Mut Express Universal Fast Mutagenesis Kit

C216

Vazyme

Instruction for Use

Version 24.1

Contents

01/Product Description

Mut Express Universal Fast Mutagenesis Kit is based on ClonExpress homologous recombination technology, capable of introducing site-directed mutations to 1 - 5 discontinuous sites on the target plasmid simultaneously in a single operation. This kit integrates the amplification module of 2 × Phanta Flash Master Mix and rapid cloning module of 2 × CE Mix V3. The 2 × Phanta Flash Master Mix features high fidelity and rapid amplification characteristics, and it is a one-tube mix, requiring only the addition of primer and template for amplification. When performing site-directed mutagenesis using the Mut Express Universal site-directed mutagenesis kit, the primer design is flexible, and the Input amounts of template are extremely low, which facilitates the complete degradation of the original methylated template. The 2 × CE Mix V3 can accommodate 1 - 5 site-directed mutations. If the amplification products are specific, the *Dpn* I digestion products can be used directly for recombination reactions without DNA purification, making it the preferred kit for DNA multi-site mutations.

02/Components

	Components	C216-01 (10 rxns)	C216-02 (20 rxns)
☐	2 × Phanta Flash Master Mix	1.25 ml	2 × 1.25 ml
■	Dpn I (10 U/μl)	50 μl	100 μl
■	2 × CE Mix V3	50 μl	100 μl

03/Storage

Store at -30 ~ -15°C and ship at ≤0°C.

04/Applications

◇ Mutation: 1 - 5 mutation sites (multiple bases within 50 bp can be counted as 1 mutation site).

◇ Micro-fragment insertion: Insert a specific sequence of no more than 50 bp at any position on the vector.

◇ Deletion: Perform deletion on specific sequences on the vector.

▲ When using this kit for site-directed mutagenesis of plasmids, please use host strains without methylase defects (such as XL10, DH5α, JM109) to amplify the original plasmid!

05/Self-prepared Materials

Original plasmids and mutant primers;

Competent cells: chemically competent cells prepared from cloning strains;

Other materials: ddH_2O, PCR tubes, PCR machine, etc.

06/Mechanism & Workflow

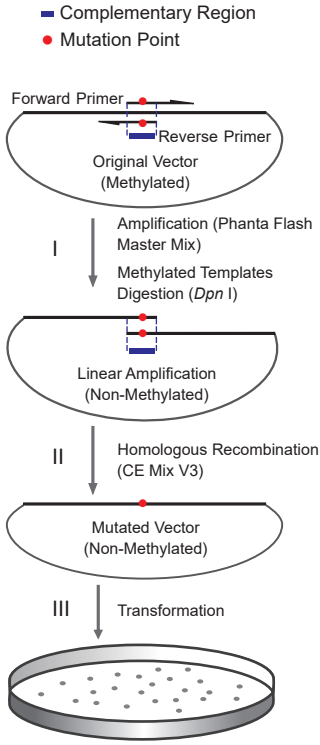

Fig 1A. Flow Diagram of Single-base Continuous Multi-base Mutation

I. Amplification to introduce mutations: design reverse complement primers to amplify the original plasmid by Inverse PCR, and digest the amplification product using *Dpn* I.

II. Recombination reaction: take a certain amount of the amplification product and react at 50°C for 5 min under the catalysis of CE Mix V3 to complete the recombination reaction.

III. Transformation to competent cells: transform the recombinant product directly, and hundreds of clones will grow on the plate for positive screening later.

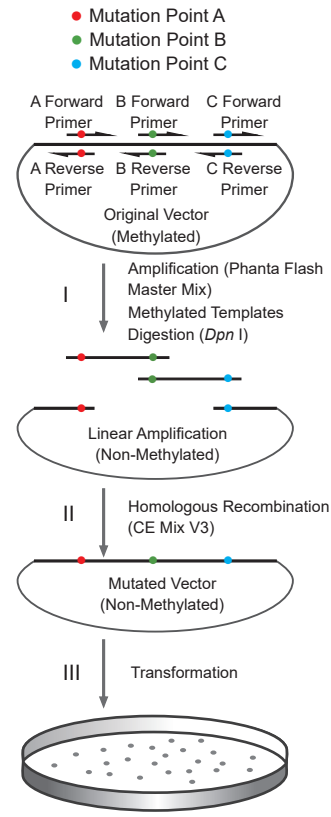

Fig 1B. Flow Diagram of Discontinuous Triple-base Mutation

I. Amplification to introduce mutations: design reverse complement primers at Mutation Sites A, B and C. Use two pairs of primers for cross amplification of AF + BR to obtain fragment AB, and BF + CR to obtain fragment BA, and CF + AR to obtain fragment CA, and digest the amplification product using *Dpn* I.

II. Recombination reaction: mix fragments AB, BC and CA proportionally and react at 50°C for 15 min under the catalysis of CE Mix V3 to complete the recombination reaction.

III. Transformation to competent cells: transform the recombinant product directly, and hundreds of clones will grow on the plate for positive screening later.

07/Experiment Process

07-1/Primer Design Guidance

1. Primer design for single-base (or continuous multi-base) site-directed mutagenesis

To introduce a single base or multiple bases within 50 bp of site-directed mutation into a plasmid, it is only necessary to design a pair of primers to amplify the plasmid by Inverse PCR.

The primers are designed as the following: **5' - 15 - 21 bp reverse complementary region + at least 15 bp non-complementary region - 3'**

- ▲ The GC content of the reverse complementary region should be 40% - 60%, avoid selecting regions with repeated sequences, the Tm value from the mutation site to the primer 3' end >60°C is preferred, the bases within the region from the mutation site to the primer 5' end should not be included in the calculation.
- ▲ The mutation site can be placed within the complementary region (point mutations need to be introduced on both primers), or it can be placed in the non-complementary region of either primer (only one primer needs to introduce a point mutation). Please do not place the mutation site at the end of the primer.

Recommended to log in to Vazyme Group official website - Product Services - Molecular Biology - Resource Support - Experimental Tools - CE Design Primer Design Guidance software (https://crm.vazyme.com/cetool/en-us/simple.html), automatically generate amplification primers. If manual design, refer to the following example:

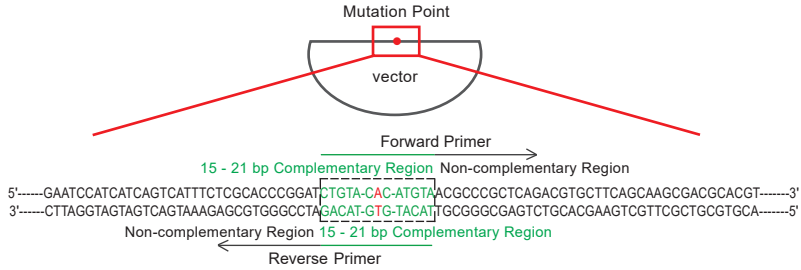

Fig 2. Primer Design for Single-base (or Continuous Multi-base) Site-directed Mutagenesis

2. Primer design for discontinuous multi-base site-directed mutagenesis (with discontinuous three-base mutagenesis as an example).

To introduce site-directed mutagenesis at three discontinuous sites of the plasmid, only three pairs of primers are needed to amplify the plasmid in segments.

The primers are designed as the following: **5' - 15 - 21 bp reverse complementary region + at least 15 bp non-complementary region - 3'.**

▲ The GC content of the reverse complementary region should be 40% - 60%, avoid selecting regions with repetitive sequences, the Tm value from the mutation site to the primer 3' end >60°C is preferred, the bases within the region from the mutation site to the primer 5' end should not be included in the calculation.

▲ The mutation site can be placed within the complementary region (point mutations need to be introduced on both primers), or it can be placed in the non-complementary region of either primer (only one Primer needs to introduce a point mutation). Please do not place the mutation site at the end of the primer.

Recommended to log in to Vazyme Group official website - Product Services - Molecular Biology - Resource Support - Experimental Tools - CE Design Primer Design Guidance software (https://crm.vazyme.com/cetool/en-us/simple.html), automatically generate amplification primers. If manual design, refer to the following example:

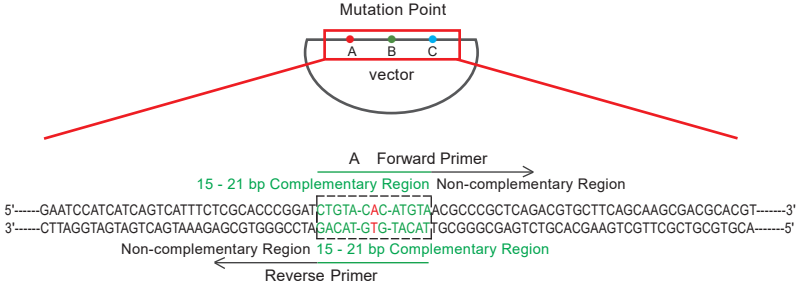

Fig 3. Primer Design for Discontinuous Multi-base Site-directed Mutagenesis

07-2/Amplification of Target Plasmid

Use 2 × Phanta Flash Master Mix to amplify the target plasmid. After thawing the PCR related components, please mix thoroughly. Return to -20°C promptly after use. The recommended reaction system is as follows:

Components	Volume	
ddH_2O	Up to 50 μl	
2 × Phanta Flash Master Mix	25 μl	☐
Template DNA*	Optional	
Primer 1 (10 μM)	2 μl	
Primer 2 (10 μM)	2 μl	

* Do not use dUTP, or primers and templates containing uracil;
As long as the plasmid can be amplified normally, the amount used should be reduced as much as possible. It is recommended to use ≤1 ng of freshly prepared plasmid as a template.

Recommended PCR conditions:

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	35
Annealinga	62 ~ 68°C	5 sec	
Extensionb	72°C	5 - 10 sec/kb	
Final Extension	72°C	1 min	1

* Extension time set according to the length of the target fragment, if the target fragment length ≤10 kb, it is recommended that the extension time be 5 sec/kb; if the target fragment length >10 kb, it is recommended that the extension time be 10 sec/kb.

After the reaction ends, take a small amount of amplification products for agarose gel electrophoresis detection. If the target plasmid is correctly amplified, then proceed to the next step experiment.

07-3/*Dpn* I Digestion of Amplification Products

Due to 07-2/Amplification of Target Plasmid containing the original template plasmid, to prevent the formation of false positive conversion progenies after conversion, *Dpn* I digestion must be performed before Recombination cyclization to remove the methylated template plasmid. The recommended reaction system is as follows:

Components	Volume
Dpn I (10 U/μl)	1 μl
Amplification Product	40 - 50 μl

Please pipette up and down to mix thoroughly, then centrifuge it briefly to the bottom of the tube. Incubate at 37°C for 15 min*.

* If the input amount of the original template plasmid >1 ng, then it is necessary to appropriately extend the digestion time to 1 h.

▲ As 07-2/Amplification of Target Plasmid is specific, *Dpn* I digestion products do not require purification, and can be directly used for subsequent recombination reactions, but the volume of the digestion products added should not exceed 1/5 of the recombination reaction volume. If amplification is not specific, agarose gel electrophoresis should be performed after *Dpn* I digestion is complete and gel extraction and purification target amplification products.

07-4/Determination of Digest Product Concentration

Concentration determination:

If the digestion products have been subjected to Gel extraction and purification using high-quality kits, and no obvious contaminant bands or Smear residues are detected by electrophoresis, concentration determination can be performed using instruments based on absorbance such as OneDrop. However, the concentration value is reliable only when the A260/A280 ratio is between 1.8 - 2.0. It is recommended to use NanoDrop, OneDrop, Qubit, PicoGreen, etc., for concentration determination.

07-5/Amount of Digested Product Used

1. The amount of *Dpn* I digestion products for single-base (or continuous multi-base) site-directed mutagenesis

The optimal amount of DNA for the recombination reaction system of single-base site-directed mutagenesis is 0.03 pmol. The corresponding molar mass of DNA can be roughly calculated according to the following formula:

The optimal mass of *Dpn* I digestion products = [0.02 × fragment base pairs number] ng (0.03 pmol)

2. The amount of *Dpn* I digestion products for discontinuous double-base site-directed mutagenesis

The optimal molar ratio for two-fragment *Dpn* I digestion products of the recombination reaction of discontinuous double-base site-directed mutagenesis is 1:2, with 0.03 pmol of digestion products for the longer fragment and 0.06 pmol for the shorter fragment. The corresponding molar mass of DNA can be roughly calculated according to the following formula:

The optimal mass of *Dpn* I digestion products for the longer fragment = [0.02 × number of fragment base pairs] ng (0.03 pmol)

The optimal mass of *Dpn* I digestion products for the shorter fragment = [0.04 × number of fragment base pairs] ng (0.06 pmol)

For example, if fragment AB is 1 kb and fragment BA is 5 kb, the optimal input of *Dpn* I digestion products should be: 0.04 × 1,000 = 40 ng for fragment AB; 0.02 × 5,000 = 100 ng for fragment BA.

3. The amount of *Dpn* I digestion products for discontinuous multi-base site-directed mutagenesis

The optimal amount of DNA for the recombination reaction system of multi-base site-directed mutagenesis is 0.03 pmol. The corresponding molar mass of DNA can be roughly calculated according to the following formula:

The optimal mass of *Dpn* I digestion products = [0.02 × fragment base pairs number] 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 input of *Dpn* I digestion products should be: 0.02 × 1,000 = 20 ng for fragment AB; 0.02 × 2,000 = 40 ng for fragment BC; 0.02 × 5,000 = 40 ng for fragment CA.

a. Both excessive and insufficient DNA input amounts will reduce the cyclization efficiency. Please prepare the reaction system as strictly as possible according to the recommended amount.

b. The input amount of *Dpn* I digestion products should range between 10 - 100 ng. When the optimal DNA input amount calculated using the above formula falls outside this range, select the minimum or maximum input amount within the range.

c. If *Dpn* I digestion products are used directly in the recombination reaction without purification, the total input amount should not exceed 1/5 of the reaction volume, that is, 2 μl. Please note that recombination efficiency may decrease; it is recommended to purify the digestion product before recombination.

07-6/Recombination Reaction

The unique primer design of Mut Express (refer to 07-1/Primer Design Guidance) allows the digestion products to be efficiently recombined at the target mutation region under the catalysis of 2 × CE Mix V3 to achieve in vitro cyclization of linearized DNA.

1. Prepare the following reaction system on ice:

a. For single-base (or continuous multiple bases) site-directed mutagenesis:

Components	Recombination Reaction
Dpn I digestion product	X μl
2 × CE Mix V3	5 μl
ddH_2O	Up to 10 μl

b. For discontinuous double-base site-directed mutagenesis:

Components	Recombination Reaction	Negative Control[a]
Dpn I digested product for AB fragment[b]	X μl	X μl
Dpn I digested product for BA fragment[b]	Y μl	Y μl
2 × CE Mix V3	5 μl	0 μl
ddH_2O	Up to 10 μl	Up to 10 μl

c. For discontinuous multiple base site-directed mutagenesis:

Components	Recombination Reaction	Negative Control[a]
n *Dpn* I digested products[c]	Z_1 - Z_n μl	Z_1 - Z_n μl
2 × CE Mix V3	5 μl	0 μl
ddH_2O	Up to 10 μl	Up to 10 μl

a. Excessive amplification template amount or incomplete *Dpn* I digestion can easily cause high false positive background, therefore it is recommended to set a negative control.

b. X/Y is calculated according to the formula based on the relative size of AB and BA segments to obtain the specific dosage.

c. Z is the amount of each digestion product calculated based on the formula.

2. Mix the reaction system thoroughly by gently pipetting (DO NOT VORTEX), and centrifuge it briefly to collect the reaction solution to the bottom of the tube.

3. Single-base site-directed mutagenesis and discontinuous double-base site-directed mutagenesis recombination reaction: Incubate at 50°C for 5 min and immediately chill the tube at 4°C or on ice;
   Discontinuous multi-base site-directed mutagenesis recombination reaction: Incubate at 50°C for 15 min and immediately chill the tube at 4°C or on ice.
   ▲ It is recommended to perform the reaction on an instrument with more precise temperature control, such as a PCR machine.
   ▲ This product is compatible with the input amount of 0.003 - 0.25 pmol vectors and inserts, so when the total volume of vectors and inserts is >5 μl, the input amount can be appropriately reduced, but the reaction time should not exceed the recommended time.
   ▲ The recombinant product can be stored at -20°C for one week. Thaw the product before transformation.

07-7/Transformation of Recombinant Product

1. Thaw the cloning competent cells (e.g., Fast-T1 Competent Cell) on ice.
2. Pipette 5 - 10 μl of the recombination products into 100 μl of competent cells, flick the tube wall to mix thoroughly (DO NOT VORTEX), and then place the tube still on ice for 30 min.
   ▲ The volume of recombination products should be ≤1/10 of the volume of competent cells.
3. Heat shock at 42°C water bath for 30 sec and then immediately place on ice for 2 - 3 min.
4. Add 900 μl of SOC or LB liquid medium (without antibiotics). Then, shake at 37°C for 1 h at 200 - 250 rpm.
5. Preheat the corresponding resistant LB solid medium plates in a 37°C incubator.
6. Centrifuge the culture at 5,000 rpm (2,400 × g) for 5 min, discard 900 μl of supernatant. Then, use the remaining medium to suspend the bacteria and use a sterile spreading rod to gently spread on an agar plate which contains the appropriate selection antibiotic.
7. Incubate at 37°C for 12 - 16 h.

07-8/Identification of Recombinant Product

After overnight culture, if the number of clones on the transformation plate is significantly higher than the negative control, several single-cell clones can be picked and inoculated into LB liquid culture medium containing appropriate antibiotics for overnight culture, then plasmids are extracted for sanger sequencing.

08/FAQ & Troubleshooting

◇ Plasmid template cannot be amplified normally

It is recommended to use the Primer Design Guidance software "CE Design", select the corresponding module for design.

① Primer Design Guidance is incorrect: Check the primer design guidance.
② Incorrectly prepared amplification system: Repeat the experiment.
③ Inappropriate amplification reaction conditions: Adjust the amplification program.
④ Low-quality template plasmid: Long-term storage and repeated freeze-thaw cycles may cause breakage, nicking, or degradation of template plasmids. Therefore, use freshly prepared plasmids as the template.

◇ Few or no clones are formed on the plate

① Primer Design Guidance is incorrect: The primer includes 15 - 20 bp homology arms (excluding restriction sites) and the content of GC is 40% - 60%.
② Add insufficient or excessive amounts of DNA or not an appropriate ratio: Please use the amount and ratio according to specifications recommended.
③ Impurity of DNA inhibits the recombination reaction: The total volume of unpurified DNA should be ≤2 μl (1/5 of the total volume of the reaction system). It is recommended that the linearized vector and PCR product are purified by gel extraction. Then, dissolve the purified product in ddH_2O.
④ The low efficiency of competent cells: Make sure the transformation efficiency of competent cells is $>10^8$ cfu/μg. The simple test can be performed. Transform the 0.1 ng of plasmids and take the 1/10 for spreading plates. If 1,000 clones are grown, the estimated transformation efficiency is 10^8 cfu/μg. The transformation volume of recombinant products should be ≤1/10 of the volume of competent cells; otherwise, the transformation efficiency will be reduced. Select competent cells used for cloning (such as DH5α/XL10/Fast-T1). Do not select competent cells used for expression.

◇ Site-directed mutagenesis is not done correctly

① Incorrect primer design: Check the primer design guidance.
② The templates used for amplification reaction are not methylated: *Dpn* I can only recognize methylated DNA. Please use the plasmids amplified from the host strain without defects in methylase as the PCR template.
③ Excessive template plasmids used in the amplification reaction: For most plasmids, 1 ng of input template is sufficient for the amplification. Excessive amounts of template will lead to incomplete *Dpn* I digestion and reduce the success rate of mutagenesis.

◇ Mutations at non-target sites

① Template plasmids carrying unknown mutations: Check if the template plasmid sequence is correct by sequencing.
② Excessive amplification cycles: To prevent non-target mutations during amplification, the number of amplification cycles should be ≤35, If the amplification efficiency is good, the recommended number of amplification cycles should be ≤30.

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