GenieClone Site-Directed Mutagenesis Kit v2

Code: MORV0006 **Code: MORV0006-25**
Pack Size: 10 Reactions **Pack Size: 25 Reactions**

AssayGenie

1. Introduction

GenieClone Site-Directed Mutagenesis Kit v2 is designed for rapid site-directed mutagenesis of 3 to 5 separate mutations in the same reaction in less than 3 hours.

Based on GenieClone rapid cloning technology, this kit uses homologous recombination to replace the conventional annealing ring-forming reactions meaning much less template and a more flexible primer design strategy. With seamless splicing of the two PCR products by GenieClone technology, the kit can complete up to two separate mutations with a single amplification reaction. DpnI digested products of the specific amplicons can be directly added to the recombination reaction without purification. The highly optimized reaction buffer, fast protocol and superior site-directed mutagenesis efficiency make GenieClone Site-Directed Mutagenesis Kit v1 make an excellent choice for introducing 3 to 5 mutations at discontinuous sites simultaneously on the same plasmid.

Key Features

- Introduce 3 - 5 separate mutations in the same reaction.
- Site-directed mutagenesis can be performed on up to 5 discontinuous sites (more than 50 bp apart) simultaneously on the target plasmid.
- Single kit for cloning and mutagenesis
- Includes Genie Fusion Ultra High-Fidelity DNA Polymerase providing high-fidelity PCR with extremely low mutation rates & excellent long fragment amplification for any plasmid amplification up to 20 kb as well as GC-rich templates.
- Amplification is carried out exponentially and the template usage is extremely low, which is beneficial to the complete degradation of the original methylation template.
- DpnI eliminates contamination of the original template and decreases background.
- Amplified products can be directly used in the recombination reaction after DpnI treatment.
- The reaction mixture can be used to directly transform chemically competent *E.coli* cells.

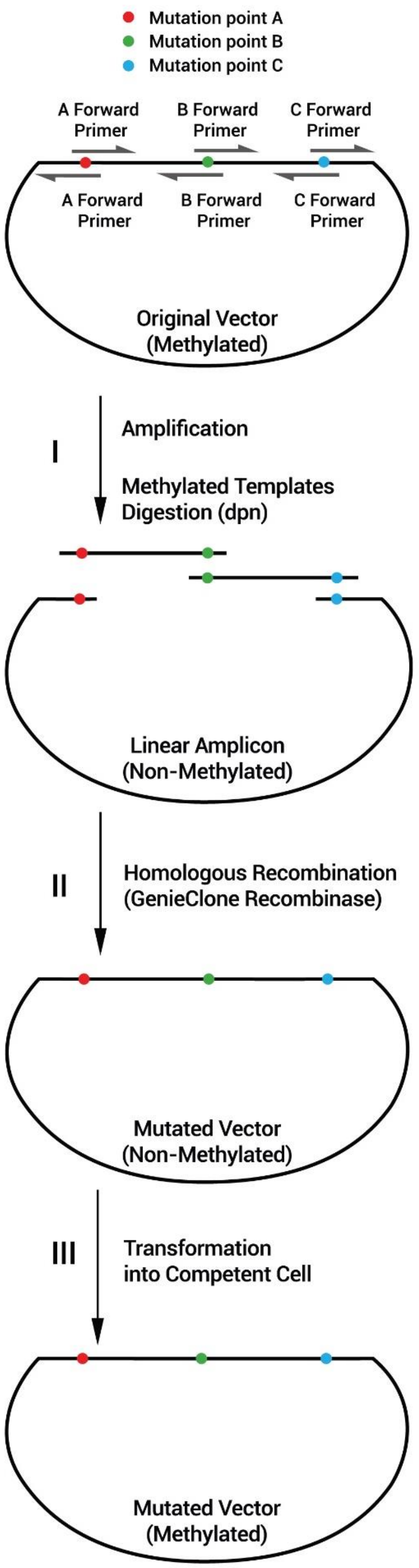
2. Kit Contents

Components	MORV0006 – 10 rxn	MORV0006 – 25 rxn
2x Max Buffer	1.25 ml	3 x 1.25 ml
dNTP Mix (10mM each)	50 µl	125 µl
Genie Fusion Ultra High-Fidelity DNA Polymerase	50 µl	125 µl
DpnI (10U/µl)	50 µl	125 µl
5 x CE Buffer	40 µl	100 µl
GenieClone Recombinase	20 µl	50 µl

3. Storage

Store the product at -20°C, and it will be valid for 1 year.

4. Multi-Site | Site-Directed Mutagenesis (50bp+ between two mutation sites)



The protocols for three to five bases (50bp+ between two mutation sites) site-directed mutagenesis are similar. Below is the process for introducing 3 mutations:

4.1 Overview of experimental process (Figure 1)

1) Primer design (refer to 4.2);
2) Amplification of the target plasmid (Figure 1, I, refer to 4.3);
3) Amplification products treated by DpnI to remove the methylated template plasmid (Figure 1, I, refer to 4.4);
4) Recombination reaction (Figure 1, II, refer to 4.5);
5) Transformation with the recombination reaction products; plating of the transformants; Colony identification (Figure 1, III, refer to 4.6).

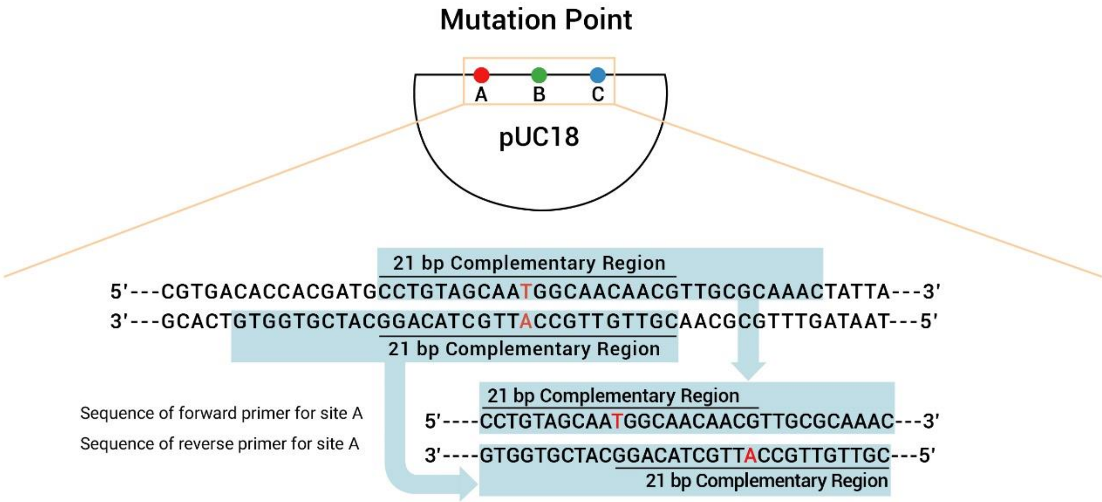
Figure 1: Separate three base site-directed mutations

Choose the mutation sites A, B, and C as the boundary to divide the vector into fragments AB, BC, and CA. Design reverse complementary primers containing three mutation sites. Amplify fragment AB (A Forward Primer and B Reverse Primer), fragment BC (B Forward Primer and C Reverse Primer) and fragment CA (C Forward Primer and A Reverse Primer) with original plasmid as template (Figure 1, I). Amplification product treated by DpnI (Figure 1, I) and used directly in the recombination reaction (Figure 1, II). Recombination product is transformed directly to complete the multiple base site-directed mutagenesis (Figure 1, III).

4.2 Primer design

To introduce site-directed mutations of three separate nucleotides in the plasmid, three pairs of primers to amplify the plasmid in three parts are needed. The basic principles for primer design is as follows:

The 5' ends of reverse and forward primers comprise 15-21 bp reverse complementary regions. The mutation sites can be in the complementary region (the mutation should be introduced in both primers) or in the non-complementary region of any one of primers. The mutation site should NOT be at the end of the primer. Figure 2 shows the detail of primer design, illustrated with the case of introducing three base mutations into vector pUC18.



Note: Primer design of primers at mutation sites B and C is the same as site A. Calculation of the Tm value of primer should be based on the region between the mutation site to the 3 ' end of the primer. Tm value should exceed 60°C by adjusting the length of primer. Please note that the region between the mutation site to the 5 ' end of the primer should not be included for calculation of the Tm value.

4.3 Target Plasmid amplification

The plasmid is divided in to fragments AB, BC and CA by mutation sites A, B, C. Use Genie Fusion Ultra High-Fidelity DNA Polymerase to amplify the three fragments. The primers to amplify AB are forward primer of mutation site A and reverse primer of mutation site B; The primers to amplify BC are forward primer of mutation site B and reverse primer of mutation site C. The primers to amplify CA are forward primer of mutation site C and reverse primer of mutation site A.

Use Genie Fusion Ultra High-Fidelity DNA Polymerase to amplify the target plasmid. Each component should be mixed well after thawing and placed back at -20 °C after use. The recommended reaction system is as follows:

ddH_2O	Up to 50 µl
2 x Max Buffer	25 µl
dNTP Mix (10mM each)[a]	1 µl
Template DNA [B]	Optional
Primer 1 (10µM)	2 µl
Primer 2 (10 µM)	2 µl
Genie Fusion Ultra High-Fidelity DNA Polymerase [c]	1 µl

a. Do not use dUTP, nor any primer and template containing uracil.
b. Given the normal amplification of the plasmid, use as little template as possible. 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 Genie Fusion Ultra High-Fidelity DNA Polymerase is 0.5 U to 2 U per 50 µl. No more than 2 U per 50 µl is recommended especially when the amplicon is longer than 5 kb.

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

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

a. For most plasmids, the appropriate denaturation temperature is 95℃.
b. Genie Fusion Ultra High-Fidelity DNA Polymerase can promote the 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 find the optimal temperature for primer binding to template. Too long annealing time may cause dispersed amplification products.
c. Long extension time can improve the yield of the amplification products.
d. In order to prevent the introduction of non-target mutation, we recommend that the amplification cycle is less than 35 cycles. If the amplification efficiency is good, we recommend the amplification cycle to be less than 30. After the PCR reaction, a small amount of amplification products are run via gel electrophoresis. If the target plasmid is correctly amplified, continue with the next step.

4.4 DpnI Treatment

The amplification product of step 4.3 includes original template plasmid. DpnI treatment before recombination cyclization is required to prevent false-positive transformants. The recommended reaction system is as follows:

Dnpl	1 µl
Amplification product	40 ~ 50 µl

Place the reaction mixture at 37℃ for 1 to 2 hours. If the amplification product of 4.3 is a single band, the DpnI digested products can be used in subsequent recombination reaction without purification. If the product is not a single band, gel extraction purification should be performed before the next step.

4.5 Recombinant reaction

The 5’ ends of forward and reverse primers share a complete reverse complementary sequence, and thus homologous recombination can occur between 5’ end and 3’ end of the amplification product catalyzed by GenieClone Recombinase to complete the amplification product cyclization. The following components are added sequentially to the bottom of a 1.5 ml sterile Eppendorf tubes or PCR tube on ice-water bath. If liquid adheres to the wall of the tube, collect the liquid to the bottom of tube by brief centrifugation.

ddH_2O	Up to 20 µl
5 × CE Buffer	4 µl
DpnI digested fragment	X ng
DpnI digested fragment BC	X ng
DpnI digested fragment CA	X ng
GenieClone Recombinase	2 µl

The optimal amount of DNA fragments in the GenieClone Recombinase multi-base mutation recombination reaction system is 0.03 pmol of each fragment. The corresponding mass of DNA fragments can be calculated roughly by the following formula:

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

For example, if fragment AB is 1 kb, fragment BC is 2 kb, and fragment CA is 5 kb the optimal amount of fragment AB digested by DpnI is 20 ng (0.02× 1000 = 20 ng), fragment BC is 40 ng (0.02 × 2000 = 40 ng), and fragment CA is 100 ng (0.02× 5000 = 100 ng)

Note: Suboptimal DNA amounts will reduce the cyclization efficiency. Confirm the DNA concentration by gel electrophoresis in advance, and mix the components strictly in accordance with the recommended amount. When the calculated optimal amount is less than 20 ng or more than 200 ng, then add 20 ng or 200 ng. When the product digested by DpnI is used directly in the recombination reaction, the volume of product should be less than 1/5 of the total volume.

After addition of all the components, mix the reaction system by gently pipetting up and down several times with a pipette and avoid air bubbles (do not vortex or shake vigorously). Incubate the tube at 37℃ for 30 min. After the incubation, place the tube on ice for 5 min. The product can be transformed directly or stored at -20℃.

4.6 Transformation and identification of clones

Add 20 µl of the cooled reaction mixture to 200 µl of competent cells. Mix gently by flicking the tube and place the tube on ice for 30 min. Heat shock the reaction by incubating the tube at 42℃ for 45 ~ 90 sec and incubate on ice for 2 min. Add 900 µl of SOC or LB medium and incubate at 37℃ for 10 min.
Incubate by shaking (150 rpm) for 45 min at 37℃ and plate 100 µl of the bacterial culture on a selective plate. Incubate overnight at 37℃.

Note: We recommend competent cells whose transformation efficiency is higher than 10^8 cfu/µg. If not, centrifuge your bacterial culture at 5,000 rpm for 3min to collect bacteria, resuspend with 100 µl of LB medium, and then plate all the bacterial cells.

5. Notes

The following table lists main considerations when using this kit for site-directed mutagenesis (Table 1):

Experimental procedure	Correct	Incorrect
The selection of reverse complementary region of primers	Choose a region of non-repetitive sequence with evenly distributed GC content. When GC content of the selected region is 40% ~ 60%, the recombination cyclization will reach maximum efficiency.	Do not select a region with repetitive sequence or high GC or high AT content
Primer design	Design as shown in Figure II or IV.	The reverse complementary region is shorter than the recommended length or including incorrect sequence.
The selection of experiment scheme	For two mutation sites, if the distance of two mutation site is more than 50 bp, choose the protocol 5. If the distance is shorter than 50 bp, choose protocol 4.	Ignore the distance of two mutation sites and choose the protocol 5.
Amplification of the plasmids	Perform highly specific amplification.	Amplification product is not specific with many non-specific PCR products
Template	Use as little template as possible with optimal PCR amplification products	Use too much template.
The template of PCR should be methylated plasmid	Use the host strain with methylase (e.g. Top10, DH5α, JM109) to extract the original plasmids.	Use the host strain without methylase to extract the original plasmid.
The quality of template	Long-term storage, repeated freezing and thawing may cause the breakage, open-loop or degradation of plasmids. So, we recommend to use freshly prepared plasmids as template.	Use the plasmids with long-term storage, repeated freezing and thawing as templates.
Purification of DpnI treated products	If the product is not a single band, gel extraction is required.	Gel extraction is not done when the product is not a single band.
DNA quantification of DpnI digested products	Quantify by agarose gel electrophoresis.	Quantify by absorbance assay.
Preparation of the recombination reaction	Prepare reaction on ice. Prepare the reaction with the recommended optimal amount of DNA with optimal ratio. When the DpnI digested product is used directly in the recombination reaction, the volume of product should be less than 1/5 of the total volume.	Prepare reaction at room temperature. Use random amount of DNA. When the DpnI digested product is used directly in the recombination reaction, the volume of product is more than 1/5 of the total volume.
Recombination reaction	Put tubes at 37°C for 30 min in the instrument with precise temperature control. (PCR instrument or water bath)	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 on ice 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 on ice before transformation. Keep the product at -20°Cfor long-term storage.	The cooled products are placed at room temperature for a long time before transformation. Store at 4°C for a long time before the transformation.

6. Troubleshooting

1. Plasmids cannot be amplified.

a) Primer design is wrong: re-check the primer design.

b) The amplification reaction mixture was not correctly prepared: repeat experiment.

c) The amplification reaction is not optimized: the concentration of Mg^{2+}, the amount of enzyme and the amplification program need to be optimized.

d) The quality of template is poor: long-term storage, repeated freeze-thawing can cause the breakage, open-loop or degradation of the plasmids. Use freshly prepared plasmids as templates.

2. There are no or few colonies on the plate.

a) The efficiency of the competent cells is too low. Use freshly prepared competent cells or competent cells stored properly and make sure the transformation efficiency of competent cells is more than 10^7 cfu/μg by.

b) The amount of DNA or ratio of fragments is suboptimal in the recombination reaction. Add the amount of DNA as recommended. Check the concentration of DpnI treated product. DNA concentration must be measured agarose gel electrophoresis and not by any other method such as an absorbance assay.

c) The DNA in the recombination cyclization contains impurities inhibiting the reaction; or the volume of unpurified DpnI treated product is more than one fifth of total volume. Perform gel extraction of DpnI treated products. Try to avoid complexing agent (e.g. EDTA) in the recombination reaction. Therefore, we recommend that the purified DNA should be dissolved in ddH_2O of pH 8.0 instead of TE buffer.

d) Addition of too much DNA to the competent cells: the volume of DNA should not exceed 1/10 the volume of competent cells, otherwise it will reduce the transformation efficiency.

e) Transformation inhibition: High concentration of input DNA can inhibit the transformation. In this case, one fifth of the DNA should be used for transformation.

3. Incorrect site-directed mutation

a) The primers are not designed correctly. Check the primer design

b) The template plasmids are not methylated. DpnI can only recognize methylated DNA. Purify the template plasmids from the host strains with functional methylases.

c) Too much plasmid used as template. For most plasmids, 1 ng of DNA is enough template for the PCR reaction. Too much plasmid will lead to incomplete digestion by DpnI, which reduces the successful rate of mutation introduction.

4. Mutations at a non-target site

a) The template plasmid carries some unknown mutations: confirm the sequence of the template.

b) Too many amplification cycles: to prevent non-target mutations during the amplification, the number of amplification cycle should not exceed 30 when the amplification efficiency is good.

5. Other Notes

a) When choosing the reverse complementary region of primers, avoid repetitive sequences. When GC content is 40% to 60%, cyclization recombination efficiency is maximized. If the GC content is higher than 70% or less than 30%, the cyclization efficiency will be significantly inhibited.

b) The double-base mutation strategy can also be used for single base mutations (one of the two sites will not undergo base modification). Therefore, if the amplification cannot be carried out in single base mutation, try to use the double base mutation strategy

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