

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

Introduction

Site-directed mutagenesis is one of the most essential techniques used 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 by Agilent Technologies is based on amplification of a circular plasmid with a pair of complementary primers that overlap each other completely. The use of these primers has been reported to lead to poor PCR yield. By using partially overlapping primers with 3'-overhangs [1–3], the success rate has been improved. Alternatively, phosphorylated non-overlapping primers can be used for amplification followed by a ligation step. As the 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 minimizing the number 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™ II DNA Polymerase (Table 1). Platinum SuperFi II DNA Polymerase is a proofreading DNA polymerase that combines superior fidelity with trusted Invitrogen™ Platinum™ hot-start technology to enable the highest success in PCR. Featuring >300x *Taq* fidelity, Platinum SuperFi II DNA Polymerase helps ensure an extremely low number of unwanted secondary mutations. Using Platinum SuperFi II 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 of 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
<ul style="list-style-type: none"> Overlapping mutagenic primers with 3'-overhangs 	<ul style="list-style-type: none"> Phosphorylated mutagenic primers Invitrogen™ Anza™ T4 DNA Ligase Master Mix (Cat. No. IVGN2104) Invitrogen™ Anza™ T4 PNK Kit (Cat. No. IVGN2304)
<ul style="list-style-type: none"> Invitrogen™ Platinum™ SuperFi™ II PCR Master Mix (Cat. No. 12368010) or Platinum SuperFi II DNA Polymerase (Cat. No. 12361010) Invitrogen™ dNTP Mix (10 mM each, Cat. No. 18427013) Plasmid DNA template Invitrogen™ E-Gel™ EX Agarose Gels, 1% (Cat. No. G402001) Invitrogen™ E-Gel™ 1 Kb Plus Express DNA Ladder (Cat. No. 10488091) Invitrogen™ One Shot™ TOP10 Chemically Competent <i>E. coli</i> (Cat. No. C404003) Optional: Invitrogen™ Anza™ 10 DpnI (Cat. No. IVGN0106) 	

Reaction conditions for PCR with master mix

Component	50 µL rxn	Final conc.
2X Platinum SuperFi II 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
Water, nuclease-free	to 50 µL	—

* Provides 1.5 mM MgCl₂ 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 Platinum SuperFi II buffer).

Reaction conditions for PCR with stand-alone enzyme

Component	50 µL rxn	Final conc.
5X SuperFi II 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 II DNA Polymerase	1 µL	—
Water, nuclease-free	to 50 µL	—

or

* Provides 1.5 mM MgCl₂ 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 Platinum SuperFi II buffer).

Cycling protocols

PCR cycles	Step	2-step protocol (for primers >30 nt in length*)		3-step protocol	
		Temp.	Time	Temp.	Time
1	Initial denaturation	98°C	30 sec	98°C	30 sec
25–30	Denature	98°C	5–10 sec	98°C	5–10 sec
	Anneal	—	—	60°C	10 sec
	Extend	72°C	15–30 sec/kb	72°C	15–30 sec/kb
1	Final extension	72°C	5 min	72°C	5 min
		4°C	Indefinitely	4°C	Indefinitely

* Without the mismatched sequences.

Mutagenesis—protocol A

Protocol A describes the most rapid and efficient method to introduce substitutions, deletions, and insertions into plasmid DNA. Point mutations are created by designing two mutagenic primers that are partially complementary with 3'-overhangs (Figure 1). Exponential amplification using Platinum SuperFi II 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.

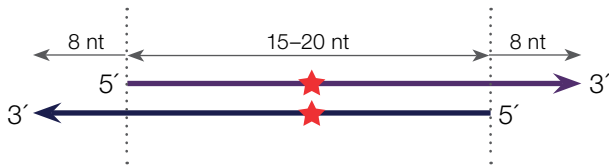


Figure 2. Design of partially overlapping primers.

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 II PCR Master Mix or Platinum SuperFi II 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 Platinum SuperFi II buffer).

It is recommended to take a 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 an E-Gel EX Agarose Gel.

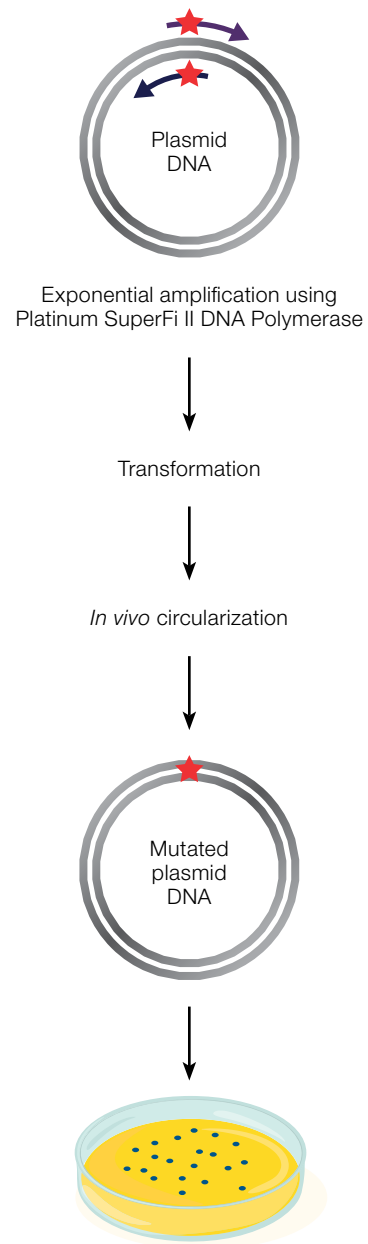


Figure 1. Site-directed mutagenesis using partially overlapping primers.

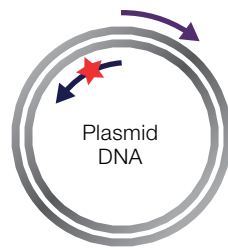
Transformation

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.

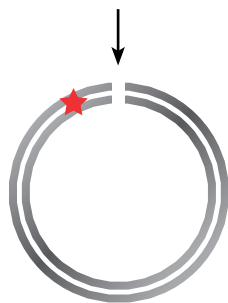
Mutagenesis—protocol B

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 II 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.

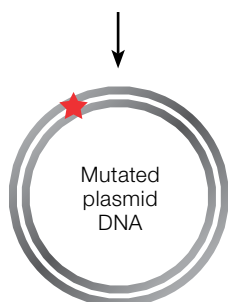
Phosphorylated non-overlapping primers



Exponential amplification using Platinum SuperFi II DNA polymerase



Ligation



Transformation

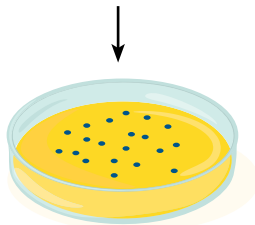


Figure 3. Site-directed mutagenesis using phosphorylated non-overlapping primers.

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 nt. 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 nt.

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

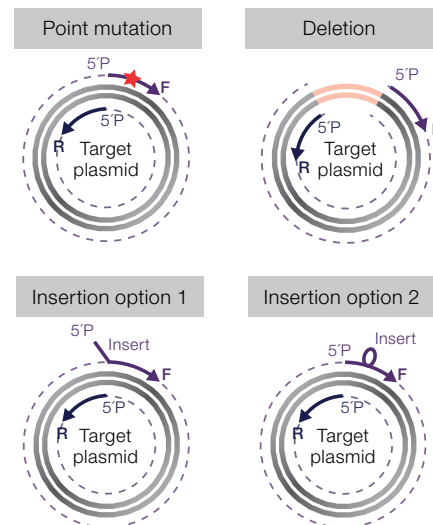


Figure 4. Design of non-overlapping primers.

PCR

PCR amplification is carried out using Platinum SuperFi II PCR Master Mix or Platinum SuperFi II DNA Polymerase, 0.5 μ M of each mutagenic primer, and 0.01–1 ng of plasmid DNA template per 50 μ L 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 II buffer).

It is recommended to take a 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 an E-Gel EX Agarose Gel.

Ligation and transformation

15 μ L of the PCR reaction is mixed with 5 μ L 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 μ L of the ligation reaction mixture is added per 50–100 μ L of chemically competent *E. coli* cells.

Results

To demonstrate the efficiency of site-directed mutagenesis using Platinum SuperFi II DNA Polymerase, *lacZa* function was restored using protocol A or protocol B. The template plasmid, which contains a stop codon (TAA) at position 8 in the *lacZa* gene, forms white colonies on lysogeny broth (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 μ L of PCR reaction. The cycling protocol was: 1 cycle at 98°C for 30 sec; 25 cycles at 98°C for 10 sec, 60°C for 10 sec, 72°C for 45 sec; 1 cycle at 72°C for 5 min. Samples after PCR were diluted 20-fold and analyzed on an E-Gel EX 1% Agarose Gel. 1 μ L 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 and IPTG.

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

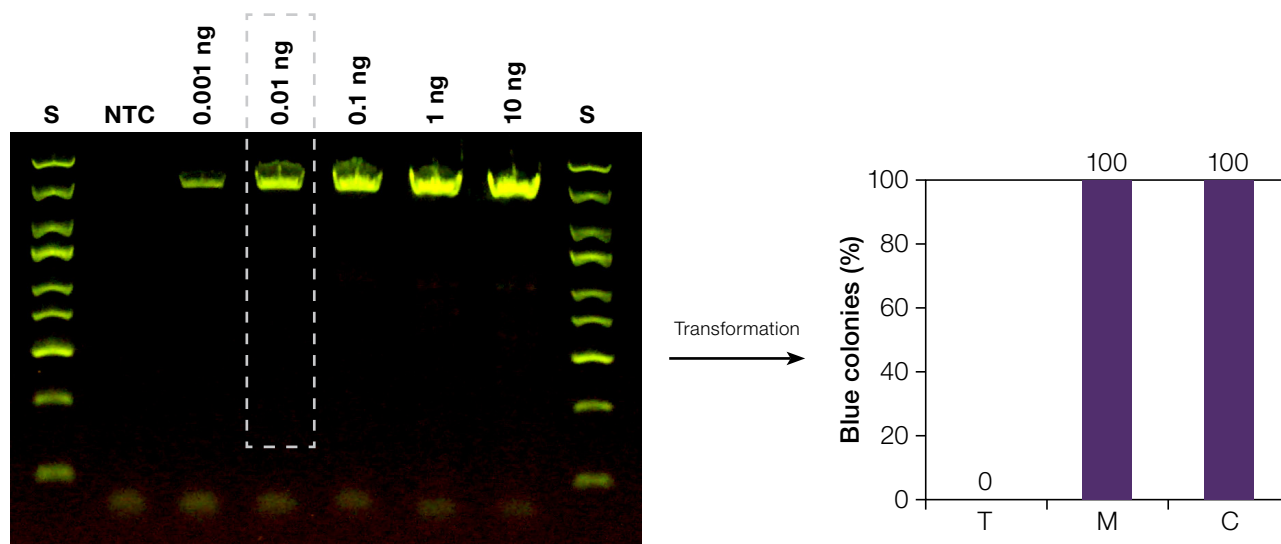


Figure 5. Mutagenesis efficiency using protocol A (using partially overlapping primers). Blue colony color indicates successful site-directed mutagenesis to restore *lacZa* function. The number of blue colonies obtained was normalized to the total number of transformants obtained. S: E-Gel 1 Kb Plus Express DNA Ladder used as size standard, NTC: no-template control, T: initial template plasmid, M: product after PCR using mutagenic primers, and C: control wild-type plasmid.

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 μ L of PCR reaction. The cycling protocol was: 1 cycle at 98°C for 30 sec; 25 cycles at 98°C for 10 sec, 60°C for 10 sec, 72°C for 45 sec; 1 cycle at 72°C for 5 min. 15 μ L of the PCR reaction from 0.01 ng of template DNA was used for ligation with 5 μ L of Anza T4 DNA Ligase Master Mix. 1 μ L 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 and IPTG. About 94% of colonies obtained were blue, indicating successful mutagenesis to restore *lacZa* function. This shows that the efficiency of mutagenesis using Platinum SuperFi II DNA Polymerase and non-overlapping primers can reach >90% (Figure 6).

Conclusions

This application note provides two fast and easy methods to introduce site-directed mutations into plasmid DNA. Platinum SuperFi II 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

- Zheng L, Baumann U, Reymond JL (2004) An efficient one-step site-directed and site-saturation mutagenesis protocol. *Nucleic Acids Res* 32(14):e115.
- 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.
- 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.

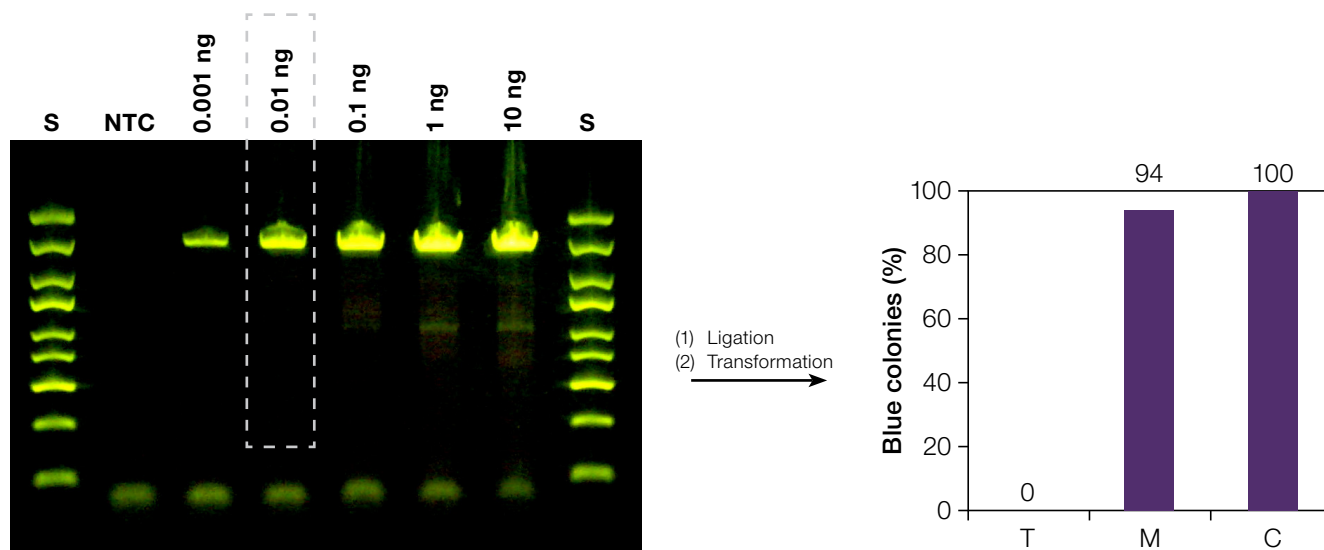


Figure 6. Mutagenesis efficiency using protocol B (using non-overlapping primers). Blue colony color indicates successful site-directed mutagenesis to restore *lacZa* function. The number of blue colonies obtained was normalized to the total number of transformants. S: E-Gel 1 Kb Plus Express DNA Ladder used as size standard, NTC: no-template control, T: initial template plasmid, M: product after PCR using mutagenic primers and ligation, and C: control wild-type plasmid.

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