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PRODUCT INFORMATION

Thermo Scientific Phusion Green Hot Start II High-Fidelity PCR Master Mix

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Lot __ Expiry Date __

Store at -20 °C

Ordering information

Component	#F-566S 100 rxns 100 x 50 µL rxns	#F-566L 500 rxns 500 x 50 µL rxns
2X Phusion Green Hot Start II High-Fidelity PCR Master Mix	2 × 1.25 mL	10 × 1.25 mL
100% DMSO	500 µL	2 × 500 µL
Water, nuclease-free	2 × 1.25 mL	10 × 1.25 mL

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For Research Use Only. Not for use in diagnostic procedures.

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1.Introduction

Thermo Scientific™ Phusion™ Hot Start II 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 II DNA Polymerase is equal to that of Phusion DNA Polymerase (4.4×10^{-7} in Phusion HF-buffer) when determined with a modified *lac*I-based method[1]. 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 II High-Fidelity DNA Polymerase is capable of amplifying long amplicons such as the 7.5 kb genomic and 20 kb λ DNA.

Phusion Hot Start II DNA Polymerase combines the DNA polymerase and a reversibly bound, specific Affibody™ protein[2,3], which inhibits the DNA polymerase activity at ambient temperatures, thus preventing 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 II DNA Polymerase does not require any separate activation step in the PCR protocol.

Phusion Hot Start II 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 Green Hot Start II High-Fidelity Master Mix is a convenient 2X mix containing Phusion Hot Start II DNA Polymerase, nucleotides and optimized reaction buffer including $MgCl_2$. Only template and primers need to be added by the user. The master mix also includes a density reagent and two tracking dyes for direct loading of PCR products on a gel. The dyes do not interfere with PCR performance and are compatible with downstream applications such as DNA sequencing, ligation and restriction digestion. For applications that require PCR product analysis by absorbance or fluorescence excitation, we recommend using the colorless Phusion Hot Start II High-Fidelity PCR Master Mix (#F-565S) or purifying the PCR product prior to analysis.

2. Important Notes

- 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 DNA Polymerases produce blunt end DNA products. See section 6 for cloning recommendations.

3. Setting up PCR reactions using Phusion Green Hot Start II High-Fidelity PCR Master Mix

Carefully mix and centrifuge all tubes before opening to ensure homogeneity and improve recovery. When using Phusion Green Hot Start II High-Fidelity PCR Master Mix, it is not necessary to perform the PCR setup on ice.

Due to the unique nature of Phusion DNA Polymerases, optimal reaction conditions may differ from standard enzyme protocols. Phusion DNA Polymerases tend 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 in section 5 when running your reactions. Following the guidelines will ensure optimal enzyme performance.

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	
2X Phusion Green HS II HF Master Mix	10 µL	25 µL	1X
Forward primer *	X µL	X µL	0.5 µM
Reverse primer *	X µL	X µL	0.5 µM
Template DNA	X µL	X µL	
(DMSO, optional)**	(0.6 µL)	(1.5 µL)	(3%)

* 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 > 20 kb.

Table 2. Cycling instruction

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	98 °C	5–10 s	98 °C	5–10 s	25–35
Annealing (see 5.3)	–	–	X °C	10–30 s	
Extension (see 5.4)	72 °C	15–30 s/kb	72 °C	15–30 s/kb	
Final extension	72 °C 4 °C	5–10 min hold	72 °C 4 °C	5–10 min hold	1

4. Notes about reaction components

4.1. Phusion Green Hot Start II High-Fidelity PCR Master Mix

Phusion Green Hot Start II High-Fidelity PCR Master Mix contains all the necessary reaction components except for template DNA and primers. The master mix provides 1.5 mM $MgCl_2$ and 200 µM of each dNTP in final reaction concentration. Phusion Hot Start II DNA polymerase concentration is optimized to give good results in most reactions. When PCR reaction is set up according to the instructions, the final concentration of Phusion enzyme is 1 U in 50 µL reaction (0.4 U in 20 µL reaction).

4.2. Template

For low complexity DNA (e.g. plasmid, lambda or BAC DNA) it is recommended to use 1 pg–10 ng per 50 µL reaction volume. For high complexity genomic DNA, the amount of DNA template should be 10–250 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.

4.3. 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. DMSO may also be required for supercoiled plasmids to relax for denaturation. 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.[4]

5. Notes about cycling conditions

5.1. Initial denaturation

Denaturation should be performed at 98 °C. Due to the high thermostability of Phusion DNA Polymerases 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 time and the length of the initial denaturation time can be extended up to 3 minutes.

5.2. Denaturation

Keep the denaturation as short as possible. Usually 5–10 seconds at 98 °C is enough for most templates. 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 DNA Polymerases may be significantly different than annealing temperature with other DNA polymerases. **Always use the Tm calculator and instructions on our website: www.thermofisher.com/tmcalculator** to determine the Tm values of your 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).

Two-step cycling without annealing step is also recommended for high Tm primer pairs.

5.4. 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 s per 1 kb. For high complexity genomic DNA 30 s per 1 kb is recommended.

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6. Cloning recommendations

Blunt end cloning is recommended when cloning DNA fragments amplified with Phusion DNA Polymerases. If TA cloning is required, it is necessary to add A overhangs to the 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 and make sure that there are no pipetting errors. • Titrate template amount. • Template DNA may be damaged. Use carefully purified template. • Increase extension time. • Increase the number of cycles. • Optimize annealing temperature.	• Titrate DMSO (2–8%) in the reaction. • Denaturation temperature may be too low. Optimal denaturation temperature for most templates is 98 °C or higher. • Optimize denaturation time. • Check the purity and concentration of the primers. • Check primer design.
Non-specific products - High molecular weight smears	
• Shorten extension time. • Reduce the total number of cycles. • Increase annealing temperature or try 2-step protocol.	• Vary denaturation temperature • Decrease primer concentration.
Non-specific products - Low molecular weight discrete bands	
• Increase annealing temperature • Shorten extension time. • Titrate template amount.	• Decrease primer concentration. • Design new primers.

8. References

1. Frey M. & Suppmann B. (1995) *Biochemica* 2: 34–35.
2. Nord K. *et al.* (1997) *Nature Biotechnol.* 15: 772–777.
3. Wikman M. *et al.* (2004) *Protein Eng. Des. Sel.* 17: 455–462.
4. Chester N. & Marshak D.R. (1993) *Analytical Biochemistry* 209: 284–290.

CERTIFICATE OF ANALYSIS

DNA amplification assay
Performance in PCR is tested by the amplification of a 7.5 kb fragment of genomic DNA.

Quality authorized by:  Jurgita Zilinskiene