Guidelines for PCR Optimization with Phusion High-Fidelity PCR Master Mix

Overview

Protocol

1. **Enzyme:**
   In Phusion PCR Master Mix the enzyme concentration is optimized to give good results in most reactions. When pipetted accordingly to the instructions the final concentration is 1 U of enzyme in 50 µl reaction (0.4 U in 20 µl reaction).
   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. Any remaining Phusion DNA Polymerase will degrade the A overhang, thus creating blunt ends again.
2. **Buffers:**
   The F-531 PCR Master Mix contains Phusion HF Buffer. The F-532 Phusion PCR Master Mix contains Phusion GC Buffer. 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 Master Mix with HF Buffer should be used as a default 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 secondary structures.
3. **Mg^{2+} and dNTP concentration:**
   The Phusion Master Mix provides 1.5 mM $MgCl_2$ and 200 µm of each dNTP in final reaction concentration.
4. **Template:**
   General guidelines are 1 pg – 10 ng / 50 µl reaction with low complexity DNA (e.g. plasmid, lambda of BAC DNA); 50-250 ng / 50 µl reaction with high complexity genomic DNA.
5. **PCR additives:**
   The recommended reaction condition 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 PCR Master Mix.
   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.
6. **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.

   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 Extension	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	70°C 4°C	5-10 min hold	1

7. **Initial denaturation:**
   Denaturation should be done at 98°C. 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.
8. **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.
9. **Primer annealing:**
   The Phusion DNA Polymerase has the ability to stablize primer-template hybridization. As a basic rule, for primers >20 nt, anneal for 10-30 seconds at Tm +3°C of the lower Tm primer. The Tm's should be calculated with the nearest neighbor method as results from primer Tm calculations can vary significantly depending on the method used. For primers $\leq$ 20 nt, 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.
10. **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 of BAC DNA) use extension time 15 seconds per 1 kb. For high complexity genomic DNA 30 seconds per 1 kb is recommended.