Phusion PCR Protocol

Introduction

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Materials

› Reagents

- › Phusion DNA Polymerase
- › 5X Phusion HF Buffer
- › 10mM dNTPs
- › DMSO

› Nucelic Acids:

- › Template DNA <250ng
- › 10μM Primer Solutions, (Total Rxn Volume)/20 mL needed per use

› Equitment

- › Ice Bath / Ice Block
- › Vortexer
- › Thermocycler
- › Mini Centrifuge

› Time:

- › Dependant on Template DNA and Primers, Usually 6-10 Hours

Procedure

Protocol

1. Assemble reaction according to table listed below

   CRITICAL We recommend assembling all reaction components on ice and quickly transferring the reactions to a thermocycler preheated to the denaturation temperature (98°C).
   Note: All components should be mixed and centrifuged prior to use.
   CRITICAL It is important to add Phusion DNA Polymerase last in order to prevent any primer degradation caused by the 3′→ 5′ exonuclease activity.
   Note: Phusion DNA Polymerase may be diluted in 1X HF or GC Buffer just prior to use in order to reduce pipetting errors.
   Note: Protocols with Phusion DNA Polymerase may differ from protocols with other standard polymerases. As such, conditions recommended below should be used for optimal performance.

Reaction Setup

	A	B	C	D	E	F	G	H
1	Fixed				Variable Input	Values Used:		
2	Reaction Size/ Component	20 µl Reaction	50 µl Reaction	Final Concentration	50			
3	Nuclease-free water (µl)	to 20 µl	to 50 µl		32	#VALUE!		
4	5X Phusion HF or GC Buffer (µl)	4 µl	10 µl	1X	10			
5	10 mM dNTPs (µl)	0.4 µl	1 µl	200 µM	1			
6	10 µM Forward Primer (µl)	1 µl	2.5 µl	0.5 µM	2.5			
7	10 µM Reverse Primer (µl)	1 µl	2.5 µl	0.5 µM	2.5			
8	Template DNA (ng)	variable	variable	< 250 ng	<250 ng			
9	DMSO (optional) (µl)	(0.6 µl)	(1.5 µl)	3%	1.5			
10	Phusion DNA Polymerase (µl)	0.2 µl	0.5 µl	1.0 units/50 µl PCR	0.5			

Note: Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Overlay the sample with mineral oil if using a PCR machine without a heated lid.

2. Transfer PCR tubes from ice to a PCR machine with the block preheated to 98°C and begin thermocycling:

3. Set up thermocycler according to the following table:

Thermocycling Conditions

	A	B	C	D	E	F	G	H
1	STEP	TEMP	TIME					
2	Initial Denaturation	98°C	30 seconds					
3	25-35 Cycles	98°C	5-10 seconds					
4		45-72°C	10-30 seconds					
5		72°C	15-30 seconds per kb					
6	Final Extension	72°C	5-10 minutes					
7	Hold	4-10°C						
8	Min DNA Length (kb):	6	**Note: Min should be no less than half the max if possible**					
9	Max DNA Length (kb):	10						
10	Annealing Temp (°C):	60						
11	Step	Temp (°C)	Min Time (Sec)	Max Time (Sec)	Time Used:			
12	Initial Denaturation	98	30	30				
13	25-35 Cycles	98	5	10				
14		60	10	30				
15		72	150	180				
16	Final Extension	72°C	300	600				
17	Hold	4-10°C						
18	Total Time (hr):		8.3	14.2				

4. **General Guidelines:**

   Template:
   Use of high quality, purified DNA templates greatly enhances the success of PCR. Recommended amounts of DNA template for a 50 µl reaction are as follows:

Table1

	A	B
1	DNA	Amount
2	genomic	50 ng–250 ng
3	plasmid or viral	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.

5. Primers:

Oligonucleotide primers are generally 20–40 nucleotides in length and ideally have a GC content of 40–60%. Computer programs such as Primer3 can be used to design or analyze primers. The final concentration of each primer in a reaction using Phusion DNA Polymerase may be 0.2–1 µM, while 0.5 µM is recommended.

6. Mg^{++} and additives:

   Mg^{++} is critical to achieve optimal activity with Phusion 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. This can also be affected by the presence of chelators (e.g. EDTA). Mg^{++} can be optimized in 0.5 mM increments using the $MgCl_2$ provided.
   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.
   CRITICAL It is important to note that if a high concentration of DMSO is used, the annealing temperature must be lowered as it decreases the primer T_m (2). Phusion DNA polymerase is also compatible with other additives such as formamide or glycerol.

7. Deoxynucleotides:

   The final concentration of dNTPs is typically 200 µM of each deoxynucleotide. Phusion cannot incorporate dUTP.

8. Phusion DNA Polymerase Concentration:

   We generally recommend using Phusion DNA Polymerase at a concentration of 20 units/ml (1.0 units/50 µl reaction). However, the optimal concentration of Phusion 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 for amplicons longer than 5 kb.

9. 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. GC buffer should be used in experiments where HF buffer does not work. Detergent-free reaction buffers are also available for applications that do not tolerate detergents (e.g. microarray, DHPLC).

10. Denaturation:

    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.

11. Annealing:

    Annealing temperatures required for use with Phusion tend to be higher than with other PCR polymerases. **The NEB T_m calculator should be used to determine the annealing temperature when using Phusion.** Typically, primers greater than 20 nucleotides in length anneal for 10–30 seconds at 3°C above the T_m of the lower T_m primer. If the primer length is less than 20 nucleotides, 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.

12. 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.

13. Cycle number:

    Generally, 25–35 cycles yields sufficient product.

14. 2-step PCR:

    When primers with annealing temperatures ≥ 72°C are used, a 2-step thermocycling protocol is recommended.

Thermocycling conditions for a routine 2-step PCR

	A	B	C
1	STEP	TEMP	TIME
2	Initial Denaturation	98°C	30 seconds
3	25-35 Cycles	98°C 72°C	5-10 seconds 15-30 seconds per kb
4	Final Extension	72°C	5-10 minutes
5	Hold	4-10°C	

15. PCR product:

    The PCR products generated using Phusion DNA Polymerase have blunt ends; if cloning is the next step, then blunt-end cloning is recommended. If TA-cloning is preferred, then DNA should be purified prior to A-addition, as Phusion 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).