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PRODUCT INFORMATION

Thermo Scientific
Phusion Site-Directed Mutagenesis Kit

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#F-541

Lot _ Expiry Date _

Store at -20°C

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For Research Use Only. Not for use in diagnostic procedures.

CERTIFICATE OF ANALYSIS

The Phusion Site-Directed Mutagenesis Kit has been tested using the control plasmid and control primer mix provided in the kit. The control reaction gave an efficiency rate of over 90%.

Quality authorized by:  Jurgita Zilinskiene

Rev.5

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COMPONENTS OF THE KIT

The Thermo Scientific™ Phusion™ Site-Directed Mutagenesis Kit contains reagents for a total of 20 mutagenesis reactions (including control reactions), and control plasmid and primers for 10 reactions.

Component	#F-541 20 rxns
Phusion Hot Start II DNA Polymerase, 2 U/µL	10 µL
5X Phusion HF Buffer	1.5 mL
dNTP Mix 10 mM each	20 µL
FastDigest™ DpnI	20 µL
Control plasmid (in TE buffer) 5 ng/µL	20 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 AAG CTT GGC GTA 3', 25 µM each	10 µL
T4 DNA Ligase	15 µL
5X Rapid Ligation Buffer	200 µL

STORAGE

All components of the kit should be stored at -20°C.

INTRODUCTION

Site-directed mutagenesis is widely used in the study of gene and protein functions. With the Thermo Scientific 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 Thermo Scientific Phusion Hot Start II High-Fidelity DNA Polymerase for exponential PCR amplification of dsDNA plasmid to be mutated. The mutagenesis protocol comprises four 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. Digestion of parental methylated and hemimethylated DNA with FastDigest DpnI.
3. Circularization of mutated PCR products by ligation with T4 DNA Ligase.
4. *E.coli* transformation with the ligation mix.

Phusion Hot Start II 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 II 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 nonspecific products and unwanted degradation of primers prior to the first cycle of PCR. The Phusion Site-Directed Mutagenesis Kit includes Phusion Hot Start II DNA Polymerase, 5X Phusion HF Buffer, dNTPs, FastDigest DpnI, T4 DNA Ligase, 5X Rapid Ligation Buffer and a control plasmid with control primer mix. The T4 DNA Ligase included in the kit enables direct ligation without extra purification steps before or after the ligation. DNA isolated from almost all *E. coli* strains is dam methylated and therefore susceptible to *Dpn* I digestion, giving the user an option to use any cells available in the laboratory.

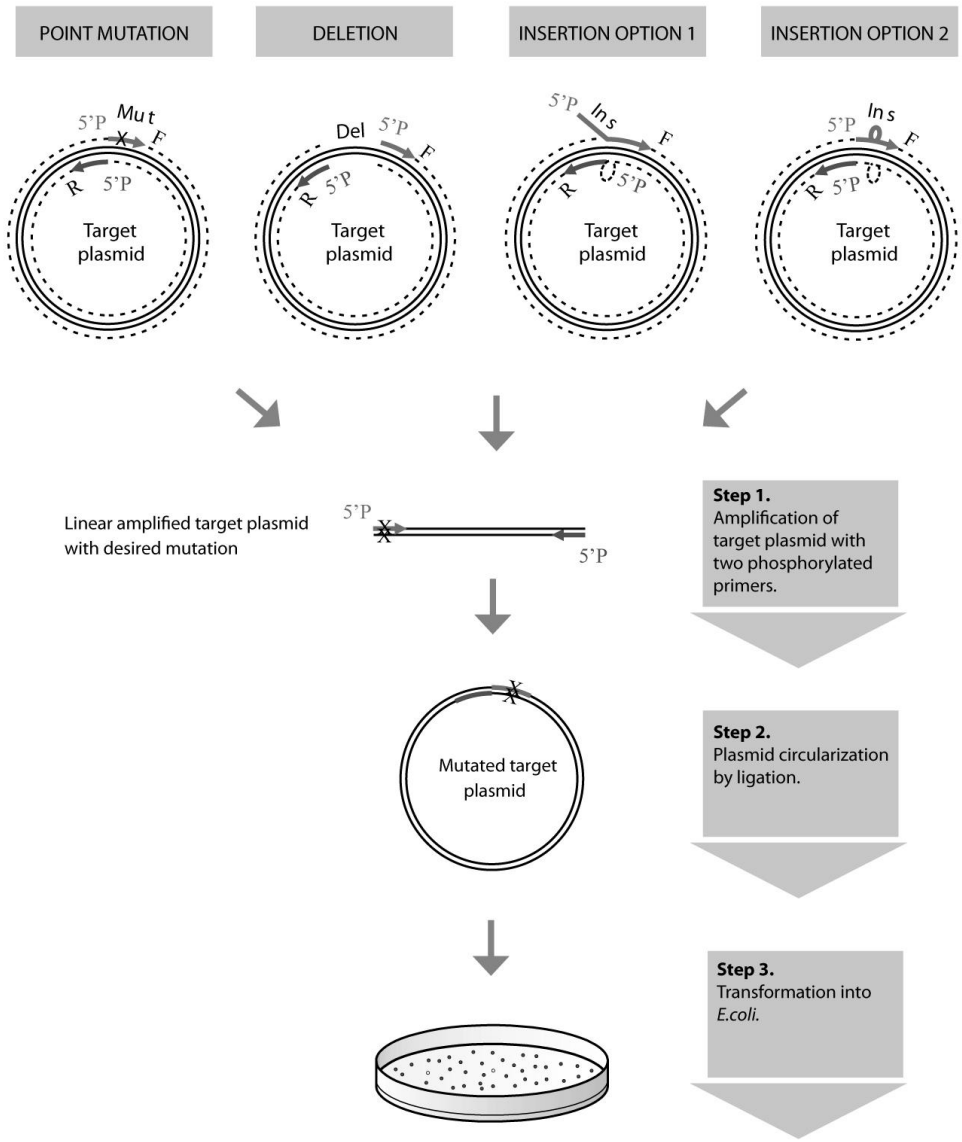

Figure 1. Flow chart of the Phusion Site-Directed Mutagenesis Kit protocol.
F = 5′ phosphorylated forward primer, R = 5′ phosphorylated reverse primer

1.1 Materials needed but not supplied with the kit

- Target plasmid DNA: for instructions, see section 2.3.
- 5'-Phosphorylated mutagenic primers: for instructions, see sections 2.1 & 2.2. Competent cells: for instructions, see section 3.3.
- 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).

2. General considerations

2.1 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 to eliminate the need for a separate phosphorylation step before direct ligation. It is recommended to use commercial 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 subsequently 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 II DNA Polymerase has the ability to stabilize primer-template hybridization. Note that the optimal annealing temperature for Phusion Hot Start II DNA Polymerase may differ significantly from that of *Taq*-based polymerases. Always use the Tm calculator and instructions on website: www.thermofisher.com/tmcalculator to determine the Tm values of primers and optimal annealing temperature. 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 3a). Note that the optimal annealing temperature may differ from the instructions above, if there are mismatches in the middle of the primer sequence. Some experimental optimization may be required.

2.2 Designing mutations

See Fig. 1 for a schematic presentation on how to introduce different types of mutations.

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 last chapter in section 2.1 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, Deletion, 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 last chapter in section 2.1 for instructions on 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, Insertion option 1, 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 last chapter in section 2.1 for instructions on determining the annealing temperature.
2. For short insertions, a stretch of mismatched nucleotides is designed in the middle of the primer (see Fig. 1, Insertion option 2, 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 last chapter in section 2.1 for instructions on determining the annealing temperature.

2.3 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. Plasmids up to 10 kb in length can be successfully mutagenized using this kit.

The optimal range of plasmid DNA amount is 0.1-10 ng of plasmid template in a 50 µL PCR reaction. If necessary, the amount of plasmid template in the mutagenesis reaction can be titrated.

2.4 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 *lacZα* and thereby forms white colonies on LB-ampicillin agarplates containing X-Gal and IPTG. The control primer mix reverts the internal *lacZα* stop codon mutation into a functional leucine codon and also introduces a *Hind* III site. Thus a successful mutagenesis control reaction forms blue colonies on LB-ampicillin agar-plates containing X-Gal and IPTG. Bacterial strains containing the ∆*(lacZ)M15* mutation should be used for blue/white screening of transformants using the control system.

2.5 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 II DNA Polymerase ensures high fidelity for the exponential amplification, thus minimizing 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[3].

Table 1. The estimated precentage of PCR products having an unwanted secondary mutation after 25 PCR cycles.

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

3. Mutagenesis protocol

3.1 PCR

Carefully mix and centrifuge all tubes before opening to ensure homogeneity and improve recovery. Due to the hot start modification in Phusion Hot Start II 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 2a. Pipetting instructions for the mutagenesis reaction: add items in this order.

Component	50 µL rxn	Final conc.
H_2O	add to 50 µL	
5X Phusion HF Buffer	10 µL	1X
10 mM dNTPs	1 µL	200 µM each
Forward primer (see 2.1)	X µL	0.5 µM
Reverse primer (see 2.1)	X µL	0.5 µM
Template DNA	X µL	See 2.3
Phusion Hot Start DNA Polymerase (2 U/µL)*	0.5 µL	0.02 U/µL

Table 2b. Pipetting instructions for the control reaction: add items in this order.

Component	50 µL rxn	Final conc.
H_2O	35 µL	
5X Phusion HF Buffer	10 µL	1X
10 mM dNTPs	1 µL	200 µM each
Control primer mix	1 µL	0.5 µM
Control plasmid	2 µL	10 ng
Phusion Hot Start DNA Polymerase (2 U/µL)*	0.5 µL	0.02 U/µL

*Due to the unique nature of Phusion Hot Start II DNA Polymerase, optimal reaction conditions differ from standard enzyme protocols. Phusion Hot Start II DNA Polymerase tends to work better at elevated denaturation and annealing temperatures due to higher salt concentrations in its buffer (see last chapter in section 2.1 for instructions on determining the annealing

temperature). We recommend 25 cycles for optimal efficiency. Please pay special attention to the conditions given in Tables 3a and 3b when running your reactions. For GC rich templates include 2-8% DMSO as a PCR additive, which aids in the denaturing of template with high GC content.

Table 3a. 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/kb	25
Final extension	72°C 4°C	5–10 min hold	1

* See last chapter in section 2.1 for instructions on determining the annealing temperature.

Table 3b. 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	5 min hold	1

The PCR product can be stored at −20°C if it is not used for ligation immediately.

Gel electrophoresis

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 the sample band intensity with known amount of DNA standard.

3.2 DpnI digestion of parental plasmid DNA

Due to the exponential nature of the PCR reaction the background after ligation and transformation is generally not a problem if low amounts of template are used. However, digestion of Dam-methylated parental plasmid DNA with FastDigest DpnI enzyme allows an efficient mutagenesis without titrations of template DNA.

For DpnI digestion, after PCR add 1 µL of FastDigest DpnI enzyme directly to the mutagenesis reaction and incubate at 37°C for 15 minutes. There is no need to inactivate DpnI prior to ligation.

3.3 Ligation

The PCR product is circularized using T4 DNA Ligase in a 5 minute reaction. Most ligations performed using the T4 DNA Ligase and 5X Rapid Ligation Buffer 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 proceed overnight at 25°C.

Prepare 10 µL of the ligation mix:

1. Take 10-20 ng of PCR product from the mutagenesis reaction after DpnI digestion. This usually equals to 1−5 µL. Do not use more than 5 µL of PCR reaction mix for the 10 µL ligation reaction, because higher volumes of the PCR mix lower the ligation efficiency.

2. Add 2 µL of 5X Rapid Ligation Buffer.
3. Adjust the reaction volume to 9.5 µL with H_2O and mix.
4. Add 0.5 µL of T4 DNA Ligase and mix thoroughly.
5. Centrifuge briefly and incubate at room temperature (25°C) for 5 minutes.
6. Chill on ice, then transform or store at −20°C.
7. Do not heat inactivate. Heat inactivation dramatically reduces transformation efficiency.

3.4 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 1−5 µL of the reaction mixture per 50−100 µL of competent *E. coli* cells. Do not heat inactivate the ligation mix, because this dramatically reduces transformation efficiency.

Electroporation: Electroporation can increase the transformation efficiency by several logs. Before electrotransformation, purify the ligation mix using spin columns (e.g., Thermo Scientific™ GeneJET™ Gel Extraction and DNA Cleanup Micro Kit, #K0831) or chloroform extraction. Use 1 µL of the purified ligation mix per 50 µL of electrocompetent *E. coli* cells.

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

3.5 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 common 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 2.1. 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.

4. Troubleshooting

No 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 or too high. Titrate template amount.
- Increase extension time.
- Decrease annealing temperature.
- Check the purity and concentration of the primers.
- If the primers were phosphorylated with T4 polynucleotide kinase (Appendix II), purify after phosphorylation.
- Check primer design (see sections 2.1 & 2.2).
- Titrate DMSO (2-8 %) in the reaction using GC rich DNA as a template.

Missing nucleotides at the ligation site

- Inadequate primer quality. Make sure that primers are complete and purified as described in section 2.1.

The desired mutation is absent from the transformants

- Make sure that the primers contain the desired mutation.
- Reduce template amount (see section 2.3).
- 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 selection pressure against mutation.

Few or no colonies

- Check 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 3.3).
- Increase the amount of ligation mix used for transformation (see section 3.4).
- Make sure that the primers are phosphorylated (see section 2.1).
- Excessive incubation times and heat inactivation reduce the ligation efficiency. Follow the guidelines in section 3.3.
- 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.

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 using Thermo Scientific T4 PNK, #EK0031.

1. Prepare the following reaction mixture in a microcentrifuge tube:
   250 pmol oligonucleotide,
   5 µL 10X reaction buffer A for T4 Polynucleotide Kinase,
   5 µL 10 mM ATP,
   2 µL T4 Polynucleotide Kinase 10 U/µL,
   H_2O to a final volume of 50 µL.
2. Incubate the reaction at 37°C for 30 minutes.
3. Inactivate the T4 Polynucleotide Kinase at 75°C for 10 minutes. Alternatively, PNK reaction can be phenol-extracted and ethanol precipitated or purified using a spin column kit.
4. The reaction products can be stored at −20°C or added directly to the mutagenesis reaction. Use 5 µL of 5´-phosphorylated oligonucleotide (5 µM) for mutagenesis reaction (*see* section 3.1).

5. References

1. Nord K. et al. (1997) Nature Biotechnol 15: 772−777.
2. Wikman M. et al. (2004) Protein Eng Des Sel 17: 455−462.
3. Frey M. & Suppmann B. (1995) Biochemica 2: 34−35.

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