Muta-direct™ Site-Directed Mutagenesis Kit

Primer Design

Primers need to be designed before the experiment begins. Below are some points we suggest you to check when you design the primers:

1. Design two strands of primers (forward and reverse). Normally, the primer size is 25~45mer and we recommend length of 30~35mer. In this step, locate the target nucleotide at the center of primer.
2. Calculate the Tm value to find if it reaches 78℃. If the Tm value is under 78℃, adjust the length of primer to reach 78℃ (Minimum GC ratio is 40%).
3. Make sure the primers are at least FPLC or OPC grade (desalting is insufficient). Although most of the manufacturers use OPC purification, it is still necessary to check this point.

Tm formula: **Tm = 0.41(% of GC) – 675/L + 81.5**

L: length of the primer

% of GC: GC ratio of the primer

Primer Design Example

Following is an example of primer design:

Case of **G**CG → **A**CG.

5' CCTCCTTCAGTATGTAG**G**CGACTTACTTATTGCGG-3'

1. First, locate A (or T) to the center of which you want to mutate, and then design 30mer for both the forward and the reverse primer:

   Primer #1: 5'-CCTTCAGTATGTAG**A**CGACTTACTTATTGC-3'

   Primer #2: 5'-GCAATAAGTAAGTCG**T**CTACATACTGAAGG-3'

2. This primer contains 40% of GC and L value is 30. Calculate the Tm value, and the result is 75.5℃ (Tm=0.41×40-675/30+81.5), which is below 78℃. So, this is not an appropriate primer.

3. Next, we adjust the length of the primers:

   Primer #1: 5'-*CCT*CCTTCAGTATGTAG**A**CGACTTACTTATTGC*GG*-3'

   Primer #2: 5'-*CC*GCAATAAGTAAGTCG**T**CTACATACTGAAGG*AGG*-3'

5mer are added to each original primer (italic, underlined). As a result, the GC ratio is 45.7% while the length is 35, and the Tm value is 80.952℃ (Tm=0.41×45.7-675/35+81.5),

and now they can be used for this experiment.

Muta-direct™ PROTOCOL

[A] Induction of Mutagenesis (PCR Reaction)

In this step, you will proceed PCR reaction with Muta-direct™ Enzyme and induce mutagenesis at the target site using the designed primers.

1. Design each primer for site-directed mutation.

   Refer to Primer Design Guide on the 1st page.

2. Prepare plasmid DNA as a template.

   We recommend you to use *dam*$^+$ bacteria (ex. DH5 strain) as host. You can also use other *dam*$^+$ bacteria (except JM110 and SCS110 strain). In case of *end*$^+$ strain, sometimes the colony number could be low, but the mutation efficiency would not be affected. For plasmid DNA extraction, we recommend our SiMax™ Plasmid DNA Miniprep Kit.

3. [Option] Control reaction (50 µl reaction volume)

10 × reaction buffer	5 µl
pUC18 control plasmid (10 ng/µl, total 20 ng)	2 µl
Control primer mix (20 pmol/µl)	2 µl
dNTP mixture (each 2.5 mM)	2 µl
dH_2O	38 µl
Muta-direct™ Enzyme	1 µl

4. Sample reaction (50 µl reaction volume)

10 × reaction buffer	5 µl
Sample plasmid (10 ng/µl, total 20 ng)	2 µl
Sample primer (F) (10 pmol/µl)	1 µl
Sample primer (R) (10 pmol/µl)	1 µl
dNTP mixture (each 2.5mM)	2 µl
dH_2O	38 µl
Muta-direct™ Enzyme	1 µl

5. PCR procedure

   Follow the PCR reaction condition described below. The final extension step can be omitted.

Cycles	Temperature	Reaction Time
1 cycle	95℃	30 sec
15 cycles	95℃	30 sec
	55℃	1 min
	72℃	1 min per plasmid Kb

6. Put it on ice for 5 minutes, then store at RT. Avoid frequent freeze-thawing.

[Note] In the PCR condition described above, control the PCR cycle number.

Please be noted that the mutagenesis efficiency could be very low in a case that more than 4 nucleotides are mutated.

Mutation	Cycles
1~2 nucleotide	15 cycles
3 nucleotides	18 cycles

[B] Selection of mutated plasmid

In this step, you will select mutated plasmid DNA by digestion of the methylated plasmid with Mutazyme™ Enzyme after PCR reaction.

1. Prepare the product from above PCR reaction.
2. Incubate the sample at 37℃ for 1 hour with 1μl (10U/μl) Mutazyme™ Enzyme.

[Note] Sometimes the Mutazyme™ Enzyme couldn't react well with the sample if using inappropriate amount of plasmid DNA. We suggest you to follow the procedure in this guide for a high mutation efficiency. If the mutation efficiency is still low, you can prolong the reaction time or increase the amount of the Mutazyme™ Enzyme.

[C] Transformation

This step recovers the nick on the plasmid DNA after reaction. When you transform the plasmid into *E. coli*, use *dam*$^+$ strain competent cell like DH5a.

1. Add 10 μl plasmid sample to 50 μl competent cell, then put it on ice for 30 minutes.
2. Follow the general steps of transformation method.

SEQUENCING ANALYSIS

- White colonies on LB plate resulted by Muta-direct™ protocol are supposed to be 100% mutated.
- To further confirm, sequencing analysis is recommended to be performed afterward.

MUTAGENESIS EXAMPLE

G**G**C→G**A**C [Reaction Mixture]:

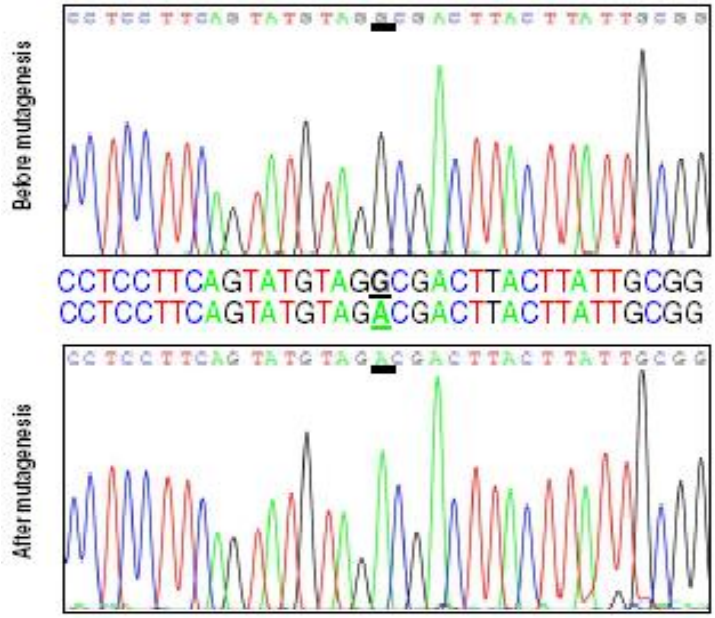
10×reaction buffer	5 μl
Sample plasmid (6.3Kb) (10ng/μl, total 20ng)	2 μl
Sample primer (F) (10pmol/μl)	1 μl
Sample primer (R) (10pmol/μl)	1 μl
dNTP mixture (2.5mM each)	2 μl
dH_2O	38 μl
Muta-direct™ Enzyme	1 μl

[PCR Condition]

Cycles	Temperature	Reaction Time
1 cycle	95℃	30 sec
15 cycles	95℃ 55℃ 72℃	30 sec 1 min 1 min per plasmid Kb

[Sequencing Analysis]

Sequencing result of mutated plasmid



TROUBLESHOOTING

Trouble	Solution
No colonies	Check the PCR amplification by gel running. If the problem is in the PCR reaction step, adjust annealing temperature.
	Check the efficiency of the competent cell.
Low mutation efficiency	Mutazyme™ Enzyme treatment step might be inappropriate. As this template plasmid can directly transform to the competent cell, the mutation efficiency could be low. Increase the volume of Mutazyme™ Enzyme or extend reaction time.
	Check the amount of template plasmid. Excessive plasmid can lead to low efficiency.
Mutant error	Check the quality of the synthesized primers.