TIANGEN®

# Fast Site-Directed Mutagenesis Kit

For efficient introduction of single or multiple mutations on target gene

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# Fast Site-Directed Mutagenesis Kit

**Cat. No. 4992901**

## Kit Contents

| Contents | 4992901 (20 rxn) |
|---|---|
| FastAlteration DNA Polymerase (1 U/μl) | 20 μl |
| 5× FastAlteration Buffer | 200 μl |
| *Dpn* I restriction enzyme (20 U/μl) | 20 μl |
| 4.5 kb Control plasmid (5 ng/μl) | 40 μl |
| Control primers (5 μM, each) | 80 μl |
| FDM competent cells | 20×50 μl |
| Handbook | 1 |

## Storage

FDM competent cells: Please store the FDM competent cells at -90~-65°C right after you receive it. Cells would be stable for 6 months at -90~-65°C.

All other components should be stored at -30~-15°C, at which they are stable for one year.

## Introduction

*In vitro* site-directed mutagenesis is a crucial technique for the modification and optimization of target gene, studying regulatory site of promoter and the complicated relationship between protein's structure and function. The Fast Site-Directed Mutagenesis Kit allows site-specific mutation at target gene, including single and multiple mutations, insertion or deletion mutations. The Fast Site-Directed Mutagenesis Kit can promise a 90% or even higher mutation rate for introducing a single mutation to target gene. Different from previous low efficient mutagenesis kit, this Fast Site-Directed Mutagenesis Kit does not require multi-round PCR or sub-clones which cost lots of time and labor; only four steps are required to construct mutants (Figure 1).

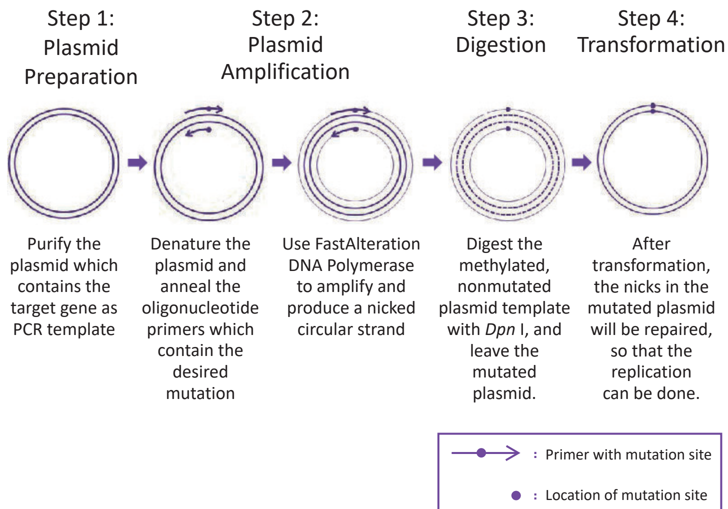


**Figure 1. Overview of the Fast Site-Directed Mutagenesis system**

FastAlteration DNA Polymerase in this kit is a DNA polymerase with high fidelity, high speed and high sensitivity. It could amplify up to 10 kb of plasmid at 15-30 sec/kb. FDM competent cells could digest methylated plasmid within the cell, it means that they could degrade those plasmids which are failed to be degraded by *Dpn* I and then ensure a higher positive rate. Meantime, this kit also contains control plasmid and primers to help customers to locate experimental problems.

## Features

1. **Simple and fast:** Only four steps are required to obtain mutants. Unlike traditional strand displacement amplification (SDA), this kit does not require multi-round PCR or the construction of sub-clones.
2. **High-efficiency primers:** Part-overlapping primers were designed to generate more desired mutated plasmids by PCR amplification.
3. **Wide applications:** Both single and multiple mutations can be applied by using this kit, and the mutation sites can be up to five within a single plasmid.
4. **High suitability:** Size of the target plasmid can be up to 10 kb, which means almost all the common used plasmids suit to this kit.
5. **High mutation rate:** Methylated plasmid can be digested both *in vivo* and *in vitro* by using this kit, ensuring higher mutation rate. And for single mutation, the mutation rate is higher than 90%.

## Principles of primer design

1. Both of the mutagenic primers must have the desired mutation sites on them, and the primers' sequences should be complementary with the target plasmid except for the mutation site.
2. If there is only one mutation site in a primer, it should be designed as what is showed in Figure 2-A. This kind of primer should contain two parts: 5' overlapping area and 3' extension area. Primers should be around 30 bases in length which include 15-20 bases of 5' overlapping area and at least 10 bases of 3' extension area. The mutation site should locate at the downstream of forward primer's overlapping area and at the 5' end of reverse primer.
3. If there are 2-5 mutation sites in a primer, it should be designed as what is showed in Figure 2-B. The sequences of this primer pair are completely complementary and contain two parts: mutation area and non-mutation area. The length of primer should be around 40 bases, within which the mutation area should be 15-20 bases and the non-mutation area should be at least 10 bases. 2-5 mutation sites could be applied depending on the experimental purpose.
4. Primers must be purified either by high performance liquid chromatography (HPLC) or by polyacrylamide gel electrophoresis (PAGE).

5. The following formula is commonly used for estimating the Tm of primers:

$$Tm = 81.5 + 0.41(\%GC) - 675/N - mismatch\%$$

(N stands for the length of primer)

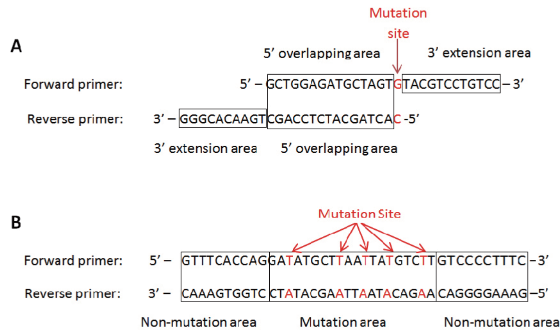


**Figure 2. Principles of primer design**

## Important Notes:

1. When multiple mutations are applied on a single primer, the more mutation sites it contains, the lower mutation rate occurs. Previous data suggested that when five mutation sites were designed on a single primer, the mutation rate were around 50%. We suggest increasing the clones number to verify.
2. This kit allows multiple mutations in multiple primers, which gives a wider range for mutation study in a certain gene. The upper limit of mutation sites is still five.
3. Control plasmid and primers are strongly recommended to be used for the troubleshooting reason.

## Applications

This kit could be used to optimize the target gene or vector, analyze the sites on promoter which interact with regulatory proteins, and study the relationship between the structure and function of proteins.

## Protocol

1. Set up a PCR reaction:

**Note: The reaction mentions below is just an example case, the real reaction condition should depend on the specific circumstance.**

1) Thoroughly thaw and shake the template plasmid, mutagenic primer solutions and all the other PCR reagents.

2) Prepare a reaction solution according to the following table:

| Contents | 50 μl Reaction | Final Concentration |
|---|---|---|
| DNA Template | 10~100 ng | - |
| Forward Mutagenic Primer (10 μM) | 2 μl | 400 nM |
| Reverse Mutagenic Primer (10 μM) | 2 μl | 400 nM |
| 5× FastAlteration Buffer | 10 μl | 1× |
| FastAlteration DNA Polymerase (1 U/μl) | 1 μl | 0.02 U/μl |
| RNase-Free $ddH_2O$ | Up to 50 μl | - |

3) Prepare the control PCR reaction according to the following table.

| Contents | 50 μl Reaction | Final Concentration |
|---|---|---|
| 4.5 kb Control Plasmid (5 ng/μl) | 2 μl | 0.2 ng/μl |
| Control Primers (5 μM, each) | 4 μl | 400 nM |
| 5× FastAlteration Buffer | 10 μl | 1× |
| FastAlteration DNA Polymerase (1 U/μl) | 1 μl | 0.02 U/μl |
| RNase-Free $ddH_2O$ | Up to 50 μl | - |

4) Start the PCR program showed below for the two reactions.

**Note: The PCR program settings could be changed depending on the specific circumstances.**

| Stages | Cycles | Temperature | Time | Contents |
|---|---|---|---|---|
| Initial Denaturation | 1× | 95°C | 2 min | Initial Denaturation |
| PCR Reaction | 18× | 94°C | 20 sec | Denaturation |
| | | 55°C | 10 sec | Annealing |
| | | 68°C | 2.5 min | Extension |
| Final Extension | 1× | 68°C | 5 min | Final Extension |

2. Plasmid template digestion

1) Set up the enzyme digestion reaction according to the following table:

| Contents | 51 μl Reaction | Final Concentration |
|---|---|---|
| PCR product | 50 μl | - |
| *Dpn* I restriction enzyme (20 U/μl) | 1.0 μl | 0.4 U/μl |
| Total Volume | 51 μl | - |

2) Mix the reaction gently and incubate at 37°C for 1 h.

3. Transformation

**Note: This is a general procedure which can be used both on experimental reaction and control reaction.**

1) Take a tube of FDM competent cell from -90~-65°C to ice and thaw.

2) After the cell thaw, add 5 μl of *Dpn* I (add right after the cell thaw) and keep the tube on ice for another 30 minutes.

3) Heat-shock the cell at 42°C for exactly 90 seconds and put the tube back to ice for 2 minutes right away.

4) Add 350 μl sterile SOC or LB medium (without antibiotics) to the tube, mix and then put it in a 37°C shaking incubator for 45-60 min (150 rpm) to revive the cells.

5) Plate all the transformation reaction on LB agar plates containing the appropriate antibiotic for the plasmid vector. Put the plates upside down and incubate at 37°C for 12-16 h.

6) Select the positive clone.

4. Calculate the mutation rate

   The control plasmid and primers are provided to estimate the mutation rate. Plating 20 μl of 0.2 M IPTG and 40 μl 40 mg/ml X-Gal on LB-ampicilin agar-plates, the control plate should have more than 50 colonies on it and the positive clones would show blue. The reason is that the control plasmid is gene modified in the gene coding for lacZ, and a successful mutation would turn it back to wild type, so the mutation rate can be measured by the color of colonies. The formula is:

   $$\text{Mutation rate} = \frac{\text{Number of blue colonies}}{\text{Number of all colonies}} \times 100\%$$

   **Note: Sequencing is required to prove the mutation.**

## Troubleshooting

1. Low transformation efficiency, few or no colony.

| Reason | Solution |
| --- | --- |
| Low yield of the PCR reaction | Increase the amount of digestion product used for transformation to 10 µl. |
| Insufficient template plasmid in PCR reaction system | Quantify the plasmid template by electrophoresis, then adjust the amount of template in PCR reaction. |

2. Low mutation rate or few colonies in control group.

| Reason | Solution |
| --- | --- |
| Inappropriate PCR program setting | Optimize the PCR reaction program and try again to rule out this problem. |
| Low yield of the PCR reaction | Increase the PCR reaction cycles to 25. |
| Competent cells were not stored well | Move the competent cells to -90~-65°C as soon as possible, and place it inside the refrigerator instead of near the door. |
| The amount of x-gal and IPTG is insufficient | Make sure to plate 20 µl 0.2 M IPTG and 40 µl 40 mg/ml X-Gal on LB-ampicillin agar-plates. |
| Repeated thawing and refreezing of 5× PCR buffer | This buffer contains reagent which are susceptible to degradation like dNTP. Avoid thawing and refreezing the buffer too frequently. |

3. Low mutation rate or few colonies in experiment group.

| Reason | Solution |
|---|---|
| Inappropriate PCR program setting | Optimize the PCR reaction program and try again to rule out this problem. |
| The reaction was not mixed properly | Mix the reaction properly by pipetting. |
| The reaction was not mixed properly after the addition of *Dpn* I | Mix the reaction properly by pipetting after the addition of *Dpn* I. |
| Too many plasmids in the transformation reaction | Overhigh concentration of plasmid would have a great negative effect on transformation efficiency. Increasing the amount of *Dpn I* in digesting reaction to 2 μl or increasing the digestion time to 1.5 h could be a good solution. |
| Repeated thawing and refreezing of 5× PCR buffer | This buffer contains reagent which are susceptible to degradation like dNTP. Avoid thawing and refreezing the buffer too frequently. |