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PRODUCT INFORMATION

Thermo Scientific Phusion U Hot Start DNA Polymerase

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Lot __ Expiry Date __

Store at -20 °C

Ordering information

Component	#F-555S	#F-555L
Phusion U Hot Start DNA Polymerase	100 U 50 µL	500 U 250 µL
5X Phusion HF Buffer*	2 × 1.5 mL	6 × 1.5 mL
5X Phusion GC Buffer*	1.5 mL	2 × 1.5 mL
100% DMSO	500 µL	500 µL

*Both 5X Phusion HF Buffer and 5X Phusion GC Buffer contain 7.5 mM $MgCl_2$.

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Rev.7

1. Introduction

Thermo Scientific™ Phusion™ U Hot Start DNA Polymerase is a modified Phusion DNA Polymerase which efficiently amplifies uracil-containing templates. It can be used to minimize carry-over contamination in conjunction with dUTP and uracil-DNA glycosylase (UDG) and for amplification of bisulfite-treated and damaged DNA. A unique processivity-enhancing domain makes this *Pyrococcus*-like proofreading enzyme extremely processive, accurate and rapid. Phusion U Hot Start DNA Polymerase is capable of amplifying long amplicons such as the 7.5 kb genomic and 20 kb λ DNA used in Thermo Fisher Scientific quality control assays.

Phusion U Hot Start DNA Polymerase combines a DNA polymerase and a reversibly bound, specific Affibody™ protein[1,2] which inhibits DNA polymerase and 3'→5' exonuclease activities at ambient temperatures, thus preventing the amplification of non-specific products and degradation of primers and template DNA during reaction setup. At polymerization temperatures, the Affibody molecule is released, rendering the polymerase fully active. Phusion U Hot Start DNA Polymerase does not require any separate activation step in the PCR protocol.

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

2. Important Notes

- Use Phusion U Hot Start DNA Polymerase at 0.5–1.0 U per 50 µL reaction volume. Do not exceed 2 U/50 µL (see 4.1).
- Use 200 µM of each dNTP. dUTP or dITP can be added up to 200 µM (see 4.3).
- Use 98 °C for denaturation (see 5.1 & 5.2).
- The annealing rules are different from many common DNA polymerases (such as *Taq* DNA polymerases). Read Section 5.3 carefully.
- Use 15–30 s/kb for extension. Do not exceed 1 min/kb (see 5.4).
- Phusion U Hot Start DNA Polymerase produces DNA products with blunt ends.

3. Guidelines for using Phusion U Hot Start DNA Polymerase

Carefully mix and centrifuge all tubes before opening to ensure homogeneity and improve recovery. When using Phusion U Hot Start DNA Polymerase, it is not necessary to perform 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.

Due to the novel nature of Phusion U Hot Start DNA Polymerase, the optimal reaction conditions may differ from PCR protocols for standard DNA polymerases. Due to the high salt concentration in the reaction buffer, Phusion U Hot Start DNA Polymerase tends to work better at elevated denaturation and annealing temperatures. Please pay special attention to the conditions listed below when running your reactions. Following the guidelines will ensure optimal enzyme performance. Protocols optimized for Phusion DNA Polymerase can be applied to Phusion U Hot Start DNA Polymerase reactions.

Table 1. Pipetting instructions (add items in this order)

Component	20 µL rxn	50 µL rxn	Final conc.
H_2O	Add to 20 µL	add to 50 µL	
5X Phusion HF Buffer*	4 µL	10 µL	1x
10 mM dNTPs	0.4 µL	1 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	See section 4.4 for guidelines
(DMSO***, optional)	(0.6 µL)	(1.5 µL)	(3%)
Phusion U HS DNA Polymerase (2 U/µL)	0.2 µL	0.5 µL	0.02 U/µL

* Optionally 5X Phusion GC Buffer can be used. See section 4.2. for details.
** The recommended 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 >20 kb.

Note. For prevention of carryover contamination, use Thermo Scientific™ Uracil-DNA Glycosylase (UDG, #EN0361) and dUTP (#R0133). Typically, dUTP is added to a final concentration of 200 µM. For longer amplicons, a lower dUTP concentration (100 µM) may be required for high yields.

Table 2.Cycling instructions

Cycle step	2-step protocol		3-step protocol		Cycles
	Temp.	Time	Temp.	Time	
Initial denaturation	98 °C	30 s	98 °C	30 s	1
Denaturation Annealing (see 5.3) Extension (see 5.4)	98 °C - 72 °C	5-10 s - 15-30 s/kb	98 °C X °C 72 °C	5 - 10 s 10 - 30 s 15 - 30 s/kb	25-35
Final Extension	72 °C 4 °C	5 - 10 min hold	72 °C 4 °C	5 - 10 min hold	1

Note. For carryover contamination prevention with UDG, perform UDG pre-treatment for 2 min at 50 °C and 10 min inactivation at 95 °C. To avoid PCR product degradation by UDG, which gains back its activity when the PCR mix cools below 55 °C, store the PCR reaction after cycling at -20 °C for long term, or at 4 °C for up to 2 days.

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 U Hot Start DNA Polymerase per 50 µL reaction volume gives good results, but the optimal amount can range from 0.5 to 2 units per 50 µL reaction depending on the amplicon length and complexity. **Do not exceed 2 U/50 µL (0.04 U/µL), especially for amplicons that are >5 kb.**

4.2. Buffers

Two buffers are provided with the enzyme: 5X Phusion HF Buffer (F-518) and 5X Phusion GC Buffer (F-519). HF Buffer should be used as the default buffer for high-fidelity amplification. However, GC Buffer can improve the performance of Phusion U DNA Polymerase on some difficult or long templates, such as GC-rich templates or those with complex secondary structures.

4.3. Mg^{2+} and dNTP

The concentration of Mg^{2+} is critical since Phusion U Hot Start 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 primers to incorrect template sites and decrease specificity. Conversely, inadequate Mg^{2+} may lead to lower product yield. The optimal Mg^{2+} concentration also depends on the dNTP concentration, the specific template DNA and the sample buffer composition. In general, the optimal Mg^{2+} concentration is 1.5 mM for standard PCR. This Mg^{2+} concentration is present in 1x dilution of the Phusion HF and Phusion GC buffers.

High quality dNTPs should be used for optimal performance with Phusion U Hot Start DNA Polymerase. Due to the high processivity of Phusion U Hot Start DNA Polymerase there is no advantage of increasing dNTP concentrations. For optimal results always use 200 µM of each dNTP. dUTP or dITP can be added up to 200 µM.

4.4. Template

General guidelines for low complexity DNA (e.g. plasmid, lambda or BAC DNA) are: 1 pg–10 ng per 50 µL reaction volume. For high complexity genomic DNA, the amount of DNA template should be 25–250 ng per 50 µL reaction volume. 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 content. 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 U Hot Start DNA Polymerase.

If high DMSO concentration is used, the annealing temperature must be decreased, as DMSO affects the melting point of the primers. It has been reported that 10% DMSO decreases the annealing temperature by 5.5 – 6.0°C[3].

5. Notes about cycling conditions

5.1. Initial denaturation

Denaturation should be performed at 98 °C. Due to the high thermostability of Phusion U Hot Start DNA Polymerase even higher than 98 °C denaturation temperatures can be used. We recommend a 30-second initial denaturation at 98 °C for most templates. Some templates may require longer initial denaturation time, and the length of the initial denaturation time can be extended up to 3 minutes.

5.2. Denaturation

Keep the denaturation time 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.

5.3. Primer annealing

The optimal annealing temperature for Phusion U Hot Start DNA Polymerase may differ significantly from that of *Taq*-based polymerases. **Always use the Tm calculator and instructions on website: www.thermofisher.com/tmcalculator** to determine the Tm values of primers and optimal annealing temperature.

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). A 2-step protocol is recommended when primer Tm values are at least 69 °C (> 20 nt) or 72 °C (≤ 20 nt) when calculated with Thermo Scientific Tm calculator. In the 2-step protocol the combined annealing/extension step should be performed at 72°C even when the primer Tm is > 72 °C.

5.4. 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 an extension time of 15 seconds per 1 kb. For high complexity genomic DNA, 30 seconds per 1 kb is recommended. For some cDNA templates, the extension time can be increased up to 40 seconds per 1 kb to obtain optimal results.

6. Recommendations for cloning

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 Thermo Scientific™ *Taq* DNA Polymerase (#EP0402), for example. Incubate purified PCR product with 1x Taq buffer, 2.5 mM $MgCl_2$, 0.2 mM dATP and 1 U *Taq* DNA polymerase in 10 µL reaction mixture up to 30 min at 72 °C. Before adding the overhangs it is very important to remove all the Phusion DNA Polymerase by carefully purifying the PCR product, for example using Thermo Scientific™ GeneJET™ PCR Purification Kit (#K0701). Any remaining Phusion DNA Polymerase will degrade the A overhangs, creating blunt ends again.

7. Troubleshooting

No product at all or low yield	
• Repeat the PCR and make sure that there are no pipetting errors. • Use our Tm calculator www.thermofisher.com/tmcalculator to determine optimal annealing temperature. • Use fresh high-quality dNTPs. • Sample concentration may be too low. Use more template. • Template DNA may be damaged. Use carefully purified template. • Increase extension time. • Increase the number of cycles. • Decrease 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 the alternative GC Buffer (see section 4.2). • If UDG is added, make sure that the temperature during PCR cycling is always higher than 55 °C.
Non-specific products - High molecular weight smears	
• Decrease enzyme concentration (see section 4.1). • Decrease extension time (see section 5.4). • Reduce the total number of cycles. • Increase annealing temperature or try 2-step protocol (see section 5.3).	• Vary denaturation temperature (see section 5.2). • Reduce primer concentration.
Non-specific products - Low molecular weight discrete bands	
• Increase annealing temperature (see section 5.3). • Shorten extension time (see section 5.4). • Reduce enzyme concentration (see section 4.1).	• Titrate template amount. • Decrease primer concentration. • Design new primers.

8. Component specifications

8.1. Phusion U Hot Start DNA Polymerase (F-551)

Thermostable Phusion U DNA Polymerase is isolated and purified from an *E.coli* strain expressing the cloned Phusion U DNA Polymerase gene. Phusion U 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 expressing the cloned Affibody-encoding gene.

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

Unit definition: One unit is defined as the amount of enzyme that will incorporate 10 nmoles of dNTPs into a polynucleotide fraction at 74 °C in 30 min.

Enzyme activity is assayed in the following mixture:
25 mM TAPS-HCl, pH 9.3 (at 25 °C), 50 mM KCl, 2 mM $MgCl_2$, 1 mM β-mercaptoethanol, 0.75 mM activated salmon milt DNA, 100 µM dTTP, 200 µM each dATP, dGTP, dCTP, 0.4 MBq/ml [^{3}H] dTTP.

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.

Caution: Repeated freezing and thawing of the buffer can result in precipitation of $MgCl_2$. For consistent results, heat the buffer to 90 °C for 10 min and vortex prior to use if needed, or store refrigerated.

8.4 Dimethyl sulfoxide DMSO, 100 % (F-515)

Note: The freezing point of DMSO is 18–19 °C, so it does not melt on ice.

9. References

1. Nord K. *et al.* (1997) *Nature Biotechnol.* 15: 772–777
2. Wikman M. *et al.* (2004) *Protein Eng. Des. Sel.* 17: 455–462.
3. Chester N. & Marshak D.R. (1993) *Anal. Biochem.* 209: 284–290

CERTIFICATE OF ANALYSIS

Endonuclease contamination assay
No endonuclease activity was observed after incubation of DNA polymerase with supercoiled plasmid DNA.

DNA amplification assay
Performance in PCR is tested by amplification of 7.5 kb genomic DNA and a 20 kb lambda DNA fragments.

Quality authorized by:  Jurgita Zilinskiene