Source: An E. coli strain that carries the Phusion DNA Polymerase gene.

Applications:
- High-specificity PCR
- Cloning
- Long or Difficult Amplification
- High-throughput PCR

Reagents Supplied with Enzyme:
- 5X Phusion HF Buffer
- 5X Phusion GC Buffer
- 100% DMSO
- 50 mM MgCl₂ Solution

Reaction Conditions: 1X Phusion HF or GC Buffer, DNA template, 0.5 μM primers, 200 μM dNTPs (not included), 3% DMSO (optional) and 1 unit of Phusion Hot Start Flex DNA Polymerase in a total reaction volume of 50 μl.

Unit Definition: One unit is defined as the amount of enzyme that will incorporate 10 nmol of dNTP into acid insoluble material in 30 minutes at 74°C.

Unit Assay Conditions: 25 mM TAPS-HCl (pH 9.3 at 25°), 50 mM KCl, 2 mM MgCl₂, 1 mM β-mercaptoethanol, 200 μM dNTPs including [3H]-dTTP and 400 μg/ml activated Calf Thymus DNA.

Heat Inactivation: No

Quality Control Assays

7.5 kb Genomic DNA PCR: 30 cycles of PCR amplification in a 50 μl reaction containing 50 ng genomic DNA with 1.0 unit of Phusion Hot Start Flex DNA Polymerase in the presence of 200 μM dNTPs and 1.0 μM primers in Phusion HF Buffer results in the expected 7.5 kb product.

20 kb Lambda DNA PCR: 22 cycles of PCR amplification in a 50 μl reaction containing 10 ng Lambda DNA with 1.0 unit of Phusion Hot Start Flex DNA Polymerase in the presence of 200 μM dNTPs and 1.0 μM primers in Phusion HF Buffer results in the expected 20 kb product.

Endonuclease Activity: Incubation of a 50 μl reaction in NEBuffer 2 containing a minimum of 10 units of Phusion DNA Polymerase with 200 μM dNTPs and 1 μg of supercoiled φX174 DNA for 4 hours at either 37°C or 72°C results in < 10% conversion to the nicked form as determined by agarose gel electrophoresis.

PCR
The following guidelines are provided to ensure successful PCR using Phusion Hot Start Flex DNA Polymerase. These guidelines cover routine PCR reactions. Amplification of templates with high GC content, high secondary structure, low template concentrations or long amplions may require further optimization.

Reaction Setup: Phusion Hot Start Flex DNA Polymerase is inhibited at room temperature, allowing flexible reaction set up (RT or ice). All components should be mixed and spun down prior to use. Phusion Hot Start Flex DNA Polymerase may be diluted in 1X HF or GC Buffer just prior to use in order to reduce pipetting errors. Please note that protocols with Phusion Hot Start Flex DNA Polymerase may differ from protocols with other standard polymerases. As such, conditions recommended below should be used for optimal performance.

Thermocycling Conditions for a Routine PCR:

<table>
<thead>
<tr>
<th>STEP</th>
<th>TEMP</th>
<th>TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>98°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>98°C</td>
<td>5–10 seconds</td>
<td></td>
</tr>
<tr>
<td>25–35 Cycles</td>
<td>45–72°C*</td>
<td>10–30 seconds</td>
</tr>
<tr>
<td>72°C</td>
<td>15–30 seconds/kb</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>5–10 minutes</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td></td>
</tr>
</tbody>
</table>

*To determine the optimal annealing temperature for a given set of primers, use of the NEB Tm Calculator is highly recommended (www.neb.com/TmCalculator).

General Guidelines:
1. Template:
   - Use of high quality, purified DNA templates greatly enhances the success of PCR reactions. Recommended amounts of DNA template for a 50 μl reaction are as follows:
     - Genomic DNA: 50 ng–250 ng
     - Plasmid or Viral DNA: 1 pg–10 ng
   - If the template DNA is obtained from a cDNA synthesis reaction, the volume added should be less than 10% of the total reaction volume.

2. Primers:
   - Oligonucleotide primers are generally 20–40 nucleotides in length and ideally have a GC content of 40–60%. Computer programs such as Primer3 (http://frodo.wi.mit.edu/primer3) can be used to design or analyze primers.
   - The final concentration of each primer in a PCR experiment using Phusion Hot Start Flex DNA Polymerase may be 0.2–1 μM, while 0.5 μM is strongly recommended.

3. Mg++ and additives:
   - Mg++ is critical to achieve optimal activity with Phusion Hot Start Flex DNA Polymerase. The final Mg++ concentration in 1X Phusion HF and GC Buffer is 1.5 mM. Excessive Mg++ can prevent full denaturation of DNA as well as cause non-specific binding of primers. The optimal Mg++ concentration is affected by dNTP concentration, the template being used and supplements that are added to the reaction. If chelators (e.g. EDTA) are present, it may be necessary to increase the Mg++ concentration. Mg++ can be optimized in 0.5 μM increments using the MgCl₂ provided.

(see other side)
Amplification of difficult targets, such as those with GC-rich sequences or secondary structure, may be improved by the presence of additives such as DMSO (included). A final concentration of 3% DMSO is recommended, although concentration can be optimized in 2% increments. It is important to note that if a high concentration of DMSO is used, the annealing temperature must be lowered as DMSO decreases the primer $T_m$ (2).

Phusion Hot Start Flex DNA Polymerase is also compatible with other additives such as formamide or glycerol.

4. Deoxynucleotides: The final concentration of dNTPs is typically 1.0 mM each dNTP (NEB #E0553) for use with Phusion Hot Start Flex DNA Polymerase. For other PCR polymerases, the recommended concentration of 3% DMSO is recommended, or 0.5–2 units/50 µl reaction depending on amplicon length and difficulty. Do not exceed 2 units/50 µl reaction, especially if there is a 10–20 fold excess of DMSO (included).

5. Phusion Hot Start Flex DNA Polymerase Concentration: We generally recommend using Phusion Hot Start Flex DNA Polymerase at a concentration of 20 units/ml (1.0 unit/50 µl reaction). However, the optimal concentration of Phusion Hot Start Flex DNA Polymerase may vary from 10–40 units/ml (0.5–2 units/50 µl reaction) depending on amplicon length and difficulty. Do not exceed 2 units/50 µl reaction, especially if there is a 10–20 fold excess of DMSO (included).

6. Buffers: 5X Phusion HF Buffer and 5X Phusion GC Buffer are provided with the enzyme. HF buffer is recommended as the default buffer for high-fidelity amplification. For difficult templates, such as GC-rich templates or those with secondary structure, GC buffer can improve reaction performance. Detergent-free reaction buffers are also available for applications that do not tolerate detergents (e.g. microarray, DHPLC).

7. Denaturation: Phusion Hot Start Flex DNA Polymerase does not require a separate activation step. An initial denaturation of 30 seconds at 98°C is sufficient for most amplicons from pure DNA templates. Longer denaturation times can be used (up to 3 minutes) for templates that require it.

During thermocycling, the denaturation step should be kept to a minimum. Typically, a 5–10 second denaturation at 98°C is recommended for most templates.

8. Annealing: Optimal annealing temperatures for Phusion Hot Start Flex DNA Polymerase tend to be higher than for other PCR polymerases. The $T_m$ calculator (www.neb.com/TmCalculator) should be used to determine the annealing temperature when using Phusion products. Typically, for primers > 20 nt, use a 10–30 s annealing step at 3°C above the $T_m$ of the lower primer. For primers < 20 nt, use an annealing temperature equivalent to the $T_m$ of the lower primer should be used. A temperature gradient can also be used to optimize the annealing temperature for each primer pair. For two-step cycling, the gradient can be set as high as the extension temperature.

For high $T_m$ primer pairs, two-step cycling without a separate annealing step can be used.

9. Extension: The recommended extension temperature is 72°C. Extension times are dependent on amplicon length and complexity. Generally, an extension time of 15 seconds per kb can be used. For complex amplicons, such as genomic DNA, an extension time of 30 seconds per kb is recommended. Extension time can be increased to 40 seconds per kb for cDNA templates, if necessary.


12. PCR product: The PCR products generated using Phusion Hot Start Flex DNA Polymerase have blunt ends. If cloning is the next step, then blunt-end cloning is recommended. If TA-cloning is preferred, the DNA should be purified prior to A-addition, as Phusion Hot Start Flex DNA Polymerase will degrade any overhangs generated.

Addition of an untemplated -dA can be done with Taq DNA Polymerase (NEB #M0267) or Klenow exo- (NEB #M0212).

References: