

SITE-DIRECTED MUTAGENESIS

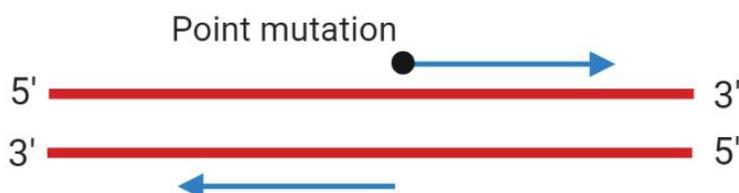
The technique described in this protocol is used to selectively generate a point mutation in your desired sequence. It can also be used to insert or delete nucleotides. To do any of the three above mentioned techniques, primers need to be designed properly. This protocol is divided into 3 sections. Each section represents a day of work in the lab, minus the first section on primer design. This protocol only works if your gene is already cloned inside a plasmid.

Primer Design

All primers are designed to have a minimum of 20 bp overlap with your desired sequence. Match the T_m of both forward and reverse primers, it does not matter if one primer is slightly longer than the other. Primers should not be higher than 50 bp. For primers to include a point mutation, deletion, or insertion, see the notes below.

a. Point mutation(s):

5' ends should be designed to be adjacent from one another, the mutation should be located at this 5' terminus on one of the primers (usually the forward).



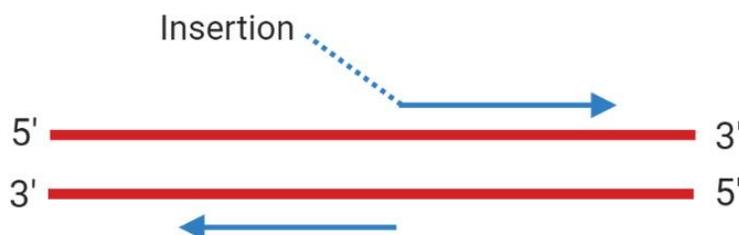
b. Deletion(s):

5' ends of primers should be designed to have a gap between them, the gap corresponds to the bases being deleted.



c. Insertion(s):

5' ends of primers should be designed to be adjacent from one another, the nucleotides to be inserted should be added to the 5' of the forward primer.



Day 1: PCR and DpnI Digestion

NOTE: PCR is done using NEB's Q5 High-Fidelity DNA polymerase. Any high-fidelity DNA polymerase will work. If using a different polymerase, please look at the manufacturer's recommended protocol for PCR amplification, primer T_m calculation and reaction mixture preparation.

1. Prepare a 50 μ L PCR reaction and a (-) control as follows (can be scaled down to 25 μ L):

Reagent	Sample (μ L)	(-) control (μ L)
H ₂ O	32.5	33
Q5 reaction buffer [5X]	10	10
dNTPs mix [10 mM/ea.]	1	1
DNA template [50 ng/ μ L]	1	1
Forward primer [10 μ M]	2.5	2.5
Reverse primer [10 μ M]	2.5	2.5
Q5 High-Fidelity DNA polymerase	0.5	-

2. Design an appropriate PCR protocol, using the below protocol as a template.

	Initial Denaturation	98 °C	30 seconds
19-24 x ↓	Denaturation	98 °C	10 seconds
	Annealing	50-74 °C ^a	30 seconds
	Extension	72 °C	30 seconds/kb ^b
	Final Extension	72 °C	2 minutes
	Hold	12 °C	∞

^a Recommend to use [NEBTm Calculator](#) to calculate theoretical annealing temperature and use the gradient heatblock feature to probe optimal annealing temperature for PCR.

^b You are performing whole plasmid PCR, make sure the extension time is enough to amplify your whole plasmid.

3. Mix 5 μ L of PCR reaction with 1 μ L of Green GoTaq [5X] reaction buffer, run all 6 μ L on an agarose gel.
4. Once you have confirmed amplification worked, perform a PCR clean-up of samples and the (-) control.
5. Prepare a 50 μ L DpnI reaction as follows and incubate at 37 °C overnight. **Template DNA should be isolated from a dam⁺ source (i.e. XL1-Blue, Top10, DH5 α).**

Reagent	Sample (μ L)	(-) control (μ L)
H ₂ O	14.5	14.5
PCR cleanup DNA	30	30
CutSmart buffer [10 X]	5	5
DpnI	0.5	0.5

Day 2: Phosphorylation/Ligation and Transformation

1. Perform a PCR clean-up or a gel extraction on the PCR samples and (-) control using the appropriate kit. Follow the manufacturer's protocol provided with the kit.
2. Prepare a 25 μL phosphorylation/ligation reaction and a (-) control as follows:

Reagent	Sample (μL)	(-) control (μL)
H ₂ O	1.5	2.5
PCR cleanup / gel extracted DNA	20	20
T4 DNA Ligase buffer [10X]	2.5	2.5
T4 PNK	0.5	-

3. Incubate reactions at 37 °C for 1 hour.
4. Add 0.5 μL of T4 DNA Ligase to each reaction, except for the (-) control. Incubate at the following times depending on the desired temperature:

Time	Temperature
2 hours	Room temperature
Overnight (16-20 hours)	16 °C

5. Transform 5-10 μL of reactions into the appropriate competent cells. Follow the transformation protocol found on the Boddy lab website. Plate the whole tube after transformation is complete.
6. Miniprep colonies and confirm mutation/insertion/deletion via sequencing.