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LiClone™ Fast Mutagenesis Kit

Cat. #: M0012   Size: 10 rxns

Introduction

LiClone™ Fast Mutagenesis Kit is is a site-directed mutagenesis system based on the LiClone homologous recombination technology for introducing single or double point mutations in plasmids. The kit integrates the LiTaq Super-Fidelity DNA Polymerase amplification system and the LiClone rapid cloning system. With superior fidelity, LiTaq significantly reduces the possibility of introducing novel mutations during amplification. Its excellent long fragment amplification ability is widely applicable to the amplification of any plasmid less than 20 kb.

The LiClone rapid cloning system replaces the conventional annealing-cyclization reactions with the more efficient homologous recombination reaction. Site-directed mutagenesis using the LiClone Fast Mutagenesis Kit allows a more flexible primer design and extremely low template input, thereby facilitating complete degradation of the initial methylated template. The highly optimized recombination reaction buffer and enhanced recombinase Exnase II significantly improve the efficiency of recombinant cyclization and tolerance to impurities. If the amplification product is highly specific, its Dpn I-digested product can be used directly in the recombination reaction without DNA purification.

Package Information

Components	M0012
2× LiClone Buffer	1.25 ml
dNTP Mix (10 mM each)	20 µl
LiTaq Super-Fidelity DNA Polymerase	20 µl
DpnI (10 U/µl)	20 µl
5× CE II Buffer	40 µl
Exnase II	20 µl

Storage

Store at -20°C

Additional Materials Required

1. Original plasmid and mutant primer

2. Chemically competent cells prepared from clonal strains: DH5α Competent Cell for routine cloning, compatible with plasmids <15 kb; XL10 Competent Cell for large fragment cloning, compatible with plasmids >10 kb; Fast-T1 Competent Cell for rapid cloning.

3. Other materials: ddH_2O, PCR tubes, PCR instrument, *etc.*

Protocol

1. Primer Design

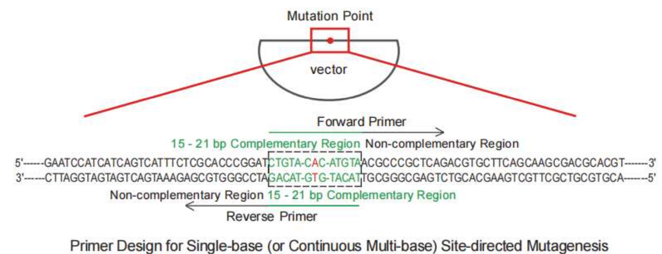
1.1 Primer design for single-base (or continuous multi-base) site-directed mutagenesis.

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'

Note: The optimal GC content in the reverse complementary region ranges between 40% - 60%. Regions with repetitive sequences should not be selected. The optimal Tm value of the region between the target mutation site and the 3' end of the primer is >60℃. The region between the target mutation site and the 5' end of the primer should not be included in the calculation.

The target mutation sites can be in the complementary region (point mutation should be introduced on both primers) or in the non-complementary region of either primer (point mutation is only required to be introduced on one primer). The mutation site should not be at the end of the primer.



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

1.2 Primer design for discontinuous double-base site-directed mutagenesis (two mutation sites more than 50 bp apart)

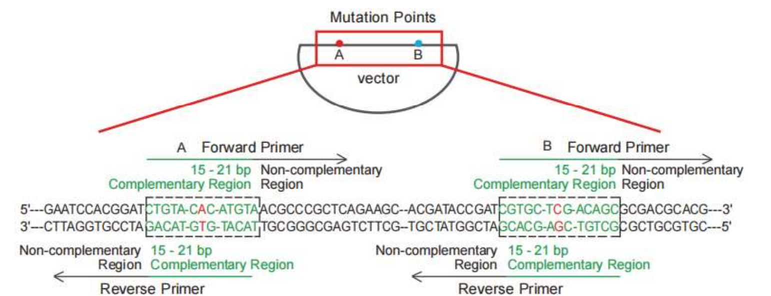
To introduce site-directed mutagenesis into two discontinuous sites that are far apart on the plasmid, it is only necessary to design a pair of reverse-complementary or partial reverse-complementary primers at each site to be mutated, then divide the plasmid into two fragments for amplification.

Note: The method is also suitable for the situation where the plasmid is too large or certain high-order structures cannot be amplified for a single site mutation. The plasmid can be amplified in fragments by adding a site "B" (without introducing any mutation) distant from the target mutation site and performing the recombination reaction following the double-base site-directed mutagenesis protocol.

The primers are designed as the following: 5' - 15 - 21-bp reverse-complementary region + non-complementary region of appropriate size (optional) - 3'

Note: The optimal GC content in the reverse complementary region ranges between 40% - 60%. Regions with repetitive sequences should not be selected. The optimal Tm value of the region between the target mutation site and the 3' end of the primer is > 60°C. The region between the target mutation site and the 5' end of the primer should not be included in the calculation.

The target mutation site can be in the complementary region (point mutation should be introduced on both primers) or in the non-complementary region of either primer (point mutation is only required to be introduced on one primer). The mutation site should not be at the end of the primer.



Schematic Diagram of Primer Design for Discontinuous Double-base Site-directed Mutagenesis

2. Amplification of Target Plasmid

Amplify the target plasmid using the LiTaq Super-Fidelity DNA Polymerase. Thoroughly mix the thawed components of the reaction system by shaking. Reagents should be returned to -20°C storage immediately after use. Recommended reaction system:

Component	Volume
ddH$_2$O	Up to 50 μl
2× Max Buffer	25 μl
dNTP Mix (10 mM each)*	1 μl
Template DNA**	X μl
Primer 1 (10 μM)***	2 μl
Primer 2 (10 μM)****	2 μl
LiTaq Super-Fidelity DNA Polymerase*	1 μl

* Please ensure that the primers and templates do not contain uracil. And do not use dUTP.

** Use the minimum possible amount of input template if it does not affect the amplification result. It is recommended to use ≤1 ng of freshly extracted plasmids as the template.

*** When amplifying the target plasmid in fragments, use Forward Primer A and Reverse Primer B to amplify fragment AB, and use Forward Primer B and Reverse Primer A to amplify fragment BA.

**** The recommended final concentration of the enzyme is 1 U/50 μl per reaction. The optimal concentration of LiTaq Super-Fidelity DNA Polymerase ranges between 0.5 - 2 U/50 μl, but should not exceed 2 U/50 μl, especially if the size of products >5 kb.

Recommended PCR conditions:

Steps	Temperature	Time	Cycle No.
Initial denaturation*	95°C	30 sec	1
Denaturation	95°C	15 sec	
Annealing*	60-70°C	15 sec	30***
Extension**	72°C	30-60 sec/kb	
Final extension	72°C	5 min	1

* LiTaq Super-Fidelity DNA Polymerase promotes the efficient annealing of templates and primers. In general, the annealing temperature is set at the Tm of primers. If required, temperature gradients can be established to determine the optimal temperature for primer-template binding. A prolonged annealing time may cause diffusion of the amplification product on the gel.

** Increasing extension time can improve amplification yield.

*** To prevent the introduction of non-target mutations during amplification, it is strongly recommended that the number of amplification cycles should be ≤35. If the amplification efficiency is superior, it is recommended that the number of amplification cycles should be ≤30.

At the end of the reaction, take a small amount of amplification products for agarose gel electrophoresis. If the target plasmid is correctly amplified, proceed to the next step.

3. *Dpn I* Digestion of Amplification Products

Since the amplification product from step 2 contains the original template plasmid, to prevent it from forming a false-positive transformant after transformation, the amplification product needs to be digested by *Dpn I* to remove the methylated template plasmid before recombination reaction.

Recommended reaction system:

Component	Volume
Dpn I digestion products	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 1 - 2 h.

Note: If the amplification product from step 08-2 is specific, the *Dpn I*-digested product can be used directly in the recombination reaction without purification, but the total input volume should not exceed 1/5 of the total reaction volume. If the amplified product is not specific, purify the target amplification product by gel extraction after *Dpn I* digestion.

4. *Dpn I*-digested product Concentration Determination

Concentration determination:

If the *Dpn I*-digested product has been purified using a high-quality gel extraction kit and no obvious non-specific bands or smears are observed in electrophoresis, its concentration can be determined using spectrophotometry-based instruments such as Onedrop. However, the concentration measured is reliable only when the A260/A280 is within the range of 1.8 - 2.0. Recommended instruments for concentration determination: Nanodrop, Onedrop, Qubit, PicoGreen.

5. *Dpn I*-digested product Input

5.1 *Dpn I*-digested product input for single-base(or continuous multi-base) site-directedmutagenesis

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

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

5.2 *Dpn I*-digested product input for discontinuous double-base site-directed mutagenesis

The optimal molar ratio for two-fragment *Dpn I*-digested products of the recombination reaction of Exnase II 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*-digested products for the longer fragment = [0.02 × number of fragment base pairs] ng (0.03 pmol)

The optimal mass of *Dpn I*-digested 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 Dpn I-digested product input should be: 0.04 × 1,000 = 40 ng for fragment AB; 0.02 × 5,000 = 100 ng for fragment BA.

Both excessive and insufficient DNA input amounts will reduce the cyclization efficiency, especially in the case of double-base mutation. Please prepare the reaction system as strictly as possible according to the recommended amount.

The input amount of *Dpn I*-digested products should range between 20 - 200 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.

If the *Dpn I*-digested 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 4 µl.

6. Recombination

The unique primer design of Mut Express (refer to 08-1/Primer Design Guidance) allows the amplified and digested products to be efficiently recombined at the target mutation region under the catalysis of Exnase II to achieve in vitro cyclization of linearized DNA.

6.1 For single-base(or continuous multi-base) site-directed mutagenesis, prepare the following reaction system on ice:

Components	Recombination Reaction	Negative Control*
Dpn I-digested product	50 - 400 ng	50 - 400 ng
5× CE II Buffer	4 µl	0 µl
Exnase II	2 µl	0 µl
ddH_2O	Up to 20 µl	Up to 20 µl

6.2 For discontinuous double-base site-directed mutagenesis, prepare the following reaction system on ice:

Components	Recombination Reaction	Negative Control*
Dpn I-digested products for AB fragment**	X µl	X µl
Dpn I-digested products for BA fragment**	Y µl	Y µl
5× CE II Buffer	4 µl	0 µl
Exnase II	2 µl	0 µl
ddH_2O	Up to 20 µl	Up to 20 µl

* Excessive amounts of amplification templates or incomplete Dpn I digestion is likely to result in a high false positive rate, so a negative control is recommended.

** The value of "X/Y" for the specific input amount is calculated using the formula based on the relative size of fragments AB and BA.

6.2 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.

6.3 Incubate at 37°C for 30 min and immediately chill the tube at 4°C or on ice.

Note: It is recommended to perform the reaction on an instrument with precise temperature control such as a PCR machine. The recombination efficiency reached the highest at about 30 min of reaction. Insufficient or too long incubation time will reduce cloning efficiency.

The recombinant product can be stored at -20°C for one week. Thaw and transform when needed.

7. Transformation

7.1 Thaw the competent cells on ice (e.g., DH5α Competent Cell).

7.2 Pipette 10 µl of the recombination products to 100 µl of competent cells, flick the tube wall to mix thoroughly (DO NOT VORTEX!), and then place the tube on ice for 30 min.

Note: The volume of recombination products should be ≤1/10 of the volume of competent cells.

7.3 Heat shock at 42°C water bath for 45 sec and then immediately place on ice for 2 - 3 min.

7.4 Add 900 µl of SOC or LB liquid medium (without antibiotics). Then, shake at 37°C for 1 h at 200 - 250 rpm.

7.5 Preheat the corresponding resistant LB solid medium plates in a 37°C incubator.

7.6 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 bent glass rod to gently spread on the plate which contains the appropriate selection antibiotic.

7.7 Incubate at 37°C for 12 - 16 h.

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8. Recombinant Product Identification

After overnight incubation, if the number of clones on the plate is significantly higher than that of the negative control, select and incubate several single clones in the LB liquid medium with appropriate antibiotics overnight, and then extract the plasmids for sequencing.

Troubleshooting

1. The plasmid template cannot be amplified normally

1.1 It is recommended to use the software CE Design for primer design. Select the appropriatemodule for primer design.

1.2 Incorrect primer design: Check the primer design guidance.

1.3 Incorrectly prepared amplification system: Repeat the experiment.

1.4 Inappropriate amplification reaction conditions: Adjust the Mg^{2+} concentration, the amountof enzyme, and the amplification program.

1.5 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.

2. Few or no clones are formed on the plate

2.1 Incorrect primer design: The primer includes 15 - 20 bp homology arms (exclude restriction sites) and the content of GC is 40% - 60%.

2.2 Add insufficient or excessive amounts of DNA or not an appropriate ratio: Please use the amount and ratio according to specification recommended.

2.3 Impurity of DNA inhibits the recombination reaction: The total volume of unpurified DNA should be ≤4 μl (1/5 of the total volume of 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 (pH 8.0).

2.4 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.

3. Few or no clones are formed on the plate

3.1 Incorrect primer design: Check the primer design guidance.

3.2 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.

3.3 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.

4. There are no or few colonies on the plate

4.1 Template plasmids carrying unknown mutations: Check if the template plasmid sequence is correct by sequencing.

4.2 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.