# Fast and efficient site-directed mutagenesis with Platinum SuperFi DNA Polymerase

## Introduction

Site-directed mutagenesis is one of the most essential techniques to study the structure-function relationship of genes and proteins. PCR-based methods such as overlap extension, inverse PCR, and megaprimer PCR were developed to introduce targeted substitutions, deletions, and insertions. The widely used QuikChange™ method (Agilent) is based on amplification of a circular plasmid with a pair of complementary primers, which are completely overlapping to each other. This primer design has been reported to lead to poor PCR yield; the success rate has been improved by using partially overlapping primers with 3´-overhangs [1–3]. Alternatively, phosphorylated non-overlapping primers can be used for amplification followed by a ligation step. As full-length plasmid sequence is amplified during site-directed mutagenesis, accumulation of PCR errors should always be taken into consideration. This is usually addressed by minimization of PCR cycles; however, low PCR yield requires digestion of template DNA (e.g., by DpnI cleavage) for efficient mutagenesis.

This application note describes two fast and easy protocols to introduce point mutations, insertions, and deletions into plasmid DNA using Invitrogen™ Platinum™ SuperFi™ DNA Polymerase (Table 1). Platinum SuperFi DNA Polymerase is a proofreading DNA polymerase that combines superior fidelity with trusted Platinum™ hot-start technology to enable the highest success in PCR. Featuring >100x *Taq* fidelity, Platinum SuperFi DNA Polymerase helps ensure an extremely low number of unwanted secondary mutations. Using Platinum SuperFi DNA Polymerase, >95% mutagenesis efficiency can be reached without any additional steps to remove template DNA. Protocol A, which uses two overlapping mutagenic primers with 3´-overhangs, is an extremely rapid and efficient method to introduce a point mutation into plasmid DNA. Protocol B, which is based on amplification using non-overlapping primers and ligation, is especially recommended for large deletions or insertions.

**Table 1. Comparison between site-directed mutagenesis protocols.**

| | Protocol A | Protocol B |
|---|---|---|
| **Primer design** | Overlapping mutagenic primers with 3´-overhangs | Phosphorylated non-overlapping primers, only one of them carrying the required mutation |
| **Applications** | Recommended to introduce point mutations into plasmid DNA | Recommended for large deletions or insertions |
| **Mutagenesis efficiency** | Up to 99% | Up to 95% |

## Materials and methods

### Materials

| Protocol A | Protocol B |
|---|---|
| • Overlapping mutagenic primers with 3´-overhangs | • Mutagenic phosphorylated primers<br>• Invitrogen™ Anza™ T4 DNA Ligase Master Mix (Cat. No. IVGN2104)<br>• Invitrogen™ Anza™ T4 PNK Kit (Cat. No. IVGN2304) |
| • Invitrogen™ Platinum™ SuperFi™ PCR Master Mix (Cat. No. 12358010) or Platinum SuperFi DNA Polymerase (Cat. No. 12351010)<br>• Invitrogen™ dNTP Mix (10 mM each, Cat. No. 18427013)<br>• Plasmid DNA template<br>• Invitrogen™ E-Gel™ General Purpose Agarose Gels, 1.2% (Cat. No. G501801)<br>• Invitrogen™ Anza™ 10 DpnI (Cat. No. IVGN0106)<br>• Invitrogen™ One Shot™ TOP10 Chemically Competent *E. coli* (Cat. No. C404003) | |

### Reaction conditions for PCR with master mix

| Component | 50 µL rxn | Final conc. |
|---|---|---|
| Water, nuclease-free | to 50 µL | — |
| 2X Platinum SuperFi PCR Master Mix* | 25 µL | 1X |
| 10 µM forward primer | 2.5 µL | 0.5 µM |
| 10 µM reverse primer | 2.5 µL | 0.5 µM |
| Plasmid DNA template** | 0.01–1 ng | Varies |
| 5X SuperFi™ GC Enhancer (optional)† | 10 µL | 1X |

* Provides 1.5 mM $MgCl_2$ in final reaction concentration.

** The amount of template DNA should be minimized for high mutagenesis efficiency. Alternatively, template DNA can be removed by Anza™ 10 DpnI digestion following PCR (DpnI is fully active in the SuperFi buffer).

† Recommended for targets with >65% GC content.

or

### Reaction conditions for PCR with stand-alone enzyme

| Component | 50 µL rxn | Final conc. |
|---|---|---|
| Water, nuclease-free | to 50 µL | – |
| 5X SuperFi™ Buffer* | 10 µL | 1X |
| dNTP Mix (10 mM each) | 1 µL | 0.2 mM each |
| 10 µM forward primer | 2.5 µL | 0.5 µM |
| 10 µM reverse primer | 2.5 µL | 0.5 µM |
| Plasmid DNA template** | 0.01–1 ng | Varies |
| Platinum SuperFi DNA Polymerase (2 U/µL) | 0.5 µL | 0.02 U/µL |
| 5X SuperFi GC Enhancer (optional)† | 10 µL | 1X |

* Provides 1.5 mM $MgCl_2$ in final reaction concentration.

** The amount of template DNA should be minimized for high mutagenesis efficiency. Alternatively, template DNA can be removed by Anza 10 DpnI digestion following PCR (DpnI is fully active in the SuperFi buffer).

† Recommended for targets with >65% GC content.

### Cycling protocols

| PCR cycles | Step | 2-step protocol | | 3-step protocol | | Long PCR (>10 kb) | |
|---|---|---|---|---|---|---|---|
| | | Temp. | Time | Temp. | Time | Temp. | Time |
| 1 | Initial denaturation | 98°C | 30 sec | 98°C | 30 sec | 95°C | 2 min |
| 25–30 | Denature | 98°C | 5–10 sec | 98°C | 5–10 sec | 95°C | 10 sec |
| | Anneal | – | – | Varies | 10 sec | – | – |
| | Extend | 72°C | 15–30 sec/kb | 72°C | 15–30 sec/kb | 68°C | 30 sec/kb |
| 1 | Final extension | 72°C | 5 min | 72°C | 5 min | 72°C | 5 min |
| | | 4°C | Indefinitely | 4°C | Indefinitely | 4°C | Indefinitely |

**Important:** Always use the $T_m$ calculator on our website at **thermofisher.com/tmcalculator** to calculate the $T_m$ of your primers and the recommended annealing temperature. Do not include the mismatched part when calculating $T_m$.

## Mutagenesis—protocol A

Protocol A is the most rapid and efficient method to introduce substitutions, deletions, and insertions into plasmid DNA. Point mutations are created by designing two mutagenic primers, which are partially complementary with 3′-overhangs (Figure 1). Exponential amplification using Platinum SuperFi DNA Polymerase leads to linear DNA molecules, which are joined through homologous recombination in *E. coli* after transformation.

### Primer design

The mutagenic primers should comprise two major parts: (1) a 5′-complementary region of 15–20 nt and (2) at least 8 non-overlapping bases at the 3′-terminus (Figure 2). The targeted mutation should be included in the middle of the complementary region in both primers. Several point mutations (substitutions, deletions, or insertions) can be introduced in the same primer. At least one G or C should be present at the 5′ and 3′ termini of the primer.

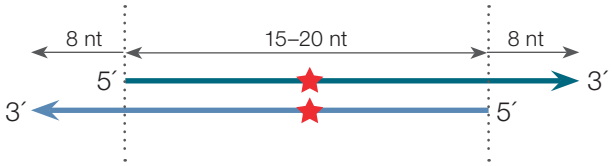


**Figure 2. Design of partially overlapping primers.**

The primers in the pair must complement each other at the 5′-terminus instead of the 3′-terminus to avoid primer self-extension. It is recommended to use primers purified by high-performance liquid chromatography (HPLC) or polyacrylamide gel electrophoresis (PAGE).

### PCR

PCR amplification is carried out using Platinum SuperFi PCR Master Mix or Platinum SuperFi DNA Polymerase, 0.5 µM of each mutagenic primer, and 0.01–1 ng of plasmid DNA template per 50 µL PCR reaction (see “Reaction conditions for PCR” and “Cycling protocols”). The amount of template DNA should be minimized for high mutagenesis efficiency. Alternatively, template DNA can be removed by Anza 10 DpnI digestion following PCR (DpnI is fully active in the SuperFi buffer).

It is recommended to take a 5 µL sample from the PCR reaction for agarose gel electrophoresis to verify the success of the amplification. The sample should be diluted 2- to 20-fold for optimal separation using E-Gel agarose gels.

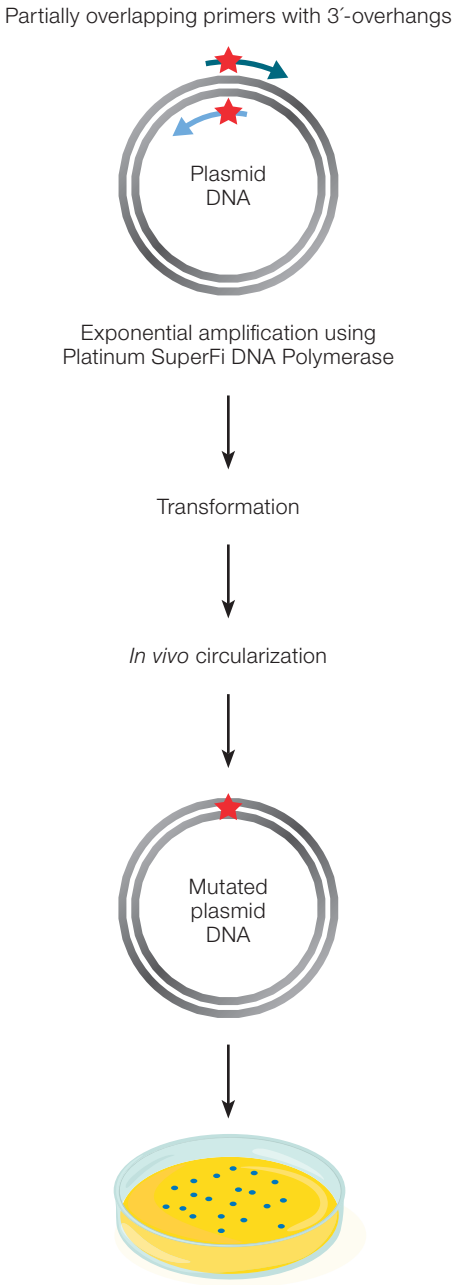


**Figure 1. Site-directed mutagenesis using partially overlapping primers.**

### Transformation

The PCR product can be used for transformation without any additional steps. Transform 1–5 µL of the PCR reaction mixture per 50–100 µL of chemically competent *E. coli* cells.

## Mutagenesis—protocol B

Protocol B is based on amplification using phosphorylated non-overlapping primers, only one of them carrying the required mutation. The two primers anneal to opposite strands on the target vector and the whole plasmid is amplified by Platinum SuperFi DNA Polymerase (Figure 3). The linear DNA obtained after exponential amplification is circularized using Anza T4 DNA Ligase Master Mix. Protocol B can be used to introduce various mutations, and is especially recommended for large deletions and insertions.

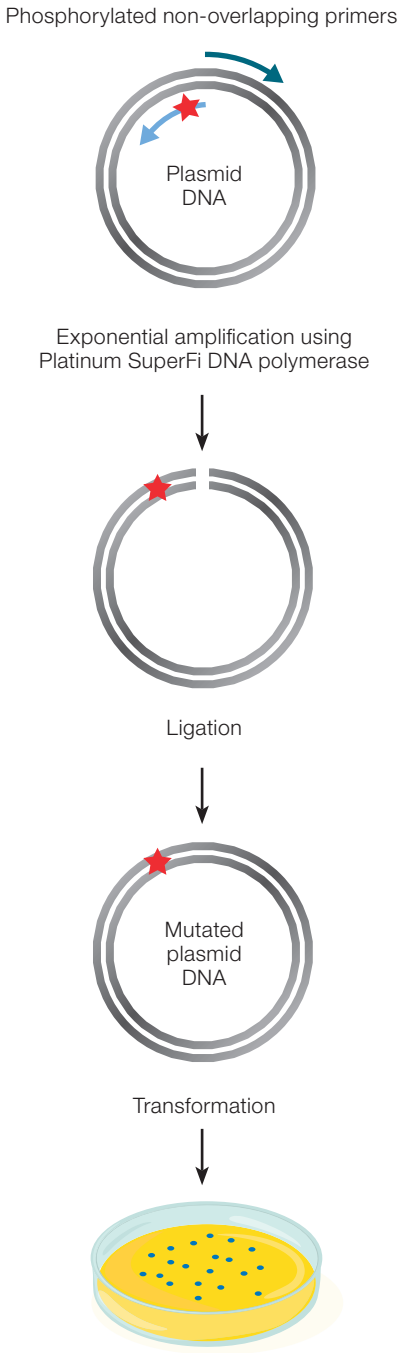


**Figure 3. Site-directed mutagenesis using phosphorylated non-overlapping primers.**

### Primer design

**Point mutations**: Point mutations are created by designing a mismatch in the mutagenic primer (Figure 4). The length of the correctly matched sequence in the mutagenic primers should be 24–30 nucleotides. The desired mutation should be in the middle of the primer with 10–15 perfectly matched nucleotides on each side. Several point mutations can be introduced in the same primer.

**Deletions**: Deletions are created by designing primers that border the deleted area on both sides (Figure 4). To generate a deletion, the primers should be perfectly matched on their entire length, which should be 24–30 nucleotides.

**Insertions**: For longer insertions, a stretch of mismatched nucleotides is designed on the 5′ end of one or both primers (Figure 4). Short insertions can be designed in the middle of the primer.

Primers can be phosphorylated using Anza T4 PNK Kit or ordered phosphorylated from the oligo supplier. It is recommended to use primers purified by HPLC or PAGE.

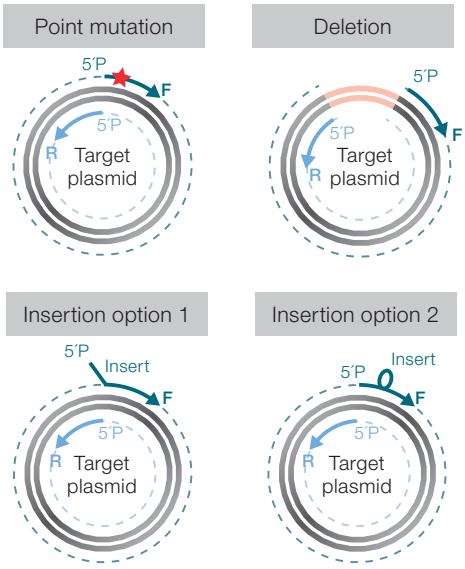


**Figure 4. Design of non-overlapping primers.**

### PCR

PCR amplification is carried out using Platinum SuperFi PCR Master Mix or Platinum SuperFi DNA Polymerase, 0.5 µM of each mutagenic primer, and 0.01–1 ng of plasmid DNA template per 50 µL PCR reaction (see “Reaction conditions for PCR” and “Cycling protocols”). The amount of template DNA should be minimized for high mutagenesis efficiency. Alternatively, template DNA can be removed by Anza 10 DpnI digestion following PCR (DpnI is fully active in the SuperFi buffer).

It is recommended to take a 5 µL sample from the PCR reaction for agarose gel electrophoresis to verify the success of the amplification. The sample should be diluted 2- to 20-fold for optimal separation using E-Gel agarose gels.

### Ligation and transformation

15 µL of the PCR reaction is mixed with 5 µL of Anza T4 DNA Ligase Master Mix by pipetting up and down, then centrifuged briefly, and incubated at room temperature for 15 minutes. For transformation, 1–5 µL of the ligation reaction mixture is added per 50–100 µL of chemically competent *E. coli* cells.

## Results

To demonstrate the efficiency of site-directed mutagenesis using Platinum SuperFi DNA Polymerase, *lacZα* function was restored using protocol A or protocol B. The template plasmid, which contains a stop codon (TAA) at position 8 in the *lacZα* gene, forms white colonies on LB agar plates containing X-gal and IPTG, while blue colonies are obtained after successful site-directed mutagenesis.

### Example experiment using protocol A

The mutagenic primers comprising a 5′ complementary region of 15 nt and 9 non-overlapping bases at the 3′-terminus were as follows (the complementary region is underlined):

Forward: 5′-GCATGTAAGCTTGGCGTAATCATG-3′
Reverse: 5′-GCCAAGCTTACATGCCTGCAGGTC-3′

PCR reactions contained 0 (no-template control), 0.001, 0.01, 0.1, 1, or 10 ng of template DNA per 50 µL of PCR reaction. The cycling protocol was: 1 cycle at 98°C for 30 sec; 25 cycles at 98°C for 10 sec, 72°C for 45 sec; 1 cycle at 72°C for 5 min. 1 µL of the PCR reaction from 0.01 ng of template DNA was directly used for transformation of One Shot TOP10 Chemically Competent *E. coli* according to the recommendations provided, and the cells were plated on LB agar plates containing X-gal.

Almost all colonies obtained were blue, indicating successful mutagenesis to restore *lacZα* function. This shows that the efficiency of mutagenesis using Platinum SuperFi DNA Polymerase and partially complementary primers with 3′-overhangs can reach 99% (Figure 5).

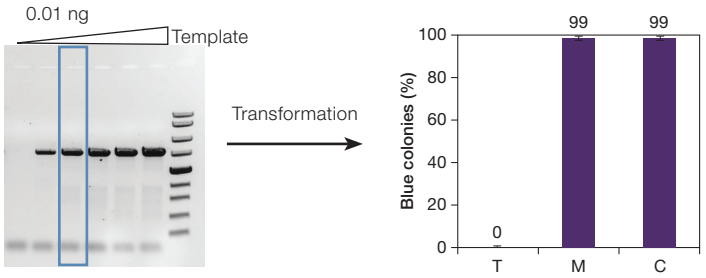


**Figure 5. Mutagenesis efficiency using protocol A.** Blue colony color indicates successful site-directed mutagenesis to restore *lacZα* function. The number of blue colonies obtained was normalized to the total number of transformants obtained. T: initial template plasmid, M: product after PCR using mutagenic primers, C: control wild-type plasmid. Thermo Scientific™ ZipRuler™ Express DNA Ladder 2 was used as a size standard.

### Example experiment using protocol B

The 5′-phosphorylated primers were as follows:

Forward: 5′-GTCGACTCTAGAGGATCCCCGGGT-3′
Reverse: 5′-CTGCAGGCATGTAAGCTTGGCGTA-3′

PCR reactions contained 0 (no-template control), 0.001, 0.01, 0.1, 1, or 10 ng of template DNA per 50 µL of PCR reaction. The cycling protocol was: 1 cycle at 98°C for 30 sec; 25 cycles at 98°C for 10 sec, 72°C for 45 sec; 1 cycle at 72°C for 5 min. 15 µL of the PCR reaction from 0.01 ng of template DNA was used for ligation with 5 µL of Anza T4 DNA Ligase Master Mix. 1 µL of the ligation reaction was used for transformation of One Shot TOP10 Chemically Competent *E. coli* cells according to the recommendations provided, and the cells were plated on LB agar plates containing X-gal.

The majority of colonies obtained were blue, indicating successful mutagenesis to restore *lacZα* function. This shows that the efficiency of mutagenesis using Platinum SuperFi DNA Polymerase and non-overlapping primers can reach 95% (Figure 6).

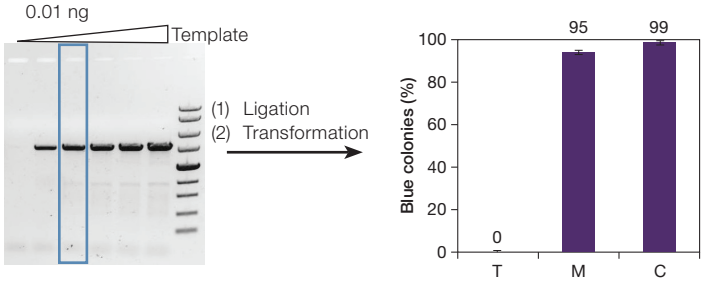


**Figure 6. Mutagenesis efficiency using protocol B.** Blue colony color indicates successful site-directed mutagenesis to restore *lacZα* function. The number of blue colonies obtained was normalized to the total number of transformants. T: initial template plasmid, M: product after PCR using mutagenic primers and ligation, C: control wild-type plasmid. ZipRuler Express DNA Ladder 2 was used as a size standard.

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## Conclusions

This application note provides two fast and easy methods to introduce site-directed mutations into plasmid DNA. Platinum SuperFi DNA Polymerase is ideally suited for these protocols due to its low error rate and high processivity, which reduces cycling times and enables high yields of longer fragments. The extremely low error rate and high PCR yields from low template amounts enable up to 99% mutagenesis efficiency without any additional steps to remove template DNA.

## References


1. Zheng L, Baumann U, Reymond JL (2004) An efficient one-step site-directed and site-saturation mutagenesis protocol. *Nucleic Acids Res* 32(14):e115.
2. Qi D, Scholthof KB (2008) A one-step PCR-based method for rapid and efficient site-directed fragment deletion, insertion, and substitution mutagenesis. *J Virol Methods* 149(1):85–90.
3. Xia Y, Chu W, Qi Q, Xun L (2015) New insights into the QuikChange process guide the use of Phusion DNA polymerase for site-directed mutagenesis. *Nucleic Acids Res* 43(2):e12.


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