Mut Express II Fast Mutagenesis Kit V2

Catalog # C214

Version 6.1

GeneBio Systems, Inc.™

Vazyme

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1. Introduction

Mut Express II Fast Mutagenesis Kit V2 is designed for rapid site-directed mutagenesis based on ClonExpress rapid cloning technology. The amplified target fragments are digested by DpnI, cyclized by ClonExpress, and then transformed to E. coli to finish the site-directed mutagenesis. The kit consists of two modules: Phanta Max Super-Fidelity DNA Polymerase amplification module and ClonExpress rapid cloning module. The ultra-fidelity of Phanta Max can significantly reduce the possibility of unexpected mutations during amplification of up to 20 kb in length. The ClonExpress rapid cloning module allows homologous recombination reactions to replace the conventional annealing ring-forming reactions, requiring much less template and more flexible primer design strategy. Due to the efficient seamless splicing of the two PCR products by ClonExpress technology, the kit can complete the two separate sites mutation by single amplification reaction. DpnI digested products of the specific amplicons can be directly added into recombination reaction without purification. Highly optimized reaction buffer, fast operating procedures and amazing site-directed mutagenesis efficiency make Mut Express II Fast Mutagenesis Kit V2 the preferred kit for DNA site mutation.

Product advantages

- Phanta Max Super-Fidelity DNA Polymerase provides high-fidelity PCR with the lowest mutation rate.
- Phanta Max Super-Fidelity DNA Polymerase with excellent long fragment amplification capability can be widely used for any plasmid amplification within 20 kb.
- 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.
- The rapid ClonExpress cloning system can efficiently cyclize PCR products.
- The amplified products can be directly used for recombinant reaction after digestion with DpnI.
- Site-directed mutations can be performed on a single site or two discontinuous sites (more than 50 bp apart) at one time on the target plasmid.

Application

DNA site-directed mutagenesis

Note: please use the methylase non-defective host strains (eg. Top10, DH5α, JM109) to extract the original plasmid when using this kit for site-directed mutagenesis of plasmid.

Version upgrade

- Amplification module is upgraded from Phanta Super-Fidelity DNA Polymerase to Phanta Max Super-Fidelity DNA Polymerase, which has stronger amplification ability, broader templates compatibility and it significantly improved the success rate of amplification with high GC template.
- Optimized primer design method for single site mutation: in this upgraded kit, we recommend “the 5’ end of reverse and forward primer should comprise at least 15-21 bp reverse complementary region (GC content 40%-60%) and at least 15 bp non-complementary region” to replace the previous requirement “the 5’ end of reverse and forward primer should comprise at least 21 bp reverse complementary region and at least 20 bp non-complementary region”. The new primer design method makes the primers 5-10 bp shorter, which not only reduces the length and the synthesis cost of primer, but also significantly improves the amplification efficiency.

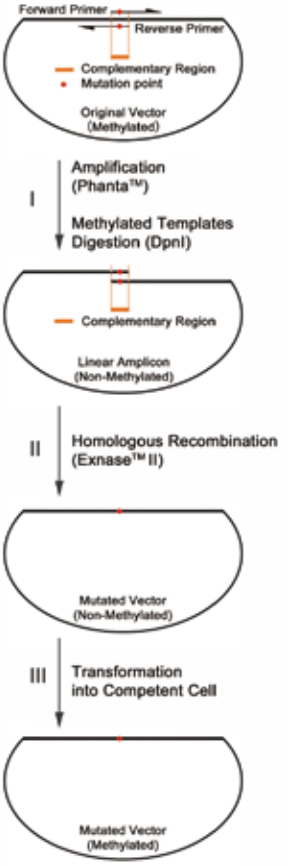
2. Package Information

Components	C214-01 (10 rxn)	C214-02 (25 rxn)
2 × Max Buffer	1.25 ml	1.25 ml
dNTP Mix (10 mM each)	20 μl	50 μl
Phanta Max Super-Fidelity DNA Polymerase	20 μl	50 μl
DpnI (10 U/μl)	20 μl	50 μl
5 × CE II Buffer	40 μl	100 μl
Exnase II	20 μl	50 μl

3. Storage

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

4. Protocols for single base (or continuous multiple bases) site-directed mutation



4.1 Overview of the 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 digested 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: Single base site-directed mutation use Mut Express II

Design partial reverse complement primers to do reversely amplification with the original plasmid as template. (Figure 1, I)

The amplification product was digested by DpnI, and then was directly used in the recombination reaction. (Figure 1, II)

The recombination product was directly transformed to complete the site-directed mutagenesis. (Figure1, III)

4.2 Primer design

To introduce site-directed mutagenesis of a single base or continuous multiple bases in the plasmid, only one pair of primers for inverse amplification of the plasmid is required. The basic principles for primer design: **the 5' ends of reverse and forward primer comprise 15-21 bp reverse complementary region (GC content 40%-60%) and at least 15 bp non-complementary region (the Tm value of the region between the mutation site and the 3' end of the primer is recommended to be higher than 60℃). The mutation site can be in the complementary region (the mutation should be introduced in both primers) or in the non-complementary region of any one of the 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 a single base mutation into vector pUC18.

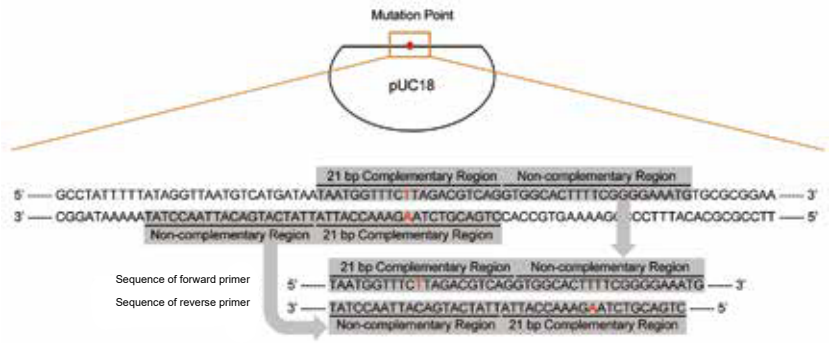


Figure 2. Schematic of introducing single base or continuous multi-bases site-directed mutation into plasmid

Note: 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℃ 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

Use Phanta Max Super-Fidelity DNA Polymerase to amplify the target plasmid. Each component should be mixed well after thawing and placed back at -20℃ in time after use. The recommended reaction system is as follows:

ddH_2O	Up to 50 μl
2 × Max Buffer	25 μl
dNTP Mix (10 mM each) [a]	1 μl
Template DNA [b]	Optional
Primer 1 (10 μM)	2 μl
Primer 2 (10 μM)	2 μl
Phanta Max Super-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 less 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 Phanta Max Super-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	
Annealing [b]	60˚C～72˚C	15 sec	30 [d]
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. Phanta Max Super-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 is subjected to gel electrophoresis. If the target plasmid is correctly amplified, please continue with the next step.

4.4 DpnI digestion of the amplification products to remove the methylated template plasmid.

The amplification product of step 4.3 includes original template plasmid, so 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:

DpnI	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 single band, the DpnI digested products can be used in subsequent recombination reaction without purification. If the product is not single band, gel extraction purification should be performed before the next step.

4.5 Recombination 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 Exnase II 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 sticks onto the wall of tubes, please collect the liquid to the bottom of tube by brief centrifugation.

ddH_2O	Up to 20 μl
5 × CE II Buffer	4 μl
DpnI digestion products	50～400 ng
Exnase II	2 μl

The optimal amount of DNA in the Exnase II single-base mutation recombination reaction system is 0.03 pmol. The corresponding mass of DNA moles can be calculated roughly by the following formula:

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

For example, to introduce a single mutation into a 5 kb plasmid, the optimal amount of DpnI digestion product is 100 ng (0.02 × 5000 = 100 ng).

Note: Too much or too little amount of DNA will reduce the cyclization efficiency. Please 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 50 ng or more than 400 ng, please add 50 ng or 400 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, please mix the reaction system by gently pipetting up and down several times with a pipette and try to avoid air bubbles (please do not vortex or shake vigorously). Incubate the tube at **37℃ for 30 min**. After the incubation, please place the tube in ice bath for 5 min. The product can be transformed directly or stored at -20℃.

4.6 Transformation, plating, colony identification

20 µl of cooled reaction mixture is added to 200 µl of competent cell. Mix gently by flicking the tube and place the tube on ice for 30 min. Incubate the tube at 42℃ for 45 ~ 90 sec for heat shock. Then incubate it in ice-water bath for 2 min. Add 900 µl of SOC or LB medium. Incubate at 37℃ for 10 min. Incubate by shaking (150 rpm) for 45 min at 37℃. 100 µl of bacterial culture is plated on selective plate. Incubate overnight at 37℃.

Note: We recommend competent cell whose transformation efficiency is higher than 10^8 cfu/µg. If not, please 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. Protocols of separate double bases site-directed mutations (the distance between two mutation sites is more than 50 bp)

5.1 Overview of the experimental process (Figure 3)

1) Primer design (refer to 5.2);

2) Separate amplification of different segments of the target plasmid (**Figure 3**, I, refer to 5.3);

3) DpnI digestion of the amplification products to remove the methylated template plasmid (**Figure 3**, I, refer to 5.4);

4) Recombination reaction (**Figure 3**, II, refer to 5.5);

5) Transformation with the recombination reaction products; plating of the transformants; Colony identification (**Figure 3**, III, refer to 5.6).

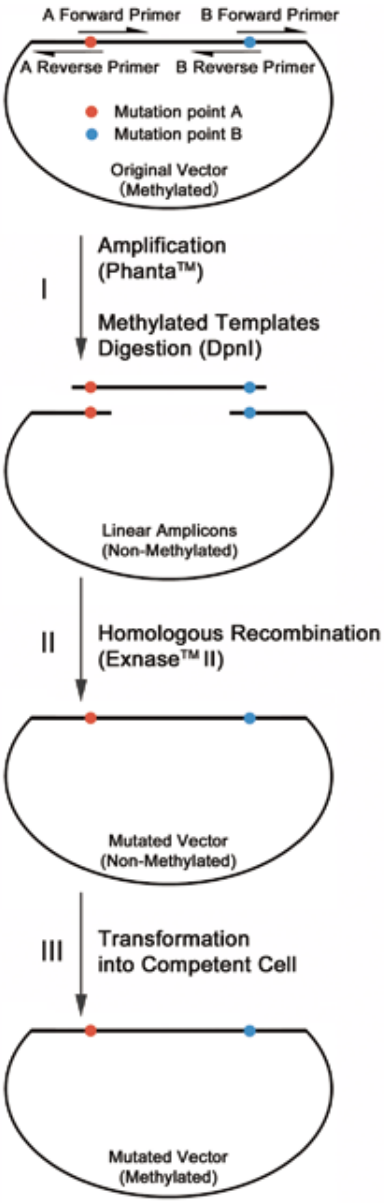


Figure 3: Overview of separate double bases site-directed mutations with Mut Express II

Choose the mutation sites A and B as the boundary to divide the plasmid into fragment AB and fragment BA. Design partially reverse complement primers contain at the two mutation sites.

Amplify fragment AB (A Forward Primer and B Reverse Primer) and fragment BA (A Reverse Primer and B Forward Primer) with the original plasmid as template. (Figure 3, I). Amplification product was digested by DpnI (Figure 3, I), and then used in the recombination reaction (Figure 3, II). Recombination product was transformed directly to complete the double base site-directed mutagenesis (Figure 3, III).

5.2 Primer design

To introduce two separate site-directed mutations in the plasmid, two pairs of primers to amplify the plasmid in two parts are needed. The basic principles for primer design: **The 5' ends of reverse and forward primers comprises 15-21 bp reverse complementary region. 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 4** shows the detail of primer design, illustrated with the case of introducing two base mutations into vector pUC18.

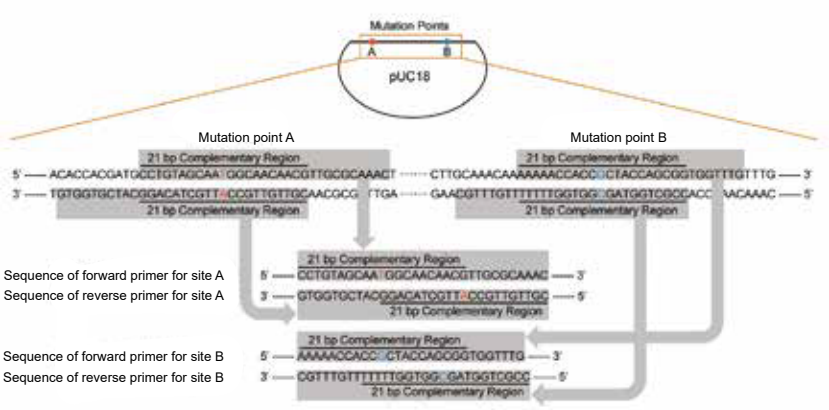


Figure 4: Schematic of primer design for introduction of separate double bases site-directed mutations

Note: 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℃ 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.

5.3 Target plasmid amplification

The plasmid is divided in to fragment AB and fragment BA by mutation sites A and B. Use Phanta Max Super-Fidelity DNA Polymerase to amplify the fragments. The primers to amplify fragment AB are forward primer of mutation site A and reverse primer of mutation site B; The primers to amplify fragment BA are forward primer of mutation site B and reverse primer of mutation site A.

Each component should be mixed well after thawing and placed back at -20℃ in time after use. The recommended reaction system is as follows:

ddH_2O	Up to 50 μl
2 × Max Buffer	25 μl
dNTP Mix (10 mM each) [a]	1 μl
Template DNA [b]	Optional
Primer 1 (10 μM)	2 μl
Primer 2 (10 μM)	2 μl
Phanta Max Super-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 less 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 Phanta Max Super-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 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	
Annealing [b]	60˚C～72˚C	15 sec	30 [d]
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. Phanta Max Super-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 is subjected to gel electrophoresis. If the target plasmid is correctly amplified, please continue with the next step.

5.4 Digested the amplification product by DpnI to remove the methylated plasmid.

The amplification product of step 5.3 includes original template plasmid, so 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:

DpnⅠ	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 single band, the DpnI digested products can be used in subsequent recombination reaction without purification. If the product is not single band, gel extraction purification should be performed before the next step.

5.5 Recombination reaction

The ends of fragments AB and BA share a region with exactly same sequence, and thus homologous recombination can occur between AB and BA catalyzed by Exnase II to complete 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 sticks onto the wall of tubes, please collect the liquid to the bottom of tube by brief centrifugation.

ddH2O	Up to 20 μl
5 × CE Ⅱ Buffer	4 μl
Dpn I digested fragment AB	20～200 ng
Dpn I digested fragment BA	20～200 ng
Exnase Ⅱ	2 μl

The optimal amount of DNA fragments in the Exnase II double-base mutation recombination reaction system is: the long fragment is 0.03 pmol and the short fragment is 0.06 pmol. The corresponding mass of DNA fragments can be calculated roughly by the following formula:

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

The optimal amount of the short fragment digested by DpnI = [0.04 × the number of base pairs of fragment] 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 × 1000 = 40 ng), and fragment BA is 100 ng (0.02 × 5000 = 100 ng)

Note: too much or too little amount of DNA will reduce the cyclization efficiency. Please 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, please 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 components, please mix the components by gently pipetting up and down several times with a pipette, and avoid air bubbles (please do not vortex or shake vigorously). Incubate the tube at **37℃ 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℃.

5.6 The transformation, plating, identification of clone

20 μl of cooled reaction mixture is added to 200 μl of competent cell. Mix gently by flicking the tube and place the tube on ice for 30 min. Incubate the tube at 42℃ for 45 ~ 90 sec for heat shock. Then incubate it in ice-water bath for 2 min. Add 900 μl of SOC or LB medium. Incubate at 37℃ for 10 min. Incubate by shaking (150 rpm) for 45 min at 37℃. 100 μl of bacterial culture is plated on selective plate. Incubate overnight at 37℃.

Note: We recommend competent cell whose transformation efficiency is higher than 10^8 cfu/μg. If not, please 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.

6. Notes

The following table lists main considerations when using Mut Express II for site-directed mutagenesis (Table 1):

Experiment procedure	Should do	Should NOT do
The selection of reverse complementary region of primers	Try to choose a region of non-repetitive sequence with evenly distributed GC. When GC content of the selected region is 40% ~ 60%, the recombination cyclization will reach maximum efficiency.	Select the region of repetitive sequence with high GC or high AT.
Primer design	Design as shown in Figure II or IV.	The reverse complementary region is shorter than the recommended length, or add the wrong 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 short than 50 bp, choose the protocol 4.	Ignore the distance of two mutation sites and choose the protocol 5.
Amplification of the plasmids	Please perform highly specific amplification.	Amplification product is not specific with many non-specific products.
The amount of template	Use as little as possible amount of template if there is no affection on the amplification yield.	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 digested products	If the product is not a single band, gel extraction operation is required.	Gel extraction operation 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 in ice-water bath. 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℃ for 30 min in the instrument with precise temperature control. (PCR instrument or water bath)	The reaction temperature is higher or lower than 37℃. 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 the ice-water bath before transformation. Please keep the product at -20℃ for long-term storage.	The cooled products are placed at room temperature for a long time before the transformation. Store at 4℃ for a long time before the transformation.

Table I: the notes of DNA site-directed mutation using Mut Expresss II

7. Trouble Shooting

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

2. There are no or few colonies on the plate.
a) The efficiency of the competent cell is very 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. Please set up a group transformed with plasmid as control to detect the transformation efficiency of competent cells.

b) The amount of DNA is not enough the recombination reaction. Or the ratio of fragments is not appropriate. Please add the amount of DNA as recommended. Please check the concentration of product digested by DpnI. The method of absorbance assay is highly vulnerable to DNA purity and pH of DNA dilutions. The measured values are often deviated from the actual concentration of DNA. So, we strongly recommend to measure the concentration of DNA by agarose gel electrophoresis.
c) The DNA in the recombination cyclization is not pure, inhibiting the reaction; or the volume of unpurified product digested by DpnI is more than one fifth of total volume. Try to do the gel extraction of DpnI digestion products. Try to avoid complexing agent (e.g. EDTA) in the recombination reaction. Therefore, we recommend that the purified DNA should be dissolved in ddH2O of pH 8.0 instead of TE buffer.
d) Add too much DNA in the competent cell: the volume of DNA should not exceed the 1/10 volume of competent cells, otherwise it will reduce the transformation efficiency.
e) The 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.

3. The site-directed mutation is not correctly introduced
a) The primers are not designed correctly. Check the primer design
b) The template plasmids are not methylated. DpnI can only recognize the methylated DNA. Please purify the template plasmids from the host strains with functional methylases.
c) Use too much plasmids as template. For most plasmids, 1 ng of DNA is enough as template for the PCR reaction. Too much plasmids will lead to incomplete digestion by DpnI, which reduces the successful rate of mutation introduction.

4. Mutations at non-target site
a) The template plasmid carries some unknown mutations: confirm the sequence of the template.
b) Too many number of 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. Special Remarks
a) When choose the reverse complementary region of primers, please avoid the 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 doesn't undergo base modification). Therefore, if the amplification cannot be carried out in single base mutation, try to use the double base mutation strategy.