TOOLSite-Directed Mutagenesis Kit

For rapid site-directed mutation at a single site or two discontinuous sites

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Introduction

TOOLSite-Directed Mutagenesis Kit is designed for rapid site-directed mutagenesis based on the new rapid cloning technology. Amplified target fragments are digested by DpnI, cyclized by Toolsitase/buffer, and then transformed to *Escherichia coli* to complete site-directed mutagenesis. The kit consists of two modules: TOOLS Ultra High Fidelity DNA Polymerase amplification module and rapid cloning module. TOOLS Ultra High Fidelity DNA Polymerase can significantly reduce the possibility of unexpected mutations during amplification of up to 20 kb. The rapid cloning module replaces conventional annealing ring-forming reactions with homologous recombination reactions, requiring considerably less template and having a more flexible primer design strategy. Because the Toolsitase technology can efficiently perform seamless splicing of two polymerase chain reaction (PCR) products, the kit can complete two separate site mutations through a single amplification reaction. DpnI-digested products of specific amplicons can be directly added into a recombination reaction without purification. Highly optimized reaction buffer, fast-operating procedures, and efficient site-directed mutagenesis make the TOOLSite-Directed Mutagenesis Kit the preferred choice for DNA site mutation.

Features

1. TOOLS Ultra High Fidelity DNA Polymerase provides high-fidelity PCR with the lowest mutation rate.
2. TOOLS Ultra High Fidelity DNA Polymerase with excellent long fragment amplification capability can be widely used for any plasmid amplification within 20 kb.
3. Amplification is conducted exponentially, and the template usage is extremely low, which is beneficial for completely degrading the original methylation template.
4. DpnI eliminates original template contamination.
5. The rapid Toolsitase cloning system can efficiently cyclize PCR products.
6. Amplified products can be directly used for recombinant reaction after digestion with DpnI.

Note: Please use methylase nondefective host strains (e.g., Top10, DH5α, and JM109) to extract the original plasmid when using this kit for the site-directed mutagenesis of plasmids.

Kit Contents

Contents	TTC-CB14 (10rxn)
2× buffer I	1.25 mL
dNTP (10 mM)	20 μL
TOOLS Ultra High Fidelity DNA Polymerase	20 μL
DpnI (10 U/μL)	20 μL
5× buffer II	40 μL
Toolsitase	20 μL

Storage

All components of the kit can be stored at −20°C for 1 year. Repeated freeze–thaw cycles should be avoided.

Work Flow

Single-base (or continuous multiple bases) site-directed mutation

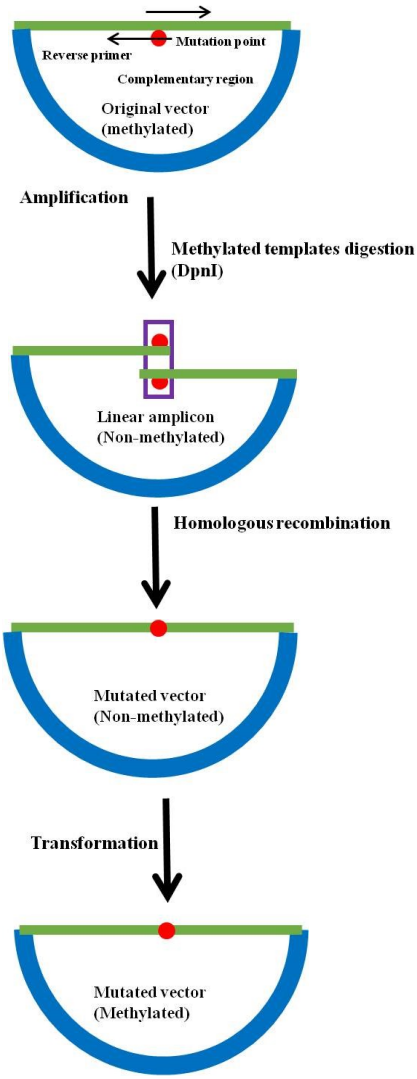


Figure 1: Single-base site-directed mutation

A design of partial reverse complement primers to perform reverse amplification with the original plasmid as the template. The amplification product was digested by DpnI, and then, it was directly used in the recombination reaction. The recombination product was directly transformed to complete site-directed mutagenesis.

Double-base site-directed mutations (the distance between two mutation sites is >50 bp).

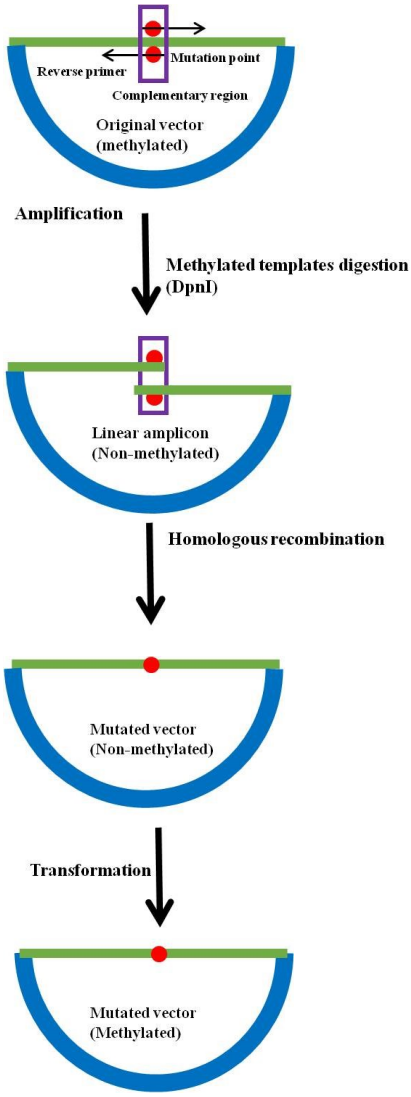


Figure 2: Overview of separate double-base site-directed mutations with this kit

Choose mutation sites A and B as the boundary to divide the plasmid into fragments AB and BA. Design partially reverse complement primers at the two mutation sites. Amplify fragments AB (forward primer A and reverse primer B) and BA (reverse primer A and forward primer B) with the original plasmid as the template.

Protocol

Single-base site-directed mutation

1. Primer design

   Only one pair of primers for the inverse amplification of the plasmid is required to introduce site-directed mutagenesis of a single base or continuous multiple bases in a plasmid. The basic principles for primer design are as follows: the 5′ ends of reverse and forward primers comprise a 15–21-bp reverse complementary region (GC content of 40%–60%) and at least a 15-bp noncomplementary region (the Tm value of the region between the mutation site and the 3′ end of the primer is recommended to be >60°C). The mutation site can be in the complementary region (the mutation should be introduced in both primers) or the noncomplementary region of any one of the primers. The mutation site should NOT be at the end of the primer. Figure 3 shows the details of primer design illustrated with the introduction of a single-base mutation into vector pUC18.

Note: Calculation of the Tm value of a primer should be based on the region between the mutation site and the 3′ end of the primer. The Tm value should exceed 60°C through adjusting the primer length. Please note that the region between the mutation site and the 5′ end of the primer should not be included for calculating the Tm value.

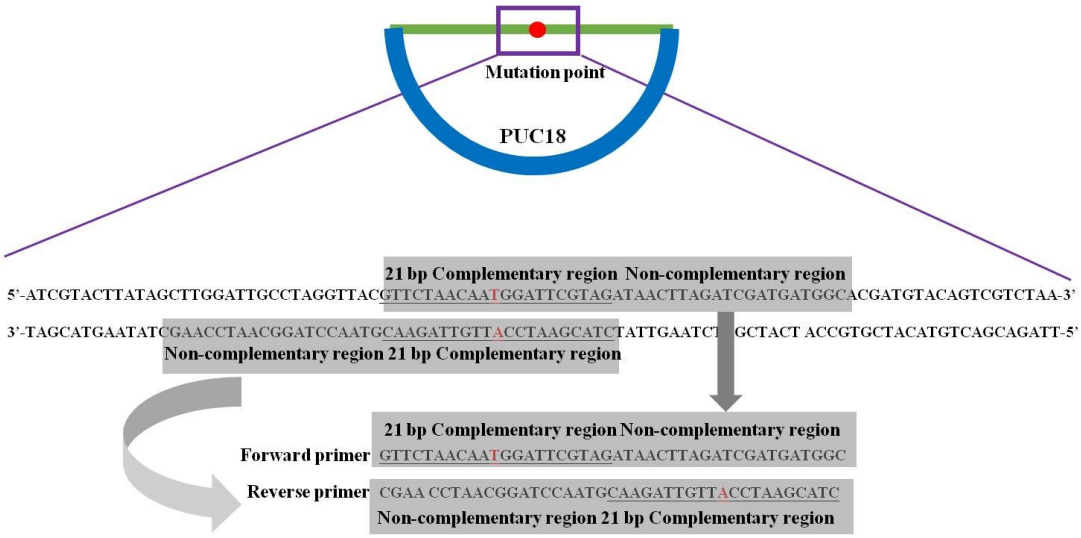


Figure 3: Schematic for introducing a single base or continuous multi-base site-directed mutation into a plasmid

2. Target plasmid amplification

Use TOOLS Ultra High Fidelity DNA Polymerase to amplify a target plasmid. Each component should be mixed well after thawing and placed back at −20°C when not in use. The recommended reaction system is as follows:

Component	Volume
ddH_2O	Up to 50 μL
2× buffer I	25 μL
dNTP (10 mM)[a]	1 μL
Template DNA[b]	Optional
Primer 1 (10 μM)	2 μL
Primer 2 (10 μM)	2 μL
TOOLS Ultra High Fidelity DNA Polymerase[c]	1 μL

a. Do not use dUTP or any primer and template containing uracil.

b. Given the normal amplification of a plasmid, use the least possible number of templates. Less than 1 ng of freshly extracted plasmid is recommended.

c. The recommended final concentration of the enzyme is 1 U/50 μL. The optimal concentration of TOOLS Ultra High Fidelity DNA Polymerase is 0.5–2 U per 50 μL. No more than 2 U per 50 μL is recommended especially when the amplicon is >5 kb.

After all components are mixed, the recommended PCR program is as follows:

Steps	Temperature	Time	Cycle number
Predenaturation[a]	95°C	30 s	1
Denaturation[a]	95°C	15 s	30[d]
Annealing[b]	60°C–72°C	15 s	
Extension[c]	72°C	30–60 s/kb	
Complete extension	72°C	5 min	1

a. For most plasmids, the appropriate denaturation temperature is 95°C.

b. TOOLS Ultra High Fidelity DNA Polymerase can promote annealing between the template and primers efficiently. In general, the annealing temperature is the Tm of primers. If required, the temperature gradient can be established to determine the optimal temperature for binding the primer to the template. Long annealing time may disperse amplification products.

c. Long extension time can improve the yield of amplification products.

d. To prevent introducing nontarget mutation, we recommend that the amplification cycle be <35. If the amplification efficiency is good, we recommend the amplification cycle be <30.

After the PCR reaction, a small amount of amplification products is subjected to gel electrophoresis.

If the target plasmid is correctly amplified, please continue with the next step.

3. Amplification products are digested by DpnI to remove the methylated template plasmid.

   The amplification product of step 2 includes the original template plasmid; thus, we need to digest the product with DpnI before recombination cyclization to prevent false-positive transformants after transformation. The recommended reaction system is as follows:

Component	Volume
DpnI	1 μL
Amplification product	40–50 μL

   Place the reaction mixture at 37°C for 1–2 hours. If the amplification product of step 2 is a single band, DpnI-digested products can be used in a subsequent recombination reaction without purification. If the product is not a single band, gel extraction purification should be performed before the next step.

4. Recombination reaction

   The 5′ ends of forward and reverse primers share a completely reverse complementary sequence; thus, homologous recombination can occur between the 5′ and 3′ ends of the amplification product catalyzed using Toolsitase to complete amplification product cyclization. The following components are added sequentially to the bottom of 1.5-mL sterile Eppendorf tubes or PCR tube on ice-water bath. If liquid sticks on tube walls, please collect the liquid to the bottom of the tube through brief centrifugation.

Component	Volume
ddH_2O	Up to 20 μL
5× buffer II	4 μL
DpnI-digested products	50–400 ng
Toolsitase	2 μL

   The optimal amount of DNA in the recombination reaction system is 0.03 pmol.

   The corresponding mass of DNA moles can be calculated roughly by using the following formula:

   Optimal amount of product digested by DpnI = [0.02 × the base pair number of the target plasmid] ng (0.03 pmol)

   For example, to introduce a single mutation in a 5-kb plasmid, the optimal amount of DpnI digestion product is 100 ng (0.02 × 5,000 = 100 ng).

Note: Too much or too little DNA will reduce cyclization efficiency. Please confirm the DNA concentration through gel electrophoresis in advance and mix the components strictly in accordance with the recommended amount. When the calculated optimal amount is <50 or >400 ng, please add 50 or 400 ng. When the product digested by DpnI is used directly in the recombination reaction, the

volume of the product should be less than one-fifth of the total volume.

After adding all the components, please mix them in the reaction system through gently pipetting up and down several times and avoid air bubbles (please do not vortex or shake vigorously). Incubate the tube at 37°C for 30 min. After incubation, please place the tube in an ice bath for 5 min.

The product can be transformed directly or stored at −20°C.

5. Transformation, plating, and colony identification

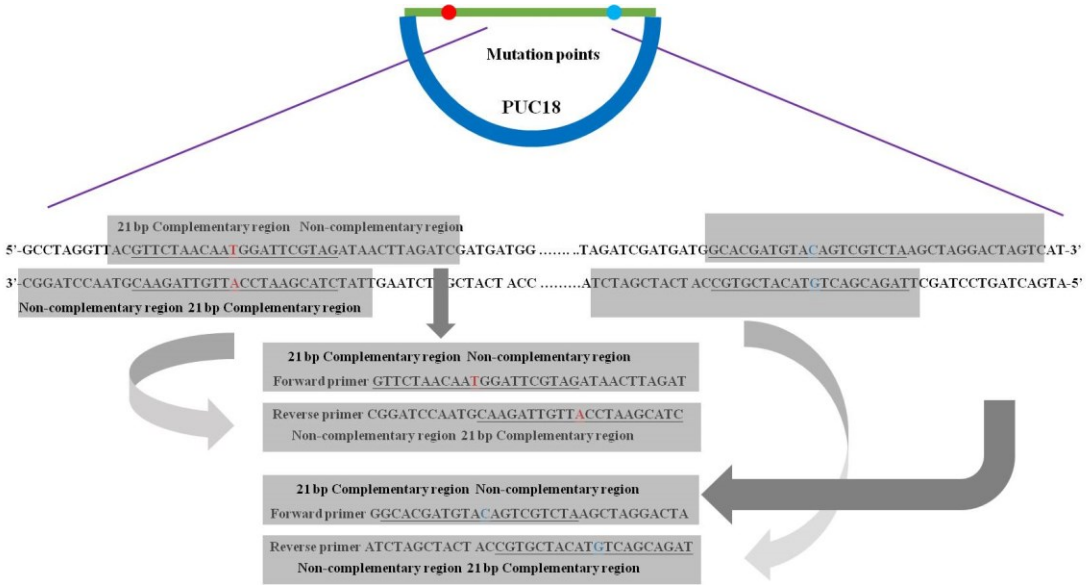
   In 20 μL of a cooled reaction mixture, add 200 μL of competent cells. Mix gently through flicking the tube, and place the tube on ice for 30 min. Incubate it at 42°C for 45–90 s for heat shock. Then, place it in ice-water bath for 2 min. Add 900 μL of SOC or LB medium. Again, incubate at 37°C for 10 min and shake (150 rpm) for 45 min at 37°C. Plate 100 μL of the bacterial culture on a selective plate. Then, incubate overnight at 37°C.

   Note: We recommend using competent cells with a transformation efficiency of $>10^8$ cfu/μg. Otherwise, centrifuge your bacterial culture at 5,000 rpm for 3 min to collect bacteria, resuspend the bacteria with 100 μL of LB medium, and then plate all the bacterial cells.

Double-base site-directed mutations

1. Primer design

   To introduce two separate site-directed mutations in a plasmid, two pairs of primers are needed to amplify the plasmid in two parts. The basic principles for primer design are as follows: The 5′ ends of reverse and forward primers comprise a 15–21-bp reverse complementary region. The mutation sites can be in the complementary region (the mutation should be introduced in both the primers) or the noncomplementary region of any one of the primers. The mutation site should NOT be at the end of the primer. Figure 4 shows the details of primer design, illustrated with the introduction of two base mutations into vector pUC18.



2. Target plasmid amplification

The plasmid is divided into fragments AB and BA through mutation sites A and B. TOOLS Ultra High Fidelity DNA Polymerase is used to amplify the fragments. The primers required to amplify fragment AB are the forward primer of mutation site A and the reverse primer of mutation site B. The primers required to amplify fragment BA are the forward primer of mutation site B and the reverse primer of mutation site A.

Each component should be mixed well after thawing and placed back at −20°C after use. The recommended reaction system is as follows:

Component	Volume
ddH_2O	Up to 50 μL
2× buffer I	25 μL
dNTP (10 mM)[a]	1 μL
Template DNA[b]	Optional
Primer 1 (10 μM)	2 μL
Primer 2 (10 μM)	2 μL
TOOLS Ultra High Fidelity DNA Polymerase[c]	1 μL

a. Do not use dUTP or any primer and template containing uracil.

b. Given the normal amplification of the plasmid, use the least possible number of templates. Less than 1 ng of freshly extracted plasmid is recommended.

c. The recommended final concentration of enzyme is 1 U/50 μL. The optimal concentration of TOOLS Ultra High Fidelity DNA Polymerase is 0.5–2U per 50 μL. No more than 2 U per 50 μL is recommended, especially when the amplicon is >5 kb.

After all components are mixed, the recommended PCR program is as follows:

Steps	Temperature	Time	Cycle number
Predenaturation[a]	95°C	30 s	1
Denaturation[a]	95°C	15 s	30[d]
Annealing[b]	60°C–72°C	15 s	
Extension[c]	72°C	30–60 s/kb	
Complete extension	72°C	5 min	1

a. For most plasmids, the appropriate denaturation temperature is 95°C.

b. TOOLS Ultra High Fidelity DNA Polymerase can promote annealing between the template and primers efficiently. In general, the annealing temperature is the Tm of primers. If required, the temperature gradient can be established to determine the optimal temperature for binding the primer to the template. Excessive annealing time may disperse amplification products.

c. Long extension time can improve the yield of amplification products.

d. To prevent introducing nontarget mutations, we recommend that the amplification cycle be <35. If the amplification efficiency is good, we recommend the amplification cycle be <30.

After the PCR reaction, a small amount of amplification products is subjected to gel electrophoresis. If the target plasmid is correctly amplified, please continue with the next step.

3. Amplification products are digested by DpnI to remove the methylated plasmid.

   The amplification product of step 2 includes the original template plasmid; thus, we must digest the product with DpnI before recombination cyclization to prevent false-positive transformants after transformation. The recommended reaction system is as follows:

Component	Volume
DpnI	1 μL
Amplification product	40–50 μL

Place the reaction mixture at 37°C for 1–2 hours. If the amplification product of step 2 is a single band, DpnI-digested products can be used in subsequent recombination reactions without purification. If the product is not a single band, gel extraction purification should be performed before the next step.

4. Recombination reaction

   The ends of fragments AB and BA share a region with exactly the same sequence; thus, homologous recombination can occur between AB and BA catalyzed using Toolsitase to complete cyclization. The following components are sequentially added to the bottom of 1.5-mL sterile Eppendorf tubes or PCR tubes on ice-water bath. If liquid sticks on the tube walls, please collect the liquid to the bottom of the tube through brief centrifugation.

Component	Volume
ddH_2O	Up to 20 μL
5× buffer II	4 μL
DpnI-digested fragment AB	20–200 ng
DpnI-digested fragment BA	20–200 ng
Toolsitase	2 μL

Optimal DNA fragments in the Toolsitase double-base mutation recombination reaction system are as follows: a long fragment of 0.03 pmol and a short fragment of 0.06 pmol. The corresponding mass of DNA fragments can be calculated roughly by using the following formula:

Optimal amount of the long fragment digested by DpnI = [0.02 × the number of base pairs of fragments] ng (0.03 pmol).

Optimal amount of the short fragment digested by DpnI = [0.04 × the number of base pairs of fragments] ng (0.06 pmol).

For example, if fragment AB is 1 kb and fragment BA is 5 kb, the optimal amount of fragment AB digested by DpnI is 40 ng (0.04 × 1,000 = 40 ng) and fragment BA is 100 ng (0.02 × 5,000 = 100 ng).

Note: Too much or too little DNA will reduce the efficiency. Confirm the DNA concentration through gel electrophoresis in advance and mix the components strictly in accordance with the recommended amount. When the calculated optimal amount is <20 or >200 ng, please add 20 or 200 ng. When the product digested by DpnI is directly used in the recombination reaction, the product volume should be less than one-fifth of the total volume.

After adding all the components, please mix them gently through pipetting up and down several times and avoid air bubbles (please do not vortex or shake vigorously). Incubate the tube at 37°C for 30 min. After the reaction, please place the tube in ice bath for 5 min. The product can be transformed directly or stored at −20°C.

5. The transformation, plating, and identification of clone

In 20 μL of a cooled reaction mixture, add 200 μL of competent cells. Mix gently through flicking the tube, and place the tube on ice for 30 min. Incubate it at 42°C for 45–90 s for heat shock. Then, place it in ice-water bath for 2 min. Add 900 μL of SOC or LB medium. Again, incubate at 37°C for 10 min and shake (150 rpm) for 45 min at 37°C. Plate 100 μL of the bacterial culture on a selective plate. Then, incubate overnight at 37°C.

Note: We recommend using competent cells with a transformation efficiency of $>10^8$ cfu/μg. Otherwise, centrifuge your bacterial culture at 5,000 rpm for 3 min to collect bacteria, resuspend the bacteria with 100 μL of LB medium, and then plate all the bacterial cells.

Other suggestions

Experiment procedure	Should do	Should NOT do
Selection of the reverse complementary region of primers	Try to choose a region containing a nonrepetitive sequence with an evenly distributed GC content. When the GC content of the selected region is 40%–60%, recombination cyclization will reach the maximum efficiency.	Select the region with a repetitive sequence with a high GC or AT content.
Primer design	Design as shown in Figure 3 or 4.	The reverse complementary region is shorter than the recommended length or adds the wrong sequence.
Experiment scheme selection	For two mutation sites, if the distance is >50 bp, choose protocol 2, and if the distance is <50 bp, choose protocol 1.	Ignore the distance of the two mutation sites, and choose protocol 2.
Plasmid amplification	Please perform highly specific amplification.	The amplification product is not specific with many nonspecific products.
Number of templates	Use the minimum possible number of templates if it does not affect the amplification yield.	Use too many templates.
PCR template should be a methylated plasmid	Use the host strain with methylase (e.g., Top10, DH5α, and JM109) to extract the original plasmid.	Use the host strain without methylase to extract the original plasmid.
Template quality	Long-term storage and repeated freezing and thawing may cause breakage, open loop, or degradation of plasmids. Hence, we recommend using freshly prepared plasmids as the template.	Use plasmids that were stored for long term and templates that were repeatedly frozen and thawed.
Purification of DpnI-digested products	If the product is not a single band, gel extraction operation is required.	Gel extraction operation is not performed when the product is not a single band.

DNA quantification of DpnI-digested products	Quantify through agarose gel electrophoresis.	Quantify through an absorbance assay.
Preparation of recombination reaction	Prepare reaction in ice-water bath. Use the recommended optimal amount of DNA at an optimal ratio. When the DpnI-digested product is used directly in the recombination reaction, the product volume should be less than one-fifth of the total volume.	Prepare the reaction at room temperature. Use a random amount of DNA. When the DpnI-digested product is used directly in the recombination reaction, the product volume is more than one-fifth of the total volume.
Recombination reaction	Incubate tubes at 37°C for 30 min in a PCR instrument or water bath with precise temperature control.	The reaction temperature is higher or lower than 37°C. The reaction time is more or less than 30 min.
Termination of recombination reaction	The tubes should be cooled down in ice-water bath for 5 min immediately after the reaction.	Put tubes at room temperature after the reaction.
Transformation	The cooled product should be transformed within an hour. The product should be kept in ice-water bath before transformation. Please keep the product at −20°C for long-term storage.	The cooled products are placed at room temperature for a long time before the transformation. For long-term storage, keep the product at 4°C.

Troubleshooting

Plasmids cannot be amplified:

1. Primer design is wrong: please check the primer design.
2. The amplification reaction mixture is not correctly prepared: please perform the experiment again.
3. The amplification reaction is not optimized: Mg^{2+} concentration, enzyme amount, and amplification program can be optimized.
4. Template quality is not good: long-term storage and repeated freezing and thawing can cause breakage, open loop, or degradation of plasmids. Please use freshly prepared plasmids as templates.

No or few colonies on the plate:

1. Competent cells have low efficiency. Use competent cells that are freshly prepared or stored properly, and make sure the transformation efficiency of the competent cells is $>10^7$ cfu/μg. Please set up a group transformed with plasmid as control to detect the transformation efficiency of competent cells.
2. The amount of DNA is insufficient for the recombination reaction or the fragment ratio is inappropriate. Please add the recommended amount of DNA. Please check the concentration of the product digested by DpnI. The absorbance assay method is highly vulnerable to the purity of DNA and the pH of DNA dilutions. The measured values often deviate from the actual DNA concentration. Hence, we strongly recommend measuring the DNA concentration through agarose gel electrophoresis.
3. DNA in recombination cyclization is impure, inhibiting the reaction, or the volume of the unpurified product digested by DpnI is more than one-fifth of the total volume. Try performing gel extraction of DpnI-digested products. Avoid using complexing agents (e.g., EDTA) in the recombination reaction. Therefore, we recommend dissolving purified DNA in ddH_2O of pH 8.0 instead of TE buffer.
4. Add excessive amount of DNA in competent cells: the DNA volume should not exceed one-tenth the volume of competent cells; otherwise, it will reduce the transformation efficiency.
5. Transformation inhibitory effect occurs: High concentration of input DNA can inhibit the transformation. In this case, one-fifth of the DNA should be used for transformation.

The site-directed mutation is incorrectly introduced:

1. Primers are not designed correctly. Check the primer design
2. Template plasmids are not methylated. DpnI can recognize only methylated DNA. Please purify the template plasmids from host strains with functional methylases.
3. Use excessive plasmids as the template. For most plasmids, 1 ng of DNA is sufficient as the template for the PCR reaction. Excessive plasmids will lead to incomplete digestion by DpnI, which reduces the successful rate of mutation introduction.

Mutations at the nontarget site:

1. The template plasmid carries some unknown mutations: confirm the template sequence.
2. Too many amplification cycles: to prevent nontarget mutations during amplification, amplification cycles should not exceed 30 when amplification efficiency is good.

Special Remarks

1. When choosing the reverse complementary region of primers, please avoid repetitive sequences. When the GC content is 40%–60%, cyclization recombination efficiency is maximized. If the GC content is >70% or <30%, the cyclization efficiency is significantly inhibited.
2. The double-base mutation strategy can be used for single-base mutations (one of the two sites do not undergo base modification). Therefore, if amplification cannot be performed in a single-base mutation, use the double-base mutation strategy.