Guidelines for PCR Optimization with Phusion High-Fidelity DNA Polymerase

Overview

Protocol

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. Any remaining Phusion DNA Polymerase will degrade the A overhang, thus creating blunt ends again.
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 ($4.4x10^{-7}$) is lower than that in GC Buffer ($9.5X10^{-7}$). Therefore the HF Buffer should be used as the default buffer for high-fidelity amplification. However, the 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.
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 primers 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 of EGTA, the apparent Mg^{2+} optimum may be shifted to higher concentration. 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 in increasing dNTP concentrations. For optimal always use 200 µm of each dNTP.
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. If cDNA synthesis reaction mixture is used as a source of template, the volume used should not exceed 10% of the final PCR reaction volume.
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 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.

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 - 15 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. For some cDNA templates, the extension time can be increased up to 40 seconds per 1 kb to obtain optimal results.