for extension. Do not exceed 1 min/kb. (See 6.4)
- Use 98°C for denaturation. (See 6.1 & 6.2)
- Anneal at Tm+3°C (> 20nt) or use 2-step protocol. (See 6.3)
- Use 200 µM of each dNTP. Do not use dUTP. (See 4.3)
- Note: Phusion DNA Polymerase produces blunt end DNA products.

2. Package Information

F-530S	**100 U (2 U/µl)** Material provided: Phusion™ DNA Polymerase 100 U (50 µl), 5x Phusion™ HF Buffer (2 x 1.5ml), 5x Phusion™ GC Buffer (1.5 ml), DMSO (500 µl) and 50 mM $MgCl_2$-solution (1.5ml).
F-530L	**500 U (2 U/µl)** Material provided: Phusion™ DNA Polymerase 500 U (250 µl), 5x Phusion™ HF Buffer (6 x 1.5ml), 5x Phusion™ GC Buffer (2 x 1.5ml), DMSO (500 µl) and 50mM $MgCl_2$- solution (2 x1.5ml).

3. Guidelines for Using Phusion™ DNA Polymerase

Phusion DNA Polymerase (2U/µl) is provided with 5x Phusion HF Buffer and 5x Phusion GC Buffer. Both buffers contain 1.5 mM $MgCl_2$ at final reaction concentrations. Separate tubes of DMSO and 50 mM $MgCl_2$ 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. PCR reactions should be set up on ice. Prepare a master mix for the appropriate number of samples to be amplified. Phusion DNA Polymerase should be pipetted carefully and gently as the high glycerol content (50 %) in the storage buffer may otherwise lead to pipetting errors. It is critical that the Phusion DNA Polymerase is the last component added to the PCR mixture, since the enzyme exhibits 3´→5´ exonuclease activity that can degrade primers in the absence of dNTPs.

Table 1. Pipetting instructions (in order).

Component	Volume / 50 µl reaction	Volume / 20 µl reaction	Final conc.
H_2O	add to 50 µl	add to 20 µl	
5x Phusion HF Buffer*	10 µl	4 µl	1x
10 mM dNTPs	1 µl	0.4 µl	200 µM each
primer A**	x µl	x µl	0.5 µM
primer B**	x µl	x µl	0.5 µM
template DNA	x µl	x µl	
(DMSO***, optional)	(1.5 µl)	(0.6 µl)	(3 %)
Phusion DNA Polymerase	0.5 µl	0.2 µl	0.02U/µl

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

** The recommendation for final primer concentration is 0.5 µM, but it can be varied in a range of 0.2-1.0 µM 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 >20kb.

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 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 > 5kb.**

When cloning fragments amplified with Phusion 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-501). However, before adding the overhangs it is very important to remove all the Phusion DNA Polymerase by purifying the PCR product carefully, as any remaining Phusion DNA Polymerase will degrade the A overhangs creating blunt ends again. A detailed protocol for TA cloning of Phusion PCR products can be found on Finnzymes' website (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 DNA Polymerase in HF Buffer (4.4 x 10^{-7}) is lower than that in GC Buffer (9.5 x 10^{-7}). Therefore, the HF Buffer should be used as the default buffer for high-fidelity amplification. However, GC Buffer can improve the performance of Phusion 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 for those cases where amplification with HF Buffer has failed. For applications such as microarray or DHPLC, where the DNA templates need to be free of detergents, detergent-free reaction buffers (F-520, F-521) are available for Phusion DNA Polymerase.

4.3 Mg^{2+} concentration and dNTP concentration

Concentration of Mg^{2+} is critical since Phusion DNA Polymerase is a magnesium dependent enzyme. Excessive Mg^{2+} stabilizes the DNA double strand and prevents complete denaturation of DNA. Excess Mg^{2+} can also stabilize spurious annealing of primer to incorrect template sites and decrease specificity. Conversely, inadequate Mg^{2+} could lead to lower product yield. The optimal Mg^{2+} concentration will also depend on the dNTP concentration, the specific template DNA and the sample buffer composition. In general, the optimal Mg^{2+} concentration is 0.5 to 1 mM over the total dNTP concentration for standard PCR. If the primers and/or template contain chelators such as EDTA or EGTA, the apparent Mg^{2+} optimum may be shifted to higher concentrations. If further optimization is needed, increase Mg^{2+} concentration in 0.2 mM steps.

High quality dNTPs (e.g. F-560) should be used for optimal performance with Phusion DNA Polymerase. Use of dUTP and other dUTP-derivatives or analogues is not recommended. Due to the increased processivity of Phusion 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); 50-250 ng/50 µl reaction with high complexity genomic DNA. If cDNA synthesis reaction mixture is used as a source of template, the volume of the template should not exceed 10 % of the final PCR reaction volume.

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 varied in 2 % increments. In some cases DMSO may also be required for supercoiled plasmids to relax for denaturation. Other PCR additives such as formamide, glycerol, and betaine are also compatible with Phusion 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[2].

5. Cycling Conditions

Due to the novel nature of Phusion DNA Polymerase, optimal reaction conditions may differ from standard enzyme protocols. Phusion 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.

	2-step protocol		3-step protocol		
Cycle step	**Temp.**	**Time**	**Temp.**	**Time**	**Cycles**
Initial denaturation	98°C	30 s	98°C	30 s	1
Denaturation Annealing (see 6.3) Extension (see 6.4)	98°C - 72°C	5-10 s - 15-30 s /1 kb	98°C X°C 72°C	5-10 s 10-30 s 15-30 s /1 kb	25-35
Final extension	72°C 4°C	5-10 min hold	72°C 4°C	5-10 min hold	1

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 DNA Polymerase even higher than 98°C denaturation temperatures can be used. We recommend 30 seconds 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 cycler.

6.3 Primer annealing

The Phusion DNA Polymerase has the ability to stabilize primer-template hybridization. As a basic rule, for primers > 20nt, anneal for 10 – 30 seconds at a Tm +3°C of the lower Tm primer. The Tm's should be calculated with the nearest-neighbor method[3] as results from primer Tm calculations can vary significantly depending on the method used. For primers ≤ 20nt, use an annealing temperature equal to the Tm of the lower Tm 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 annealing step is also recommended for high Tm primer pairs. Instructions for Tm calculation and a link to a calculator using the nearest-neighbor method can be found on Finnzymes' website (www.finnzymes.com).

6.4 Extension

The extension should be performed at 72°C. Extension time depends on amplicon length and complexity. For low complexity DNA (e.g. plasmid, lambda or BAC DNA) use extension time 15 seconds per 1kb. For high complexity genomic DNA 30 seconds per 1kb is recommended. For some cDNA templates, the extension time can be increased up to 40 seconds per 1 kb to obtain optimal results.

7. Troubleshooting

No product at all or low yield

- Repeat and make sure that there are no pipetting errors.
- Use fresh high quality dNTPs. Do not use dNTP mix that contains dUTP.
- Use more template, sample concentration may be too low.
- Template DNA may be damaged. Use carefully purified template.
- Lengthen extension time.
- Increase the number of cycles.
- Optimize 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 the denaturation time.
- Check the condition of the primers.
- Check primer design.
- Try using the 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^{2+}-concentration.
- 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.
- Optimize Mg^{2+}-concentration.
- Titrate template amount.
- Lower primer concentration.
- Design new primers.

8. Component Specifications

8.1 Phusion™ High-Fidelity DNA Polymerase (F-530)

Thermostable Phusion DNA Polymerase is purified from an *E.coli* strain expressing the cloned Phusion DNA Polymerase gene. Phusion DNA Polymerase possesses the following activities: 5´→3´ DNA polymerase activity and 3´→5´ exonuclease activity. Phusion 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, stabilizers, 200 µg/ml BSA and 50 % glycerol.

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 $MgCl_2$, 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 [α-^{32}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 assay: Performance in PCR is tested in amplification of 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 ^{3}H ssDNA ($2x10^5$ 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.

Caution: Repeated freezing and thawing of the buffer can result in the precipitation or accumulation of $MgCl_2$ 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.

8.2 5x Phusion™ HF Buffer (F-518)

The 5x Phusion HF Buffer contains 7.5 mM $MgCl_2$, which provides 1.5 mM $MgCl_2$ in final reaction conditions.

8.3 5x Phusion™ GC Buffer (F-519)

The 5x Phusion GC Buffer contains 7.5 mM $MgCl_2$, which provides 1.5 mM $MgCl_2$ in final reaction conditions.

8.4 50 mM $MgCl_2$ Solution (F-510MG)

Both Phusion Buffers supply 1.5 mM $MgCl_2$ at final reaction conditions. If higher $MgCl_2$ concentrations are desired, use 50 mM $MgCl_2$ solution to increase the $MgCl_2$ titer. Using the following equation you can calculate the volume of 50 mM $MgCl_2$ needed to attain the final $MgCl_2$ concentration: [desired mM Mg] – [1.5 mM] = µl to add to a 50 µl reaction.

For example to increase the $MgCl_2$ concentration to 2.0 mM, add 0.5 µl of the 50 mM $MgCl_2$ solution. Because the PCR reactions can be quite sensitive to changes in the $MgCl_2$ concentration, it is recommended that the 50 mM $MgCl_2$ stock solution is diluted 1:5 (to 10 mM) to minimize pipetting errors.

9. References

1. Frey & Suppmann (1995) *Biochemica* 34-35.
2. Chester & Marshak (1993) *Analytical Biochemistry* 209, 284-290.
3. Breslauer *et al.*, (1986) *PNAS* 83, 3746-3750.

Storage and shipping

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

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Finnzymes Oy warrants that its products will meet the specifications stated on the technical data section of the data sheets, and Finnzymes Oy agrees to replace the products free of charge if the products do not conform to the specifications. Notice for replacement must be given within 60 days of receipt. In consideration of the above commitments by Finnzymes Oy, the buyer agrees to and accepts the following conditions:

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TOOLS FOR MOLECULAR BIOLOGY

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