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

Overview

Protocol

1. **Phusion Flash high-fidelity PCR Master Mix:**
   Phusion Flash PCR Master Mix contains all necessary components for PCR except for template DNA and primers. The composition of the Phusion Flash PCR Master Mix is designed to give optimal results.

   When cloning fragments amplified with Phusion Flash II 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 DyNAzyme II DNA Polymerase (F-501), for example. However, before adding the overhangs it is very important to remove all Phusion Flash II DNA Polymerase by purifying the PCR product carefully. Any remaining Phusion Flash II DNA Polymerase will degrade the A overhangs, creating blunt ends again.
2. **Primers:**
   The recommendation for final primer concentration is 0.5 µm. If required, the primer concentration may be optimized between 0.2-1.0 µm.

   To shorten the time required for a PCR protocol, it is advisable to design primers suitable for a two-step PCR protocol, if possible. In a two-step PCR protocol, primer annealing and extension occur at 72°C and separate annealing step can be omitted. However, Phusion Flash PCR Master Mix can also be used when performing a PCR protocol with a separate annealing step.

   When designing primers, the Tm values should be calculated with the nearest-neighbor method because results from primer Tm calculations can vary significantly depending on the method used.
3. **Template:**
   General guidelines for low complexity DNA (e.g. plasmid, lambda of BAC DNA) are: 1 pg – 10 ng / 20 µl reaction volume with low complexity, or 2.5 pg-25 ng per 50 µl reaction volume. For high complexity genomic DNA, the amount of DNA template should be 10-100 ng per 20 µl reaction volume, or 25-250 ng per 50 µl reaction volume. 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.
4. **Initial Denaturation:**
   Denaturation should be done at 98°C. Due to the high thermostability of Phusion Flash II DNA Polymerase, even higher denaturation temperatures may be used. Initial denaturation of 10 seconds is recommended for all templates when using Phusion Flash Master Mix.
5. **Denaturation:**
   A very short denaturation step is recommended. For this step, it is usually sufficient that the reaction mixture reaches the required 98°C. If the PCR instrument used does not accept 0 seconds as a value, then a 1-second value can be programmed.
6. **Primer annealing:**
   For minimizing the total PCR cycling time, a two-step PCR protocol is recommended. It is applicable with primers whose Tm values are 69°C or 72°C (primers > 20 nt or ≤ 20 nt, respectively).

   Basic rules, for primers are: For primers > 20 nt, anneal for 5 seconds at Tm +3°C of the lower Tm primer. For primers ≤ 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).

7. **Extension:**
   The extension should be performed at 72°C. Extension time of 15 seconds per 1 kb is suitable for most templates. Some amplicons can be successfully amplified by using even shorter extension times, e.g. 1-5 seconds per 1 kb.