Protocol for Q5® Site-Directed Mutagenesis Kit (NEB #E0554)

Materials Required but not Supplied

Q5® Hot Start High-Fidelity 2X Master Mix

- Template DNA and associated forward and reverse primers
- Nuclease-free Water (NEB #B1500)

KLD Enzyme Mix

- Nuclease-free Water (NEB #B1500)

Overview

Site-directed mutagenesis (SDM) is a method to confer a desired mutation in a double-stranded DNA plasmid using custom-designed oligonucleotide primers. This protocol for the Q5® Site-Directed Mutagenesis Kit can be completed in less than two hours for moderately sized plasmids. We recommend using the NEB online primer design software NEBaseChanger®.

Protocol

Step I: Exponential Amplification (PCR)

1. Assemble the following reagents in a thin-walled PCR tube.

	25 µl RXN	FINAL CONC.
Q5 Hot Start High-Fidelity 2X Master Mix	12.5 µl	1X
10 µM Forward Primer	1.25 µl	0.5 µM
10 µM Reverse Primer	1.25 µl	0.5 µM
Template DNA (1–25 ng/µl)	1 µl	1-25 ng
Nuclease-free water	9.0 µl	

2. Mix reagents completely, then transfer to a thermocycler.

3. Perform the following cycling conditions:

Thermocycling Conditions for a Routine PCR

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds

STEP	TEMP	TIME
25 Cycles	98°C	10 seconds
	50–72°C*	10–30 seconds
	72°C	20–30 seconds/kb
Final Extension	72°C	2 minutes
Hold	4–10°C	

* For a Q5-optimized annealing temperature of mutagenic primers, please use NEBaseChanger, the online NEB primer design software. For pre-designed, back-to-back primer sets, a Ta = Tm + 3 rule can be applied, but optimization may be necessary. When using pre-designed primer sets, the Tm should be calculated based on the homologous binding region of the primer, not including any 5´ non-homologous tails.

Step II: Kinase, Ligase & DpnI (KLD) Treatment

1. Assemble the following reagents:

	VOLUME	FINAL CONC.
PCR Product	1 µl	
2X KLD Reaction Buffer	5 µl	1X
10X KLD Enzyme Mix	1 µl	1X
Nuclease-free Water	3 µl	

2. Mix well by pipetting up and down and incubate at room temperature for 5 minutes.

Step III: Transformation

1. Thaw a tube of NEB 5-alpha Competent *E. coli* (High Efficiency) cells on ice. Add 5 µl of the KLD mix from Step II to the tube of thawed cells. Carefully flick the tube 4-5 times to mix. Do not vortex.

2. Place the mixture on ice for 30 minutes.

3. Heat shock at 42°C for 30 seconds.

4. Place on ice for 5 minutes.

5. Pipette 950 µl of room temperature SOC Outgrowth Medium into the mixture.

6. Incubate at 37°C for 60 minutes with shaking (250 rpm).

7. Mix the cells thoroughly by flicking the tube and inverting, then spread 50-100 µl onto a selection plate and incubate overnight at 37°C. It may be necessary (particularly for simple substitution and deletion experiments) to make a 10- to 40-fold dilution of the transformation mix in SOC prior to plating, to avoid a lawn of colonies

Resources:

- NEBaseChanger