Dokdo™
Site Specific Mutagenesis Kit

User Manual

Cat no. EBT-5001

Storage Conditions : -20 ℃
For Research Use Only

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Overview

The Site-Specific Mutagenesis kit is used to introduce point mutations, and deletion or insertion into constructed genes. The site-specific mutagenesis is performed using Pfu Plus DNA polymerase by amplifying whole plasmid DNA with mutant complementary primer pair. Pfu Plus DNA polymerase replicates both strands of double stranded DNA without displacing the mutant primers. The basic procedure utilizes a circular double stranded plasmid DNA (dsDNA) with an insert of interest, and two synthetic oligonucleotide primers containing the desired mutation.

The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by DNA polymerase. Incorporation of the oligonucleotide primers generates a mutated plasmid containing taggered nicks. Following temperature cycling, the product is digested with Dpn I restriction endonuclease (restriction sequence: 5´-GmATC-3´) which is specific for methylated and hemimethylated parental dsDNA. The plasmid DNA isolated from *dam+ E.coli* strain is susceptible to Dpn I digestion. The Dpn I digested PCR product containing desired mutations is then transformed into *E.coli* competent cells.

Highly accurate Pfu Plus DNA polymerase and the low number of thermal cycles all contribute to the high efficiency and the decreased potential for generating unwanted mutations during the reaction.

Pfu Plus Polymerase is effective in the amplification of smaller than 12 kbp PCR products. All the components, except competent cells, are included in the kit including Pfu Plus Polymerase, Dpn I restriction enzyme, buffers, dNTP mix, control vector, and control primers. The reagents are sufficient for 50 separate reactions.

Kit Contents (EBR-5001)

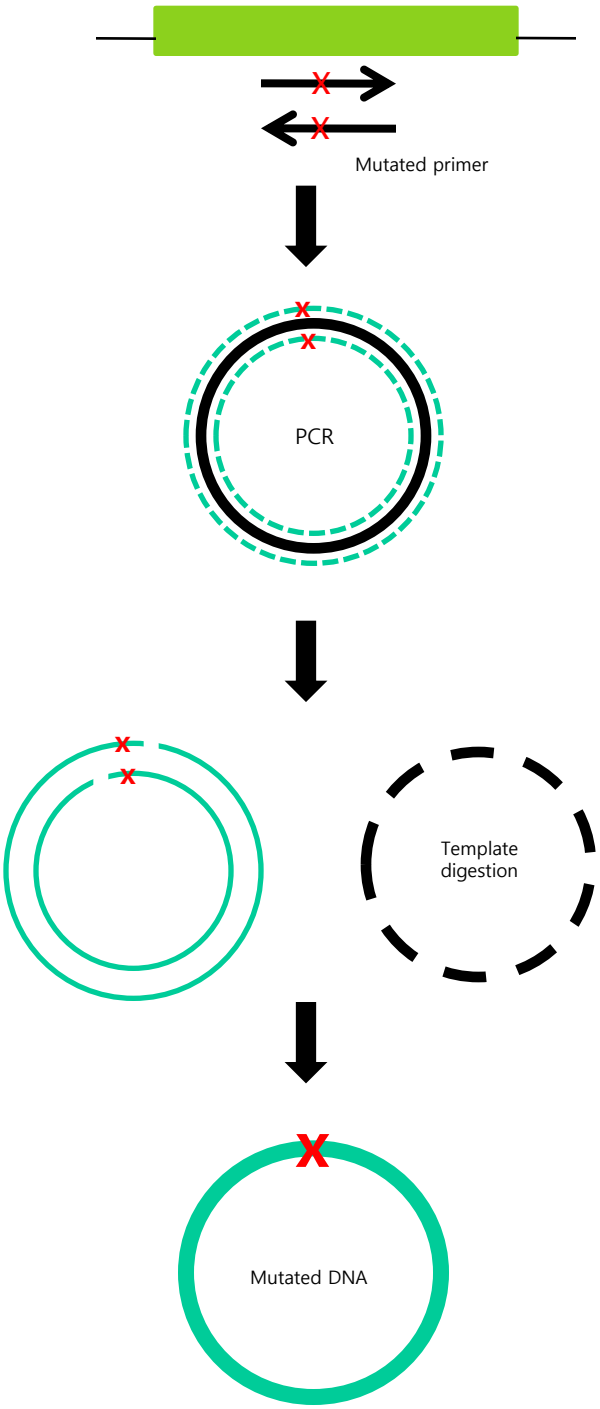
Components	Amount (50 reactions)	Storage
Pfu Plus DNA polymerase (5 U/ µl)	250 U (50ul)	- 20℃
10x reaction buffer	1ml	- 20℃
Dpn I restriction enzyme (10 U/µl)	500 U (50ul)	- 20℃
Sense primer #1 (34-mer, 10pmol/µl) 5´CCATGATTACGCCAAGCGCGTAATTAACCCTCAC 3´	10 µl	- 20℃
Antisense primer #2 (34-mer , 10pmol/µl) 5´GTGAGGGTTAATTACGCGCTTGGCGTAATCATGG 3´	10 µl	- 20℃
pBS control plasmid (5 ng/ µl)	50 ng	- 20℃
dNTP mix (each 2.5mM)	250 µl	- 20℃

Thaw the dNTP mix once, prepare single-use aliquots, and store the aliquots at –20°C. Avoid repeated freezing and thawing.

While plasmid DNA isolated from almost all of the commonly used E. coli *strains (dam+) is methylated and is a suitable template for mutagenesis, plasmid DNA isolated from the exceptional dam–* E. coli *strains, including JM110 and SCS110, is not suitable.*

Pfu plus DNA polymerase has 5-fold higher fidelity in DNA synthesis than Taq DNA polymerase.

Overview of the Site Specific Mutagenesis Method



1. Plasmid prep and primer design
   Point mutation, multiple mutation,
   Insertion, deletion primer pair design

2. PCR
   - Denature DNA template
   - Anneal mutagenic primers
     containing desired mutation
   - Extend anf corporate primers with
     high-fidelity Pfu-Plus DNA polymerase.

3. Dpn I digestion
   Digest the nonmutated parental
   DNA template with Dpn I .

4. Transformation
   transformed the mutated, nicked
   circular DNA into competent cell
   (ex, XL1 Blue)

Quality Control

The performance of Site-Specific Mutagenesis Kit is monitored routinely on a lot number. The pBS control plasmid containing β-galactosidase gene under the control of lac promoter is used to test the efficiency of mutant generation.

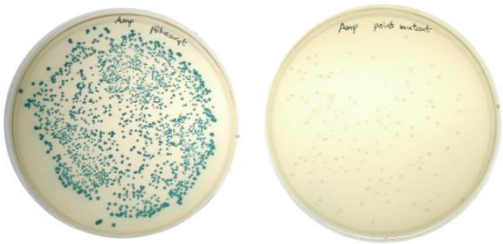
Step 1) PCR with control primer pair (#1,2) using Pfu Plus DNA Polymerase,

Step 2) Dpn I digestion of PCR products to remove template DNA,

Step 3) Transformation into host cells, and screen mutation rate by blue/white assay.

The mutated pBS plasmid contains a stop codon (TAA) at the position where a glutamine codon (CAA) would normally appear in the β-galactosidase. The white phenotype on IPTG/X-gal plate indicates that mutation is normally introduced into control β-galactosidase gene. The overall efficiency of mutagenesis is approximately 98%.

control *point mutation*



Blue colony ***white colony***

Storage Condition

DokDo™ Site specific mutagenesis kit should be stored in freezer (- 20℃). Kits can be stored for up to 12 months without showing any reduction in performance and quality.

Additional Materials Required

PCR reaction tubes

Thermal Cycler

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)

Isopropyl-1-thio-β-D-galactopyranoside (IPTG)

Protocols

Mutant Strand Synthesis (PCR reaction)

Ensure that the plasmid DNA template is isolated from a dam+ E. coli strain. Plasmid DNA isolated from dam– strains (e.g. JM110 and SCS110) is not suitable. The following protocols were optimized using thin-walled PCR tubes.

1. Synthesize two complimentary oligonucleotides containing the desired mutation, flanked by unmodified nucleotide sequence.

2. Prepare the **control PCR reaction** as follows :
   5 µl of 10× reaction buffer
   2 µl (10 ng) of pBS 3kb control plasmid (5 ng/µl)
   1 µl of control sense primer (34mer, 10pmol/µl)
   1 µl of control antisense primer (34mer, 10pmol/µl)
   5 µl of dNTP mix
   35 µl of double-distilled water (ddH_2O) to a final volume of 50 µl Then add
   1 µl of *Pfu Plus DNA polymerase (5 U/µl)*

3. Prepare the **sample PCR reaction** as follows :
   Note *Set up a series of sample reactions using various concentrations of dsDNA template ranging from 5 to 50 ng while keeping the primer concentration constant.*

   5 µl of 10× reaction buffer
   X µl (5–50 ng) of dsDNA template
   X µl of sense primer
   X µl of antisense primer
   5 µl of dNTP mix
   ddH_2O to a final volume of 50 µl Then add
   1 µl of *Pfu Plus DNA polymerase (5 U/µl)*

   * Always, *Pfu Plus DNA Polymerase* should be added last to the mixture.

4. Set PCR cycling as follows :
   Initial denature at 95°C : 3 min

	Temperature	Time
Denature	94°C	20 sec
Anneal	Tm-4°C	20 sec
Extend	72°C	30-60 sec/1kb plasmid length

   > 20 cycles reaction.
   (For the control reaction, use a 2 minute extension time and run the reaction for 25 cycles.

5. Following temperature cycling, place the reaction on ice for 2 minutes to cool the reaction to ≤37°C.
   Note *If desired, amplification may be checked by electrophoresis of 5µl of the product on a 0.7% agarose gel. A band may or may not be visualized at this stage. In either case proceed with Dpn I digestion and transformation.*

Removal of template DNA : Dpn I Digestion

1. Add 1 µl of the *Dpn I restriction enzyme (10 U/µl) directly to each* amplification reaction.

2. Gently mix each reaction mixture by pipetting the solution up and down several times. 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 template DNA (i.e., the nonmutated dsDNA).

Transformation of *E.coli* Competent Cells

Notes *Please read the Transformation Guidelines before proceeding with the transformation protocol.*

1. Gently thaw the *E.coli* competent cells (DH5a, XL1-Blue etc) on ice. For each control and sample reaction to be transformed, aliquot 50 µl of the competent cells.

2. Transfer 1-5 µl (control 5ul) of the *Dpn I-treated DNA from each control and sample* reaction to separate aliquots of the competent cells.

3. Heat shock the transformation reactions for 45 seconds at 42˚C and then place the reactions on ice for 2 min.

4. Add 0.5 ml SOC broth and incubate the transformation reactions at 37˚C for 1hr with shaking at 225-250 rpm.

5. Plate the appropriate volume of each transformation reaction (>250 ul), on agar plates containing the appropriate antibiotic for the plasmid vector. For the mutagenesis controls, spread cells on LB–ampicillin agar plates containing 80 µg/ml X-gal and 20 mM IPTG.

6. Incubate the transformation plates at 37˚C for >16 hours.

Expected Results for the Control Transformations

The expected colony number from the transformation of the pBS control mutagenesis reaction is >50 colonies. Greater than 50-80% of the colonies should contain the mutation and appear as blue colonies on agar plates containing IPTG and X-gal.

Mutagenesis efficiency (%)= (Number of white colony / Total number of colony)× 100%

Expected Results for Sample Transformations

The expected colony number is between 10 and 1000 colonies, depending upon the base composition and length of the DNA template employed. *The insert* of interest should be sequenced to verify that selected clones contain the desired mutation.

Primer Design Guidelines

The mutagenic oligonucleotide primers for use in this protocol must be designed individually according to the desired mutation. The following considerations should be made for designing mutagenic primers:

1. Both of the mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid.

2. Primers should be between 25 and 45 bases in length, with a melting temperature (*Tm) of* ≥*78* ℃. *Primers longer than 45 bases may be used,* but using longer primers increases the likelihood of secondary structure formation, which may affect the efficiency of the mutagenesis reaction.

3. The desired mutation (deletion or insertion) should be in the middle of the primer with ~10–15 bases of correct sequence on both sides.

4. The primers optimally should have a minimum GC content of 40% and should terminate in one or more C or G bases.

Troubleshooting Guide

Question 1. Low transformation efficiency or low colony number

<Check1>
Ensure that sufficient mutant DNA is synthesized in the reaction. Increase the amount of the Dpn I-treated DNA used in the transformation reaction to 4 µl.
<Check 2>
Visualize the DNA template on a gel to verify the quantity and quality. Nicked or linearized plasmid DNA will not generate complete circular product. Verify that the template DNA is at least 80% supercoiled.
<Check 3>
It is not uncommon to observe low numbers of colonies, especially when generating large mutations. Most of the colonies that do appear, however, will contain mutagenized plasmid.
<Check 4>
Low PCR DNA concentration : Ethanol precipitate the Dpn I digested PCR product, and resuspend in a decreased volume of water before transformation.

Question 2. Low mutagenesis efficiency or low colony number with the control reaction

<Check1>
Different thermal cyclers may contribute to variations in ramping efficiencies. Adjust the cycling parameters for the control reaction and repeat the protocol for the sample reactions.
<Check 2>
Ensure that competent cells are stored at the bottom of a –80˚C freezer immediately upon arrival.
<Check 3>
Verify that the agar plates were prepared correctly.
<Check 4>
For best visualization of the blue (β-gal+) phenotype, the control plates must be incubated for at least 16 hours at 37°C.
<Check 5>
Avoid multiple freeze-thaw cycles for the dNTP mix. Thaw the dNTP mix once, prepare single-use aliquots, and store the aliquots at –20°C.
<Check 6>
Adjust the cycling parameters for the sample reaction to overcome differences in ramping efficiencies of thermal cyclers.

Question 3. Low mutagenesis efficiency with the sample reaction

<Check 1>
Carefully add the Dpn I restriction enzyme in the digestion step and ensure proper mixing of all components in the reaction especially the Dpn I.
<Check 2>
Allow sufficient time for the Dpn I to completely digest the parental template; repeat the digestion if too much DNA template was present.
<Check 3>
The formation of secondary structures may be inhibiting the mutagenesis reaction. Increasing the annealing temperature up to 68˚C may help to alleviate secondary structure formation and improve mutagenesis efficiency.

Question 4. False positives

<Check 1>
Poor quality primers can lead to false positives. Check for degradation on an acrylamide gel or resynthesize the primers.
<Check 2>
False priming can lead to false positives. Increase the stringency of the reaction by increasing the annealing temperature up to 68˚C.

Question 5. Low PCR production

Customer & Technical Services

For technical assistance and more information please call one of the Elpis-Biotech., Inc.

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