

Site-directed mutagenesis protocol

Materials:

- Takara MutanBEST Kit with the following content
- A pair of primers which juxtapose their 5' ends and have contrary directions of 3' ends to import the mutation;
- Template plasmid;
- Commercial competent cells.

Procedure:

- PCR Reaction
 1. Design and synthesizè two complimentary oligonucleotides containing the desired mutation, flanked by unmodified nucleotide sequence. Purify these oligonucleotide "primers" prior to use in the following steps
 2. Prepare PCR reaction system which has the following composition(total 50 uL)

10x Pyrobest buffer II	5uL
ddNTP Mixture (2.5 mM for each)	4uL
Primer 1 (20uM)	1uL
Primer 2 (20uM)	1uL
Template plasmid	0.01~1ng
Pyrobest DNA Polymerase(5 U/uL)	0.25uL
ddH2O	up to 50 uL
 3. Proceed the reaction at the following reaction conditions
 - 94 degree 30 sec;
 - 55 degree 30 sec;
 - 72 degree 5 min;
 - Repeat the cycle above.
 - 30 cycles in total

Cycle each reaction using the cycling parameters above (For the control reaction, use

a 5-minute extension time).

4. Electrophoresis PCR reaction system in 1% agarose gel.
5. Excise the gel slice and extract the target DNA fragment.

● Blunting Kination Reaction

1. Prepare the following reaction system in a microcentrifuge tube.

DNA Fragment	around 1 pmol
10x Blunting Kination Buffer	2 uL
Blunting Kination Enzyme Mix	2 uL
ddH ₂ O	up to 20 uL

2. React for 10 min at 37 degree.
3. React for 10 min at 70 degree.

● Ligate Reaction

1. Add about 0.25 pmol (5 uL) of solution marked No.3 into a new microcentrifuge tube.
2. Add 5 uL of ligation solution I, mix gently and thoroughly.
3. Spin down the reaction mixtures in a microcentrifuge for 1 minute and immediately incubate each reaction at 37°C for 1 hour to digest the parental. React for 1 hour at 16 degree.
4. Transfer the whole reacted system into 100 ul of competent cells to transform. Swirl the transformation reactions gently to mix and incubate the reactions on ice for 30 minutes to be followed by subsequent steps. When the transformation is finished, plate the appropriate volume of each transformation reaction on agar plates containing the appropriate antibiotic for the plasmid vector.

Notes:

1. To maximize temperature-cycling performance, we strongly recommend using thin-walled tubes, which ensure ideal contact with the temperature cycler's heat blocks. The following protocols were optimized using thin-walled tubes.
2. Set up a series of sample reactions using various concentrations of dsDNA template ranging from 5 to 50 ng (e.g., 5, 10, 20, and 50 ng of dsDNA template) while keeping

the primer concentration constant.