FINNZYMES

Phusion™

Site-Directed Mutagenesis Kit

Product code: F-541

Stable for one year from the packaging date. Store at -20°C.

1. Introduction

Site-directed mutagenesis is widely used in the study of gene and protein functions. With the Phusion™ Site-Directed Mutagenesis Kit, point mutations, insertions and deletions can be introduced in any type of plasmid DNA.

This kit uses the highly processive Phusion™ Hot Start High-Fidelity DNA Polymerase for exponential PCR amplification of dsDNA plasmid to be mutated. The mutagenesis protocol comprises only three steps:

1. PCR amplificaton of target plasmid with two phosphorylated primers. The primers, one or both with desired mutation(s), are designed so that they anneal back to back to the plasmid (for schematic presentation, see Fig. 1).
2. Circularization of mutated PCR products by ligation with Quick T4 DNA Ligase (New England Biolabs).
3. Transformation to *E. coli*.

For the target plasmid, there are no requirements such as special vectors, restriction sites or methylation status. Because minute amounts of template DNA are exponentially amplified in this method, the fraction of non-mutated template is minimal and thus there is no need to destroy it in a separate step.

Phusion Hot Start DNA Polymerase ensures high fidelity for the exponential amplification, thus reducing unwanted secondary mutations and enabling amplification of large plasmids up to 10 kb. Phusion Hot Start DNA Polymerase combines the DNA polymerase and a reversibly bound, specific Affibody® protein[1,2], which inhibits the DNA polymerase activity at ambient temperatures. The hot start modification in the polymerase prevents the amplification of non-specific products and unwanted degradation of primers prior to the first cycle of PCR.

The Phusion Site-Directed Mutagenesis Kit includes Phusion Hot Start DNA Polymerase, 5x Phusion HF Buffer, dNTPs, Quick T4 DNA Ligase, 2x Quick Ligation™ Buffer and Control Plasmid with Control Primer Mix. The Quick T4 DNA Ligase included in the kit enables direct ligation without extra purification steps before or after the ligation. The kit is compatible with all competent *E. coli* cells, giving to the user an option to use any cells available in the laboratory.

2. Kit Components

Component	Concentration	F-541
Phusion™ Hot Start DNA Polymerase	2 U/µl	10 µl
5x Phusion™ HF Buffer		1.5 ml
dNTP Mix	10 mM/each	20 µl
Control Plasmid (in TE buffer)	10 pg/µl	10 µl
Control Primer Mix containing the following 5′ phosphorylated primers: Primer #1 5′ GTC GAC TCT AGA GGA TCC CCG GGT 3′ Primer #2 5′ CTG CAG GCA TGT **A**AG CTT GGC GTA 3′	25 µM each	10 µl
Quick T4 DNA Ligase (NEB)		10 µl
2x Quick Ligation™ Buffer (NEB)		100 µl

The Phusion Site-Directed Mutagenesis Kit contains reagents for 20 mutagenesis reactions including 10 control reactions.

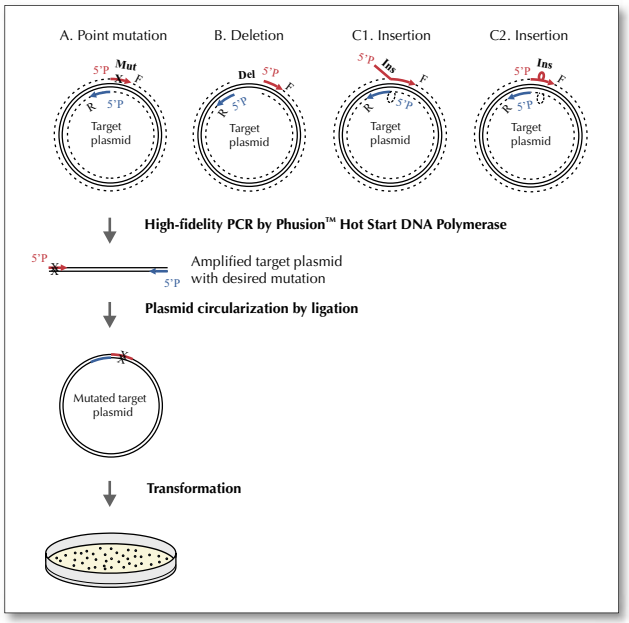

Figure 1. Flow chart of the Phusion Site-Directed Mutagenesis Kit protocol. Any target plasmid can be used as a starting material for creating point mutations, deletions or insertions. Phosphorylated primers are designed to introduce the desired mutation (A, B, C1 or C2) so that they anneal back to back to the plasmid. In the PCR reaction, Phusion DNA Polymerase extends the primers and amplifies the plasmid with the mutation. The mutated plasmid is then circularized by quick ligation and transformed into bacteria.

3. Storage and Shipping

Phusion Site-Directed Mutagenesis Kit is shipped in gel ice. Upon arrival, store the components at -20°C. The components are stable for one year from the date of packaging when stored properly.

4. Material Needed But Not Supplied With the Kit

- Target plasmid DNA: for instructions, see section 5.2.
- 5′-Phosphorylated mutagenic primers: for instructions, see section 5.1.
- Competent cells: for instructions, see section 6.3.
- SOC medium and LB agar plates with antibiotics: for media recipes, see Appendix I.
- 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)
- Isopropyl-β-D-thiogalactopyranoside (IPTG)

5. General Considerations

5.1 Primer design

The primer design depends on the type of the desired mutation. Both primers need not to be mutagenic. However, if two separate mutations are desired, two mutagenic primers are needed (see Fig. 1 for a schematic presentation).

Phosphorylation

The primers **must be phosphorylated** at the 5′ end. It is recommended to use commercially phosphorylated primers. However, a protocol for primer phosphorylation is included in Appendix II.

Primer quality

For this application it is crucial that only full-length (n) molecules of the primers are present in the reaction mixture. The presence of shorter primers (n-1, n-2 etc.), which lack nucleotides at the 5′ end, will lead to shorter PCR products and thus to missing nucleotides at the ligation site. Therefore it is recommended only to use primers purified with reverse phase high performance liquid chromatography (RP-HPLC) or with polyacrylamide gel electrophoresis (PAGE). For primers longer than 40 nucleotides, purification with PAGE is preferable.

Calculating the Tm for determining the annealing temperature

Phusion Hot Start DNA Polymerase has the ability to stabilize primer-template hybridization. The Tm's should be calculated with the nearest-neighbor method[3], because results from primer Tm calculations can vary significantly depending on the method used. Instructions for Tm calculation and a link to a calculator using the nearest-neighbor method can be found on Finnzymes' website (www.finnzymes.com). For primers longer than 20 nucleotides, use an annealing temperature 3°C higher than the Tm of the lower Tm primer given by the calculator. If the primer length is 20 nucleotides or less, use an annealing temperature equal to the Tm of the lower Tm primer given by the calculator. It is recommended to design primers so that the annealing temperature falls between 65°C and 72°C. In case the annealing temperature approaches 72°C, a two-step cycling protocol without a separate annealing step can be used when running the PCR (see Table 2A).

Point mutations

Point mutations are created by designing a mismatch in the mutagenic primer. There can be more than one mismatch in the mutagenic primer, either separated by correctly matched nucleotides or present in consecutive nucleotides. For generating point mutations, the length of the correctly matched sequence in the mutagenic primers should be in average 24-30 nucleotides. The desired mutation should be in the middle of the primer with 10-15 perfectly matched nucleotides on each side. See previous chapter for instructions on determining the annealing temperature.

Deletions

Deletions are created by designing primers that border the deleted area on both sides (see Fig. 1,B for a schematic presentation). To generate a deletion, the primers should be perfectly matched on their entire length, which should be 24-30 nucleotides. See instructions above for determining the annealing temperature.

Insertions

For generating insertions, primers can be designed in two alternative ways.

1. For longer insertions, a stretch of mismatched nucleotides is designed in the 5′ end(s) of one or both primers (see Fig. 1,C1 for a schematic presentation). If mismatched stretches are designed in the 5′ ends of both primers, they form one entire insertion when the ends of the PCR product are ligated. The Tm's should be calculated for the perfectly matched portion of the primers. See instructions above for determining the annealing temperature.
2. For short insertions, a stretch of mismatched nucleotides is designed in the middle of the primer (see Fig. 1,C2 for a schematic presentation). The length of the correctly matched sequence in the mutagenic primers should be in average 24-30 nucleotides. The desired insertion should be in the middle of the primer with 10-15 perfectly matched nucleotides on each side. See instructions above for determining the annealing temperature.

5.2 Plasmid template

The target plasmid DNA may be isolated from any source and purified using standard methods such as alkaline lysis or commercial DNA purification kits. There are no requirements for special vectors, restriction sites or methylation status. Plasmids up to 10 kb in length can be successfully mutagenized using the kit.

The amount of plasmid template in the mutagenesis reaction should be titrated as down as possible to prevent background transformants after ligation and transformation, but to still ensure PCR amplification. Generally, by using picogram amounts of plasmid template in a 50 µl PCR reaction, background transformants can be avoided. The recommended starting amount is 10 pg of plasmid template in a 50 µl PCR reaction.

5.3 Control Plasmid and Control Primer Mix

Control Plasmid and Control Primer Mix using color conversion are included in the Phusion Site-Directed Mutagenesis Kit. The reagents are sufficient for 10 mutagenesis control reactions, which can be performed along the actual mutagenesis reactions, or for troubleshooting reasons. The Control Plasmid, derived from pUC19 (2686 bp), contains a stop codon (TAA) at position 8 in the gene coding for *lacZ*α and thereby forms white colonies on LB-ampicillin agar-plates containing X-Gal and IPTG. The Control Primer Mix reverts the internal *lacZ*α stop codon mutation into a functional leucine codon and also introduces a *Hin*d III site. Thus a successful mutagenesis control reaction forms blue colonies on LB-ampicillin agar-plates containing X-Gal and IPTG. The efficiency of the mutagenesis control reaction is estimated by the number of blue (mutated) colonies divided by the total number of blue and white (unmutated) colonies. The control reaction should give an efficiency rate of over 90%.

5.4 Mutagenesis efficiency

The Phusion Site-Directed Mutagenesis Kit yields an average efficiency rate of over 80%. This high frequency means that mutants can be screened by direct sequencing. An efficiency rate of over 90% can be expected for the control reaction.

Phusion Hot Start DNA Polymerase ensures high fidelity for the exponential amplification, thus reducing unwanted secondary mutations. Due to the high fidelity, even large plasmids can be reliably amplified. The fidelity value (4.4×10^{-7}) of Phusion DNA Polymerase is determined using a *lacI*-based method[4]. Calculated from the fidelity value, the estimated percentage of the PCR products having an unwanted secondary mutation after 25 PCR cycles is shown below.

Plasmid size kb	% products having a polymerase-induced error
2.5	2.75
5	5.5
7.5	8.25
10	11

6. Mutagenesis Protocol

6.1 PCR

Instructions for the PCR amplification of both the mutagenesis and the control reaction are given below. Carefully mix and centrifuge all tubes before opening to improve recovery. Due to the hot start modification present in Phusion Hot Start DNA Polymerase, it is not necessary to perform the PCR setup on ice. Prepare a master mix for the desired number of samples to be mutagenized. The DNA polymerase should be pipetted carefully and gently, because the high glycerol content (50%) in the storage buffer may otherwise lead to pipetting errors.

Table 1a. Pipetting instructions for the mutagenesis reaction (in order).

Component	Volume / 50 µl reaction	Final conc.
H_2O	add to 50 µl	
5x Phusion HF Buffer	10 µl	1x
10 mM dNTPs	1 µl	200 µM each
primer A	x µl	0.5 µM
primer B	x µl	0.5 µM
template DNA	x µl	See 5.2
Phusion Hot Start DNA Polymerase (2 U/µl)	0.5 µl	0.02 U/µl

Table 1b. Pipetting instructions for the control reaction (in order).

Component	Volume / 50 µl reaction	Final conc.
H_2O	36.5 µl	
5x Phusion HF Buffer	10 µl	1x
10 mM dNTPs	1 µl	200 µM each
Control Primer Mix	1 µl	0.5 µM each
Control Plasmid	1 µl	10 pg
Phusion Hot Start DNA Polymerase (2 U/µl)	0.5 µl	0.02U/µl

Due to the unique nature of Phusion Hot Start DNA Polymerase, optimal reaction conditions may differ from standard enzyme protocols. Phusion Hot Start DNA Polymerase tends to work better at elevated denaturation and annealing temperatures due to higher salt concentrations in its buffer. We recommend 25 cycles for optimal efficiency. Please pay special attention to the conditions listed in Tables 2A and 2B when running your reactions.

Table 2a. Cycling instructions for the mutagenesis reaction.

Cycle step	Temp.	Time	Number of cycles
Initial denaturation	98°C	30 s	1
Denaturation Annealing Extension	98°C 65-72°C* 72°C	5-10 s 10-30 s 15-30 s /1 kb	25
Final extension	72°C 4°C	5-10 min hold	1

* See section 5.1 for determining the annealing temperature

Table 2b. Cycling instructions for the control reaction.

Cycle step	Temp.	Time	Number of cycles
Initial denaturation	98°C	30 s	1
Denaturation Extension	98°C 72°C	10 s 45 s	25
Final extension	72°C 4°C	5min hold	1

The PCR product can be stored at -20°C.

Gel electrophoresis (optional)
It is recommended to take a 5 µl sample from the PCR reaction for agarose gel electrophoresis to verify the success of the PCR amplification. The amount of the PCR product can be evaluated from the gel by comparing to known amount of standards.

6.2 Ligation

The PCR product is circularized with Quick T4 DNA Ligase in a 5 minute reaction.

1. Take 25 ng of PCR product from mutagenesis reaction, which usually equals to 1-5 µl. Adjust volume to 5 µl with H_2O.*
2. Add 5 µl of 2x Quick Ligation Buffer and mix.
3. Add 0.5 µl of Quick T4 DNA Ligase and mix thoroughly.
4. Centrifuge briefly and incubate at room temperature (25°C) for 5 minutes.**
5. Chill on ice, then transform or store at -20°C.
6. Do not heat inactivate. Heat inactivation dramatically reduces transformation efficiency.

***DNA amount**
For optimum ligation, the volume of DNA should be 5 µl before adding 2x Quick Ligation Buffer. For DNA volumes greater than 5 µl, increase the volume of 2x Quick Ligation Buffer such that it remains 50% of the reaction and correspondingly increase the volume of ligase. The amount of the PCR product from the mutagenesis reaction should be between 1-10 ng/µl for efficient ligation. If you are unsure of your DNA concentrations, perform multiple ligations with varying DNA amounts.

****Reaction time**
Most ligations performed using the Quick Ligation Kit reach an end point at 5 minutes or less at 25°C. Incubation beyond this time provides no additional benefit. In fact, transformation efficiency starts to decrease after 2 hours and is reduced by up to 75% if the reaction is allowed to go overnight at 25°C.

6.3 Transformation

Any standard *E. coli* strain that is suitable for DNA cloning can be used as a transformation host. Both electrocompetent and chemically competent *E. coli* cells may be used. Follow standard transformation protocols or proceed as instructed by the manufacturer of your competent cells. Competent cells can vary by several logs in their competence. Ligation efficiency directly correlates to the competence of the cells used for transformation.

Chemical transformation: Transform 1-10 µl of the reaction mixture per 50-100 µl competent *E. coli* cells.

Electroporation: Electroporation can increase transformation efficiency by several logs. Before using the products of a Quick Ligation reaction for electrotransformation, it is necessary to reduce the PEG concentration. We recommend spin column purification.

Incubate the plates overnight at 37°C. Alternatively, incubate at 30°C for 16 hours or 25°C for 24 hours.

6.4 Analysis of transformants

The Phusion Site-Directed Mutagenesis Kit yields an average efficiency rate of over 80%. This high frequency means that mutants can be screened by direct sequencing. Screening 3 colonies by sequencing will give a high probability of finding the desired mutation. The most frequent reason for incorrect clones is incomplete primers, which result in lacking nucleotides at the ligation site. However, these clones generally do contain the desired mutation. For instructions on primer synthesis, see section 5.1.

For the control reaction, the efficiency of mutagenesis is estimated by the number of blue (mutated) colonies divided by the total number of blue and white (unmutated) colonies. The control reaction should give an efficiency rate of over 90% when plated on LB-ampicillin agar-plates containing X-Gal and IPTG.

7. Product Qualification

The Phusion Site-Directed Mutagenesis Kit has been tested using the Control Plasmid and Control Primer Mix provided in the kit, described in section 5.3. The control reaction gave an efficiency rate of over 90%.

8. Troubleshooting

No PCR product at all or low yield

- Repeat the PCR and make sure that there are no pipetting errors.
- Plasmid template concentration may be too low. Use more template (see section 5.2).
- Lengthen extension time.
- Lower annealing temperature.
- Check purity and concentration of the primers.
- If primers were phosphorylated with T4 polynucleotide kinase (Appendix II), purify after phosphorylation.
- Check primer design (see section 5.1).

Missing nucleotides at the ligation site

- Inadequate primer quality. Make sure that primers are complete and purified as described in section 5.1.

The desired mutation is absent from the transformants

- Make sure that the primers contain the desired mutation.
- An excessive amount of target plasmid in the PCR results in background transformants. Lower template amount (see section 5.2).
- Some DNA structures, including inverted and tandem repeats, are selected against by *E. coli*. Some recombinant proteins are not well tolerated by *E. coli* and can result selection pressure against mutation.

Few or no colonies

- Ensure the transformation competence of the *E. coli* strain.
- Low yield of the PCR reaction. Increase the amount of the PCR product used for ligation (see section 6.2).
- Increase the amount of ligation mix used for transformation (see section 6.3).
- Make sure that the primers are phosphorylated (see section 5.1).
- Excessive incubation times and heat inactivation reduce the ligation efficiency. Follow the guidelines in section 6.2.
- Make sure that the transformation plates are properly prepared and contain the appropriate concentration of antibiotics and selection reagents.
- Some DNA structures, including inverted and tandem repeats, are selected against by *E. coli*. Some recombinant proteins are not well tolerated by *E. coli* and can result in poor transformation or small colonies.

9. References

1. Nord *et al.* (1997) *Nature Biotechnol* 15: 772-777.
2. Wikman *et al.* (2004) *Protein Eng Des Sel* 17: 455-462.
3. Breslauer *et al.* (1986) *Proc Natl Acad Sci U S A* 83: 3746-3750.
4. Frey & Suppmann (1995) *Biochemica* 2: 34-35.

Appendix I: Media Recipes

LB agar with antibiotics (per liter)
Tryptone 10 g
Yeast Extract 5 g
NaCl 10 g
Agar 15 g
Adjust pH to 7.0 with NaOH
Autoclave
Cool to 55°C and add the appropriate antibiotic:
e.g. ampicillin (final concentration 100 µg/ml) before pouring the plates.
For plates with blue-white color screening, add 80 µg/ml X-Gal
and 1 mM IPTG.

SOC medium (per liter)
Tryptone 20 g
Yeast Extract 5 g
NaCl 0.5 g
KCl 0.186 g
Adjust pH to 7.0 with NaOH
Autoclave
Before use add sterile solutions:
1 M $MgCl_2$ 10 ml
1 M $MgSO_4$ 10 ml
1 M Glucose 20 ml

Appendix II: 5´-Phosphorylation of Oligonucleotides

1. Add the following components to a microcentrifuge tube.
   300 pmol oligonucleotide
   5 µl 10xT4 Polynucleotide Kinase Reaction Buffer
   1 µl T4 Polynucleotide Kinase 10 U/µl (e.g. New England Biolabs M0201S/L)
   5 µl 10 mM ATP
   H_2O to a final volume of 50µl
2. Incubate the reaction at 37°C for 30 minutes.
3. Inactivate the T4 Polynucleotide Kinase at 65°C for 20 minutes. Alternatively PNK reaction can be phenol-extracted and ethanol precipitated or purified with commercial kit.
4. The reaction products can be stored at -20°C or added directly to the mutagenesis reaction.

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FINNZYMES
TOOLS FOR MOLECULAR BIOLOGY
FINNZYMES OY
Keilaranta 16 A
02150 Espoo, Finland
Tel. +358 9 584 121
Fax +358 9 5841 2200
fz@finnzymes.fi
www.finnzymes.com