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PRODUCT INFORMATION

Thermo Scientific Phusion High-Fidelity PCR Kit

Pub. No. MAN0013363
Rev. Date 27 June 2018 (Rev. C.00)

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Lot _ Expiry Date _

Store at -20 °C

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

COMPONENTS OF THE KIT

Component	#F-553S 50 rxns	#F-553L 200 rxns
Phusion DNA Polymerase, 2 U/µL	25 µL	100 µL
5X Phusion HF buffer	1 × 1.5 mL	3 × 1.5 mL
5X Phusion GC buffer	1 × 1.5 mL	1 × 1.5 mL
dNTP mix, 10 mM each	100 µL	400 µL
50 mM $MgCl_2$ solution	1.5 mL	1.5 mL
Control lambda template, 0.5 ng/mL	40 µL	40 µL
1.3 kb primers, 4 µM each	50 µL	50 µL
10 kb primers, 4 µM each	50 µL	50 µL
DNA size standard	200 µL	400 µL
DMSO	0.5 mL	0.5 mL

1.INTRODUCTION

Thermo Scientific™ Phusion™ High-Fidelity DNA Polymerase offers extreme performance for all major PCR applications. Incorporating an exciting technology, Phusion DNA Polymerase brings together a novel *Pyrococcus*-like enzyme with a processivity-enhancing domain. 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. The error rate of Phusion DNA Polymerase in Phusion HF Buffer is 4.4×10^{-7} 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.

The Phusion High-Fidelity PCR Kit includes lambda DNA control template and primers for 1.3 kb and 10 kb amplicons. The template amount is sufficient for performing 20 control reactions in 50 µL volume or 50 control reactions in 20 µL volume.

2. IMPORTANT NOTES

- Use 98 °C for denaturation (see 5.1 and 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).
- Use Phusion DNA Polymerase at 0.5–1 U per 50 µL reaction volume. Do not exceed 2 U/50 µL (see 4.1).
- Use 200 µM of each dNTP. Do not use dUTP (see 4.3).
- Note: Phusion DNA Polymerases produce blunt end PCR products.

3. GUIDELINES FOR PHUSION DNA POLYMERASE

Phusion DNA Polymerase (2 U/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 DNA amplification

Carefully mix and centrifuge all tubes before opening to ensure homogeneity and improve recovery. PCR reactions should be set up on ice. 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 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.

Due to the nature of Phusion 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 DNA Polymerase tends to work better at elevated denaturation and annealing temperatures. 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	**50 µL rxn**	**20 µL rxn**	**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	0.02 U/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 the 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 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

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 amount can range from 0.5 to 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 Thermo Scientific *Taq* DNA Polymerase, for example. However, before adding the overhangs it is very important to remove all the Phusion DNA Polymerase by purifying the PCR product carefully. Any remaining Phusion DNA Polymerase will degrade the A overhangs, creating blunt ends again.

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 (4.4×10^{-7}) is lower than that in GC Buffer (9.5×10^{-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, such as GC-rich templates or those with complex secondary structures. 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 Polymerases.

4.3. Mg^{2+} and dNTP

The 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 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 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 should be used for optimal performance with Phusion DNA Polymerase. The polymerase cannot read uracil derivatives or inosine in the template strand so the use of these analogues or primers containing them is not recommended. Due to the high processivity of Phusion DNA Polymerase there is no advantage in increasing dNTP concentrations. For optimal results always use 200 µM of each dNTP.

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 50–250 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 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 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[2].

5. NOTES ABOUT CYCLING CONDITIONS

5.1. Initial denaturation

Denaturation should be performed at 98 °C. Due to the high thermostability of Phusion 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 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 Fisher 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. Extension time depends on amplicon length and complexity. 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. AMPLIFYING CONTROL TEMPLATE

6.1. Reaction conditions

Table 3. Pipetting instructions for control reactions

Component	50 µL rxn	20 µL rxn	Final conc.
H_2O	34 µL	13.6 µL	
5X Phusion HF Buffer	10 µL	4 µL	1X
10 mM dNTPs	1 µL	0.4 µL	200 µM each
Primers*	2.5 µL	1 µL	0.2 µM
Control template DNA	2 µL	0.8 µL	
Phusion DNA Polymerase	0.5 µL	0.2 µL**	0.02 U/µL

* Either the 1.3 kb primer set or 10 kb primer set.

** Dilution of polymerase should be made to 1X reaction buffer to avoid pipetting errors.

6.2. Cycling conditions

A separate cycling protocol is given for both 1.3 kb and 10 kb control amplicons. Alternatively, both control reactions can be amplified simultaneously using the 10 kb cycling protocol.

Table 4. Cycling conditions for 1.3 kb control fragment (2-step protocol)

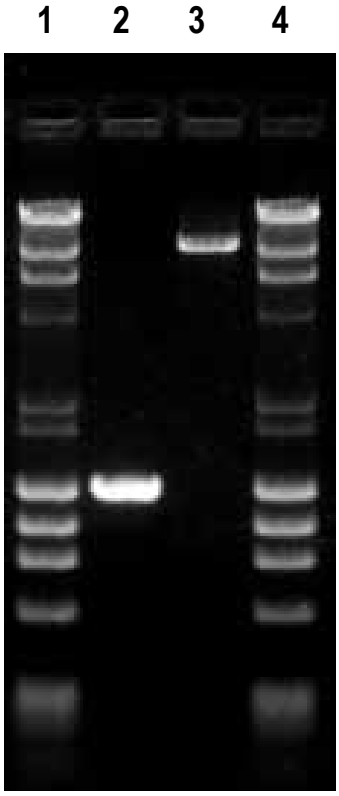
Cycle step	Temp.	Time	Cycles
Initial denaturation	98 °C	1 min	1
Denaturation Annealing/Extension	98 °C 72 °C	5 s 20 s	25
Final Extension	72 °C 10 °C	10 min hold	1

Table 5. Cycling conditions for 10 kb fragment (3-step protocol). This program can also be used if both control reactions are amplified simultaneously.

Cycle step	Temp.	Time	Cycles
Initial denaturation	98 °C	1 min	1
Denaturation Annealing Extension	98 °C 60 °C 72 °C	5 s 15 s 2 min 30 s	25
Final Extension	72 °C 10 °C	10 min hold	1

The cycling protocols above are recommendations. If you wish to run these controls together or with your experimental samples, please note that the controls have been shown to work in a variety of conditions. The 1.3 kb control has been successfully amplified with both 2- and 3-step protocols with extension times ranging from 15 s to 5 min, and cycle numbers ranging from 20 to 30. The 10 kb control has been successfully amplified with 3-step protocol with extension times ranging from 2 min to 5 min, and cycle numbers ranging from 20 to 30.

6.3. Analysis of the control reactions



In the image on the left both control reactions have been run on an ethidium bromide stained 1% agarose gel in TAE buffer. For this run 15 µL of loading dye was added to the 50 µL control PCR reactions, and 5 µL of the resulting mixtures were loaded on the gel.
Lane 1. DNA size standard
Lane 2. 1.3 kb control amplicon
Lane 3. 10 kb control amplicon
Lane 4. DNA size standard
After running your control reactions on a gel, compare the results to the image on the left to check for specificity and efficiency of the reactions.

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 contain dUTP or dITP or primers that contain uracil or inosine. • Titrate template amount. • Template DNA may be damaged. Use carefully purified template. • Increase 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. • 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 DNA is not carefully purified, inhibitors may be present – decrease the amount of DNA.
Non-specific products - High molecular weight smears	
• Decrease enzyme concentration (see section 4.1). • Make sure the extension time used was not too long (see section 5.4). • Titrate template amount. • 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) • Optimize Mg^{2+}-concentration • Decrease primer concentration.
Non-specific products - High molecular weight discrete bands	
• Increase annealing temperature (see section 5.3). • Shorten extension time (see section 5.4) • Decrease enzyme concentration.	• Optimize Mg^{2+}-concentration. • Titrate denaturation template amount. • Decrease 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 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 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.

8.4. dNTP mix (F-560)
The dNTP mix is a premixed ready-to-use solution consisting of the following compounds: dATP, dGTP, dCTP and dTTP dissolved in H_2O at 10 mM each.

8.5. 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.

8.6. Lambda DNA control template (F-304K)
The control template is bacteriophage lambda DNA (GenBank access number J02459, 48 502 bp). The concentration is 0.5 ng/µL in TE buffer.

8.7. 1.3 kb control primer mix (F-535)
This component is a mix of primers for amplification of a 1.3 kb fragment of lambda DNA. Each primer concentration is 4 µM in H_2O.

Primer #1 (27-mer)
5'-GTC ACC AGT GCA GTG CTT GAT AAC AGG-3'
Melting point: 71.0 °C
Coordinates in lambda DNA: 30 006–30 032

Primer #2 (28-mer)
5'-GAT GAC GCA TCC TCA CGA TAA TAT CCG G-3'
Melting point: 73.2 °C
Coordinates in lambda DNA: 31 325–31 352

8.8. 10 kb control primer mix (F-536)

This component is a mix of primers for amplification of a 10 kb fragment of lambda DNA. Each primer concentration is 4 µM in H_2O.

Primer #1 (22-mer)
5'-CAG TGC AGT GCT TGA TAA CAG G-3'
Melting point: 63.5 °C
Coordinates in lambda DNA: 30 011–30 032

Primer #2 (20-mer)
5'-GTA GTG CGC GTT TGA TTT CC-3'
Melting point: 63.3 °C
Coordinates in lambda DNA: 40 024–40 043

8.9. Ready-to-use DNA standard (F-303SD)

This DNA standard is a mix of lambda DNA HindIII digest and bacteriophage φX174 DNA HaeIII digest, each at 50 ng/µL (100 ng/µL total). It is supplied in 8 mM Tris-HCl (pH 8.0), 12 mM EDTA, 12% glycerol and 0.012% (w/v) bromophenol blue dye.

The DNA standard solution contains 19 fragments of the following sizes and mass amounts (per 10 µL):

Fragment	Base pairs	DNA amount ng/10 µL
1	23130	238
2	9416	97
3	6557	68
4	4361	45
5	2322	24
6	2027	21
7	1353	126
8	1078	100
9	872	81
10	603	56
11	564*	6
12	310	29
13a	281	26
13b	271	25
14	234	22
15	194	18
16a	125*	1
16b	118	11
17	72	7

Note: The cohesive areas of fragments 1 and 4 can be separated by heating at 65 °C for 5 minutes. For daily use the marker can be stored at +4 °C (at least one month). The marker is stable at -20 °C for at least one year.

* Due to the low amount of DNA these bands are almost invisible.

8.10. Dimethyl sulfioxide DMSO, 100% (F-515)

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

9. REFERENCES

1. Frey M. & Suppmann B. (1995) *Biochemica* 2: 34–35.
2. Cherster N. & Marshak D.R (1993) *Analytical Biochemistry* 209: 284-290.

PRODUCT USE LIMITATION

This product is developed, designed and sold exclusively for research purposes and in vitro use only. The product was not tested for use in diagnostics or for drug development, nor is it suitable for administration to humans or animals. Please refer to www.thermofisher.com for Material Safety Data Sheet of the product.