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Instruction manual KOD -Plus- Mutagenesis Kit 2004

F0936K

KOD -Plus- Mutagenesis Kit

SMK-101 20 reactions

Store at -20°C

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CAUTION

All reagents in this kit are intended for research purposes. Do not use for diagnosis or clinical purposes. Please observe general laboratory precaution and utilize safety while using this kit.

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[1] Introduction

Description

This kit is an inverse PCR (iPCR)-based site-directed mutagenesis kit using KOD DNA polymerase[1) 2)] as a high-fidelity PCR enzyme. This reagent was developed based on a high fidelity and efficient PCR reagent, "KOD-Plus- (Code No. KOD-201)", which consists of KOD DNA polymerase and anti-KOD DNA polymerase antibodies[3)] for Hot Start PCR.

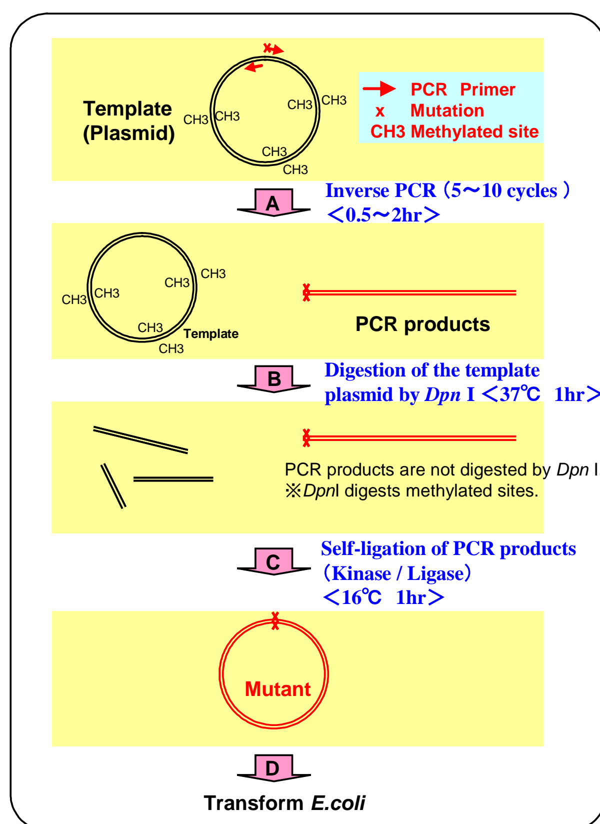
This kit enables not only the introduction of point mutations, but also the introduction of large insertions and deletions. The PCR fidelity of KOD-Plus- is greater than Taq DNA polymerase (ca. 80-fold); therefore, unexpected, 2nd-site mutations can be reduced. PCR reactions can be performed using standard PCR primers and do not require phosphorylated primers, because this protocol contains a 'Phosphorylation Step' of PCR products.

Features

- Applicable for various mutations, such as substitutions, insertions, and deletion mutations.
- High efficiency (95% maximum) can be obtained.
- Simple protocol facilitates speedy experiments. Phosphorylated primers are not required.

[2] Flow chart

The flow chart for this kit is as follows:



(A) Inverse PCR of plasmid DNA, using a mutation primer.

(B) Plasmid DNA is digested by *Dpn* I.

Note: *Dpn* I digests methylated DNA, such as plasmid DNA from typical *E. coli* cell lines (e.g. JM109 and DH5α).

(C) Self-ligation of PCR products is performed by a reaction with T4 polynucleotide kinase and ligase.

(D) Transformation of *E. coli* cells using self-ligated PCR products.

Figure 1. Flow chart of KOD -Plus- Mutagenesis Kit

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[3] Components

The KOD -Plus- Mutagenesis Kit contains enough reagents for 20 mutagenesis reactions, including 5 control reactions.

Component	Volume
KOD-Plus- (1 U/μL)	25 μL
10x Buffer for iPCR	125 μL
2 mM dNTPs	125 μL
*Dpn*I (10 U/μL)	50 μL
T4 Polynucleotide Kinase (5 U/μL)	50 μL
Ligation high (T4 Ligase + Buffer Mixture)	250 μL
Control Plasmid pAK119M (50 ng/μL)	10 μL
Control Primer #1 (10 pmol/μL)	10 μL
Control Primer #2 (10 pmol/μL)	10 μL

＜Additional Materials Required ＞

- Target plasmid DNA (plasmid DNA should be isolated from commonly used *E.coli* strains that bear dam-methylase, such as JM109 and DH5α.)
- Mutagenic primers (normal primers, **not phosphorylated primers**, should be used.)
- Competent cells
- SOC medium and LB agar plates with antibiotics
- 4% X-Gal and 100 mM IPTG (for control reaction)

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[4] Notes

1. Template plasmids

In this kit, template plasmids are digested through treatment with the restriction enzyme *Dpn* I. The template plasmid should be methylated, since *Dpn* I recognizes the G^{m6}ATC (m6 methylated) site. The “GATC” recognition sites of plasmid DNA purified from ordinary host cells (e.g., *E. coli* JM109 or DH5α) are m6-methylated by the host cell’s Dam methylase.

2. PCR primers

(1) Primer design

Mutation sites should be designed at the 5’ terminal end of primers. Specific sites should be designed at the 3’ region of the primers. The optimal length of the specific regions should be ≥ 20 mer (preferably 25 mer).
This kit includes a kination step of the PCR product; therefore, phosphorylated primers are not required.

(2) Quality of primers

Primer quality is critical for obtaining good results. HPLC-grade primers, which do not contain deficient regions at the 5’ termini, are suitable for this experiment. In the case of long primers (≥ 40 mer), PAGE-grade primers are recommended.

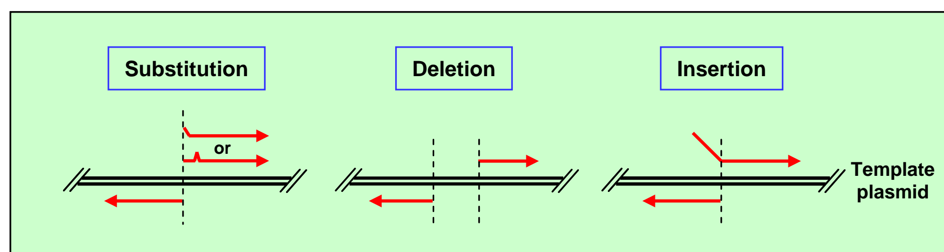

Figure 2. Examples of primer design for site-specific mutations

- **Substitution:** Mutations should be introduced around the 5’ termini of primers.
- **Deletion:** The deletion site should be introduced between the 5’ termini of primers.
- **Insertion**: The insertion site should be introduced at the 5’ termini of primers.
  In the case of a broad insertion, the insertion region can be divided into 2 parts and introduced at the 5’ termini of the forward and reverse primers.

3. PCR conditions

Specific amplification of the target sequence is critical to obtain the expected mutant clones. Consequently, a preliminary amplification test with 10~20 cycles can be helpful to confirm PCR specificity. If specific amplification is obtained, the same cycling conditions can be utilized with fewer cycles.

4. Unexpected mutations (2nd mutation)

KOD DNA polymerase exhibits excellent accuracy in PCR. However, the introduction of unexpected mutations in other primer regions cannot be excluded. Mutants should be confirmed by sequencing.

5. Control plasmid and control reaction

This kit provides a control plasmid (pAK119M), bearing the mutated LacZα gene. The 6th codon has been changed from CCA to TAA (stop codon). Because the mutated LacZα gene exhibits no activity, *E. coli* colonies expressing this gene form white colonies on X-gal agarose plates. A control reaction can be performed using this plasmid and mutation primers. This reaction reverts the stop codon (TAA) to the wild-type codon (CCA).

The control reaction flow chart using the control plasmid provided in this kit as follows:

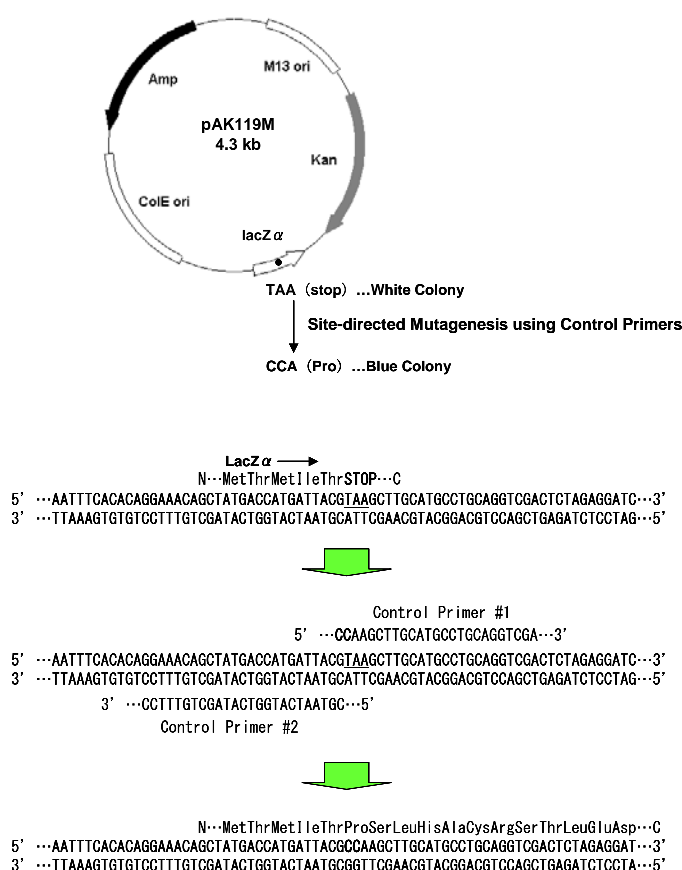

Figure 3. Flow chart of the control experiment.

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[5] Protocol

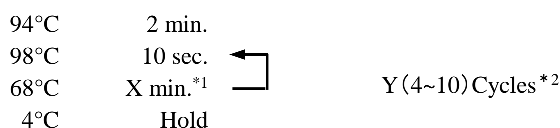
1. Inverse PCR

(1) Prepare PCR primers (10 pmol/µL) and template plasmid (50 ng/µL).

(2) Prepare the PCR reaction mixture as follows:

Component	Volume
PCR grade water	35 µL
10×Buffer for iPCR	5 µL
2 mM dNTPs	5 µL
Primer A (10 pmol/µL)	1.5 µL
Primer B (10 pmol/µL)	1.5 µL
Plasmid Template DNA (50 ng/µL)	1 µL
KOD -Plus- DNA Polymerase	1 µL
Total Volume	50 µL

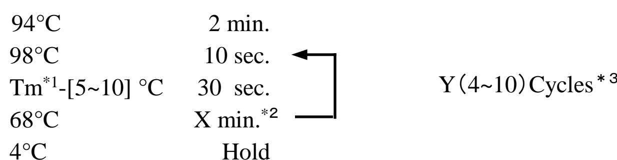
(3) Perform PCR reaction as follows:



[*1] Extension time should be 1 min/kb of plasmid length (e.g., a 5 kb-size plasmid: 5 min.).
[*2] Cycle number should be 1 cycle/kb of plasmid length (e.g., a 5 kb-size plasmid: 5 cycles).

Notes

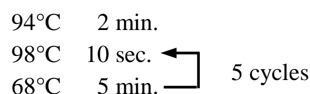
If the Tm value of the specific regions of the primers are less than 73°C, the following 3 step condition is recommended;



[*1] Tm value of the specific region of the primer.
[*2] Extension time should be 1 min./kb of plasmid length (e.g., a 5 kb-size plasmid: 5 min.).
[*3] Cycle number should be 1 cycle/kb of plasmid length (e.g., a 5 kb-size plasmid: 5 cycles).

(4) [Optional] The control reaction is as follows:

PCR grade water	35 μL
10×Buffer for iPCR	5 μL
2 mM dNTPs	5 μL
Control Primer #1 (10 pmol/μL)	1.5 μL
Control Primer #2 (10 pmol/μL)	1.5 μL
Control Plasmid pAK119M (50 ng/μL)	1 μL
KOD -Plus- (1 U/μL	1 μL
Total Volume	50 μL



2. *Dpn*I digestion of the template plasmid

(1) Add 2 μL of *Dpn*I restriction enzyme (10 U/μL) to 50 μL PCR reaction from step 1.

Notes
Add 1 μL of the *Dpn*I restriction enzymes to 25 μL PCR reaction. (half of reaction volume)

(2) Gently and thoroughly mix each reaction mixture by pipetting.

(3) Spin down the reaction mixture and incubate at 37°C for 1 hr to digest the template plasmid.

(Optional) Gel electrophoresis
To confirm PCR amplification, we recommend agarose gel electrophoresis analysis. However, in the case of 5 or less cycles of amplification, it may be only possible to detect a faint band or no band at all. If only a faint or no band is detected, please proceed to the next step.

3. Self-ligation of the PCR Product

(1) Prepare the kination/ligation reaction mixture using a fresh tube as follows:

*Dpn*I- treated PCR product	2 μL
PCR grade water	7 μL
Ligation high	5 μL
T4 Polynucleotide Kinase	1 μL
Total Volume	15 μL

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(2) Gently and thoroughly mix each reaction mixture by pipetting.

(3) Centrifuge the reaction mixture and incubate each reaction at 16°C for 1 hr.

4. Transformation

Standard *E. coli* strains can be used as the transformation host. Both electrocompetent and chemically competent *E. coli* cells can be used. The following protocol is a standard protocol using chemically prepared competent *E. coli* cells.

(1) Gently thaw the competent cells（100 μL）on ice.

(2) Add 10 μL of reaction mixture from Step 3 to the competent cells, swirl gently and incubate for 30 min. on ice.

Note
To verify transformation efficiency of the competent cells, 1 pg of pBR322 can be used as a control plasmid.

(3) Incubate for 30 seconds at 42°C and then place on ice for 2 min.

(4) Add 900 μl of SOC medium and incubate at 37°C for 1 hr with shaking.

(5) Plate appropriate volumes (10~200 μL) of transformed *E. coli* solution on agar plates containing the appropriate antibiotic for the plasmid vector.

Note
For the mutagenesis controls, spread cells onto LB-Ampicillin (or LB-Kanamycin) agar plates containing X-gal and IPTG.

(6) Incubate the plates at 37°C for ~16 hr.

Note
The expected colony number from the transformation of the pAK119M control mutagenesis reaction is between 50 and 500 when competent cells with transformation efficiency $1x10^9$ cfu/μg-pBR322 are used. Greater than 80% of the colonies should contain the mutation and appear as a blue colony on agar plates containing X-gal and IPTG.

5. Analysis of Transfomants

A typical mutagenesis efficiency of this kit is greater than 80%. Therefore, analysis of 4-8 clones is typically appropriate for sequence verification.

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[6] Troubleshooting

Symptom	Cause	Solution
Low colony number	Error of Inverse PCR reaction	Reconfirm the primer design and PCR conditions.
	Cycle number is not sufficient	Increase the cycle number up to 10 cycles.
	Transformation efficiency of the competent cells is low	Check the transformation efficiency of the competent cells using a standard plasmid such as pBR322. Competent cells showing >10^8 cfu/µg-pBR322 are suitable.
Excess colony number	Plasmids are not methylated	Use *E. coli* host bearing dam-methylase for the template plasmid preparation, such as JM109 or DH5α.
	Concentration of antibiotics is not appropriate	Use the appropriate concentration of antibiotics. Ensure antibiotics are fresh.
No mutant	Error of Inverse PCR	Reconfirm the primer design and PCR conditions.
	Plasmids are not methylated	Use *E. coli* host bearing dam-methylase for the template plasmid preparation, such as JM109 or DH5α.
Failure of the control experiment	Concentration of IPTG or X-gal is not correct.	Confirm the concentration of IPTG or X-gal.
	Host *E. coli* cell is not appropriate.	Use *E. coli* strains suitable for white/blue selection such as DH5α or JM109.

[7] References

1) Takagi M, Nishioka M, Kakihara H, Kitabayashi M, Inoue H, Kawakami B, Oka M, and Imanaka T., *Appl Environ Microbiol.*, 63: 4504-10 (1997)

2) Hashimoto H, Nishioka M, Fujiwara S, Takagi M, Imanaka T, Inoue T and Kai Y, *J Mol Biol.*, 306: 469-77 (2001)

3) Mizuguchi H, Nakatsuji M, Fujiwara S, Takagi M and Imanaka T, *J Biochem.*, 126: 762-8 (1999)

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