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1. Description

Mutan™-Express Km **Enzyme/Oligo Set..... Cat. #6090**
Vector/Host Set ..... Cat. #6091

Takara's Mutan™-Express Km is designed to efficiently introduce site-directed mutations based on the ODA method (Oligonucleotide-directed Dual Amber method). Using the simplified procedure, a desired mutation can be introduced in only three days with no ssDNA isolation necessary. This kit is helpful in the studies of the structure and function of gene and protein.

Table 1. Comparison of Takara's Site-Directed Mutagenesis Systems

Product	Mutan™-Express Km	Mutan™-Super Express Km	Mutan™-K
Cat. #	6090	RR022	6060
Principle	ODA method	ODA-LA PCR method	Kunkel method
Efficiency	70 - 95%	>80%	60 - 70%
Vector	pKF 18k-2/19k-2	pKF 18k-2/19k-2	Vectors capable of preparing ssDNA
Host strain	*sup*^{0} *mut*S	*sup*^{0}	*dut*$^-$ *ung*$^-$ *mut*S F^+
Preparation of ssDNA	no preparation	no preparation	should be prepared
Time required	3 days	2 days	4 - 5 days

2. Kit component

A. Enzyme/Oligo Set (for 20 reactions) Cat. #6090

1. Annealing buffer .....40 μl
   (200 mM Tris-HCl, pH7.5, 100 mM $MgCl_2$, 500 mM NaCl, 10 mM DTT)
2. Extension buffer.....60 μl
   (100 mM Tris-HCl, pH7.5, 20 mM DTT, 10 mM ATP, 5 mM each dNTPs (dATP, dCTP, dGTP, and dTTP))
3. T4 DNA ligase (60 units/μl).....20 μl
4. T4 DNA polymerase (1 unit/μl) .....20 μl
5. Selection Primer (5 pmol/μl) * .....20 μl
6. Control dsDNA Solution (pKF 19kM ds DNA 50 fmol/μl).....5 μl
7. Control Synthetic Oligonucleotide Solution (50 pmol/μl) * .....5 μl

* Selection primer and Control Synthetic Oligonucleotide *sup*plied with this kit are already phosphorylated at the 5'-termini to be annealed to the plus chain of *lac*Z gene of pKF18k-2/19k-2 DNA. (Refer to Fig.1)

B. Vector/Host Set Cat. #6091

1. pKF 18 k-2 DNA (10 OD/ml) .....10 μl
2. pKF 19 k-2 DNA (10 OD/ml) .....10 μl
3. *E. coli* BMH71-18 *mut*S * 1 (10% glycerol solution) .....100 μl
4. *E. coli* MV1184 * 2 (10% glycerol solution).....100 μl

* 1 : △ *(lac-proAB), supE, thi-1, mut*S *215 : : Tn10 (tetr)/F' [traD36, proAB+, lacIq, lacZ* △ *M15]*

* 2 : △ *(lac-proAB), ara, rpsL, thi(*φ*80 lacZ* △ *M15),* △ *(srl-recA) 306 : : Tn10(tetr)/ F' [traD36, proAB+, lac Iq, lacZ* △ *M15]*

3. Storage

Enzyme/Oligo set : - 20℃
Vector/Host set : - 80℃
Caution : Avoid repeating freeze-thaw cycles.

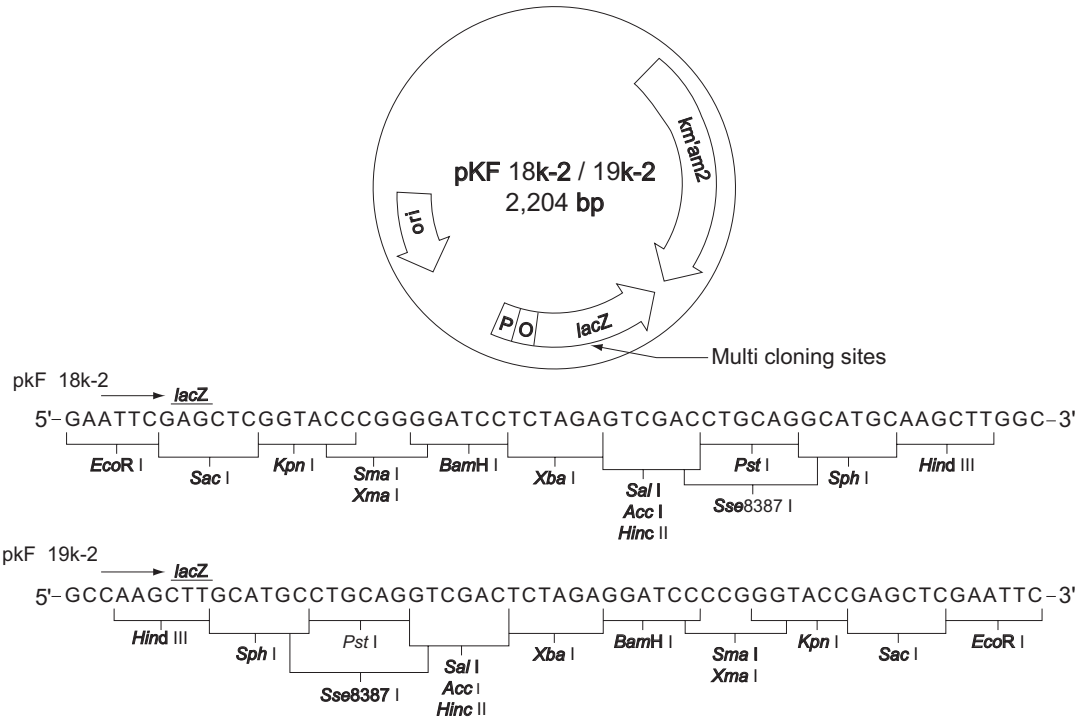


Fig. 1 Cloning site of pKF18k-2/19k-2

4. Reagents required but not *sup*plied in the kit

・M9 glucose medium :

per liter	
Na_2HPO_4	6 g
KH_2PO_4	3 g
NaCl	0.5 g
NH_4Cl	1 g

Autoclave, cool
Add 1 ml of 1 mg/ml thiamine, 1 ml of 1 M $MgSO_4$, 1 ml of 0.1M $CaCl_2$, 10 ml of 20% glucose. Each of these solutions should be autoclaved separately, and mixed under sterile conditions.

・M9 glucose plate
Agar is added to M9 glucose medium to a final concentration of 1.5%.

・LB medium :

per liter :	
Bacto tryptone	10 g
Bacto yeast extract	5 g
NaCl	5 g

Adjust to pH7.0 with NaOH. Autoclave.

・LB plate : Agar is added to LB medium to a final concentration of 1.5%.

・Antibiotics :

Tetracycline	10 mg/ml in 50% Ethanol. Store at -20℃ . Use at 10 μg/ml
Streptomycin	10 mg/ml in sterile distilled water. Store at -20℃ . Use at 30 μg/ml.
Kanamycin	25 mg/ml in sterile distilled water. Store at -20℃ . Use at 100 μg/ml.

Antibiotics dissolved in sterile distilled water should be sterilized by filtration through a 0.22-micron filter. Antibiotics dissolved in ethanol need not be sterilized. Store solutions in light-tight containers.

・TE buffer : 10 mM Tris-HCl, pH8.0, 1 mM EDTA
・Sterile distilled water
・Synthetic mutagenic oligonucleotide
・Reagents required for Mini preparations of plasmid DNA ＊
　＊ : Alkaline lysis miniprep, Boiling miniprep, or Lithium miniprep are recommended.
・0.2 M EDTA, pH8.0
・Competent cells/ Electro cells

Competent cells and electro cells with good transformation efficiency (>10^6 cfu/μg) should be used to obtain enough colonies.

・SOC medium :

per liter		
Bacto-tryptone	20 g	
Bacto yeast extract	5 g	
NaCl	0.584 g	(10 mM)
KCl	0.186 g	(2.5 mM)

Autoclave, cool
Add 5 ml of 2 M $MgCl_2$, 5 ml of 2 M $MgSO_4$, and 20 ml of 1 M glucose. Each of these solutions should be auto claved separately, and mix under sterile conditions.

・Primers for sequencing analysis
pKF18k-2/19k-2 are pUC vectors. The following primers are available for sequencing analysis of obtained mutagenized DNA.

M13 primer M1	(Cat. #3810)
M13 primer M2	(Cat. #3820)
M13 primer M3	(Cat. #3831)
M13 primer M4	(Cat. #3832)
M13 primer RV-N	(Cat. #3833)

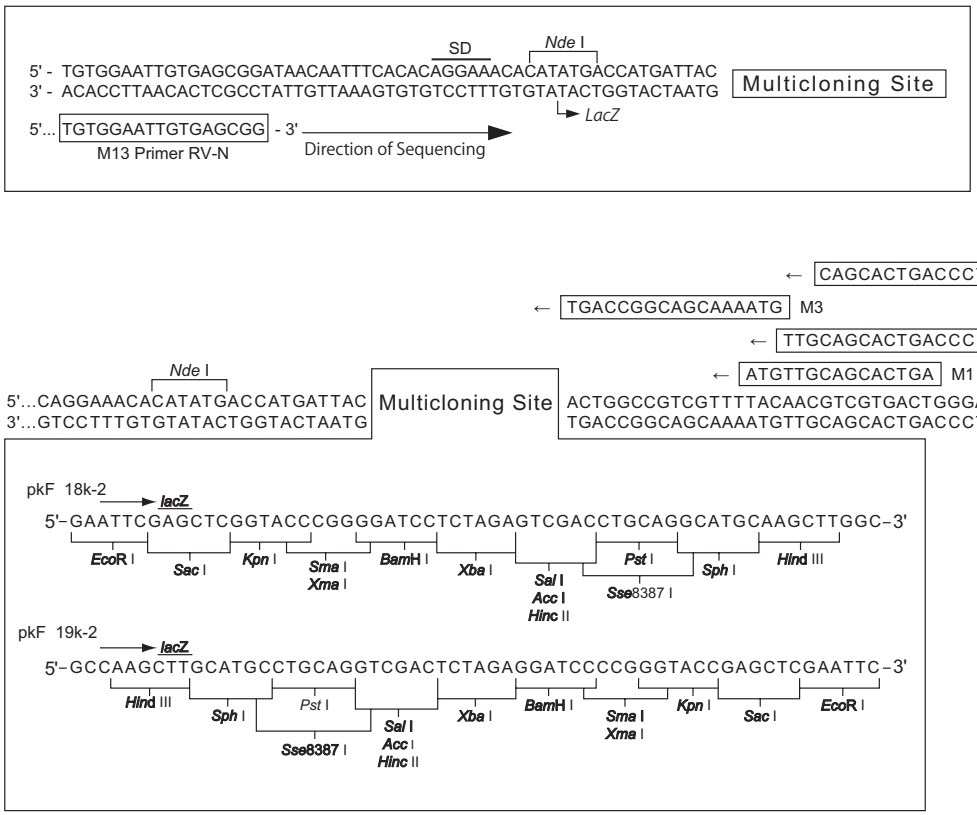


Fig. 2. Restriction Enzyme recognition site of pKF18k-2/19k-2 and primer sequences

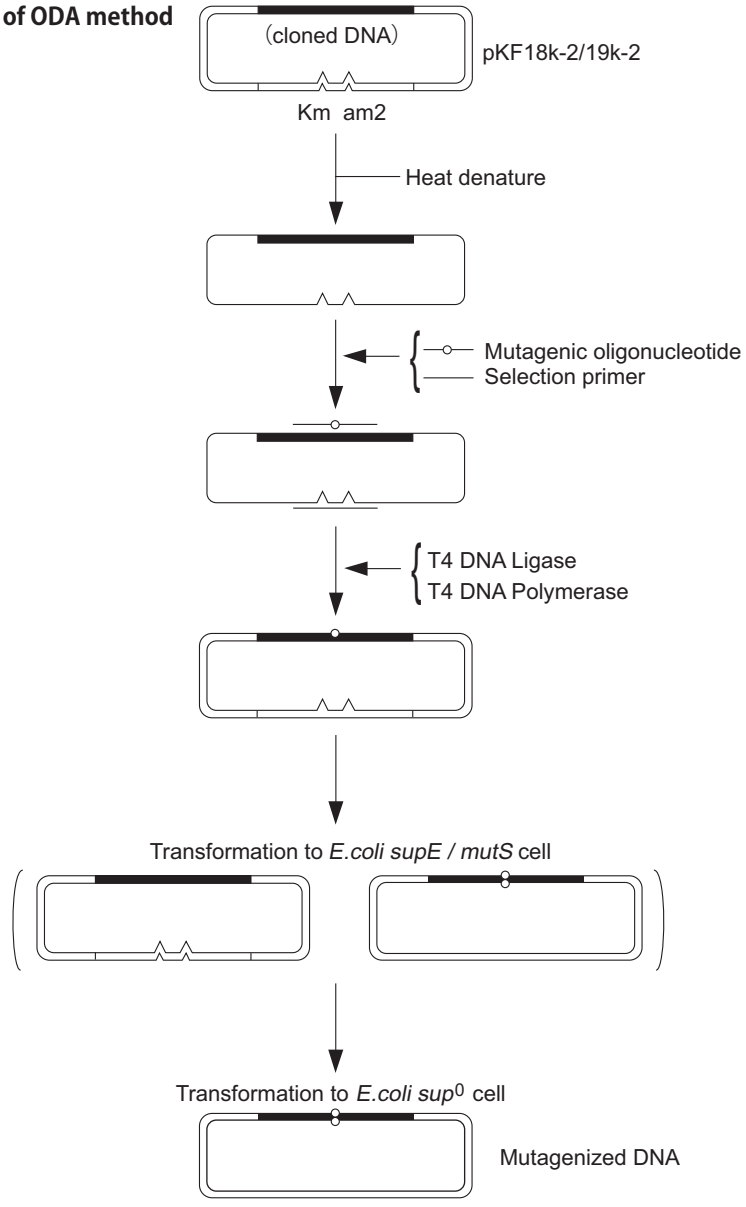
5. References

1. Hashimoto-Gotoh, T. *et al*. (1995) *Gene* **152**, 271-275.
2. Zoller, M.J. and Smith , M. (1983) *Methods in Enzymology* **100**, 468.
3. Hanahan, D. (1983) *J. Mol. Biol*. **166**, 557.
4. Sambrook, J., *et al*. (1989) *Molecular Cloning 2nd Edition* **1**. 74-**1**. 84.
5. Dower, W. J., *et al*. (1988) *Nucl.Acids.Res*. **16**, 6127.
6. Ausubel, F. M. *et al*. (1987) *Current Protocols In Molecular Biology*. **1**.8.1-**1**.8.8.
7. Sambrook, J., *et al*. (1989) *Molecular Cloning 2nd. Edition* **1**.25-**1**.31.
8. Ausubel, F. M., *et al*. (1987) *Current Protocols In Molecular Biology* **1**.6.1-**1**.6.10.

6. Principle

The principle of the Oligonucleotide-directed Dual Amber (ODA) method is shown in Fig. 3. The ODA method utilizes the pKF 18k-2/19k-2 vector containing dual amber mutations on a kanamycin-resistant gene which can only be propagated in the *sup*E host strain. The target DNA to be mutated is cloned into the multiple cloning site of pKF 18k-2/19k-2 and ssDNA is prepared by heat denaturation. An oligonucleotide containing the desired mutation along with the selection primer for revision of amber on the kanamycin gene are simultaneously hybridized to the ssDNA. A complimentary strand is synthesized by polymerase reaction. This newly synthesized strand is introduced into the *sup*E/*mut*S strain and DNA replication proceeds. By selecting DNA which is propagated only in the *sup*^{0} strain, the target DNA with the desired mutation is obtained.

Fig.3 Principle of ODA method



7. Storage of bacterial strains

BMH71-18*mut*S :
Plate on M9 glucose plate containing 10 μg/ml tetracycline overnight at 37℃. Inoculate a single colony into M9 glucose medium containing 10 μg/ml tetracycline and incubate at 37℃ overnight. Remove 1.5 ml to a cryovial, add 1.5 ml of sterilized 20% glycerol, and mix well. Store at -80℃.
(BMH71-18*mut*S is deficient in the ability to repair mismatched DNA. It works as host strain to fix mutation without repairing mismatches in mutagenized DNA.)

MV1184 :
Store in the same method as BMH71-18*mut*S. However, M9 glucose medium should contain 10 μg/ml tetracycline and 30 μg/ml streptomycin.
(MV1184 is a sup^0 strain for selection of mutagenized DNA out of template DNA fixed in *mut*S strain.

8. Design of synthetic mutagenic oligonucleotides

Synthetic oligonucleotides complimentary to the plus chain of *lac*Z gene should be prepared and they should anneal to the same chain as selection primer does. Purified synthetic DNA of sequencing grade with phosphorylated 5'-termini are used for introducing the mutation. The region to be changed should be flanked by 8 - 10 bases on both sides and the oligonucleotides should be approx. 20 bases in total length. When conducting deletion, insertion, or many base substitutions, it is advisable to design oligonucleotides to be flanked by 15 - 20 bases at both side from the mismatches and having GC rich 3' ends.

The purity of the oligonucleotide is important to obtain good results. This can be assessed by dideoxy sequencing to see whether clear ladders appear when synthetic DNA is used as sequencing primer. Store the oligonucleotides at -20℃. Avoid repeated freeze-thaw cycles.

9. Protocol for mutagenesis reaction

A. Preparation of template ds DNA and competent cells

1. Prepare template DNA by interseting the target DNA fragment into pKF18k-2 DNA (or pKF19k-2 DNA).
2. In this case, use *sup*E strain as a host, ex.JM109, and select transformants on the kanamycin-resistant media containing X-Gal and IPTG by color selection.
3. Purify plasmid DNA from the culture by minipreps.
4. Prepare competent cells from *E. coli* BMH71-18 *mut*S and *E. coli* MV1184, supplied in Vector/Host Set.
5. If using a low effeciency competent cell, enough colonies don't appear.
6. Competent cells with good transformation efficiency (>10^6 cfu/μg DNA) should be used.

B. Synthesis of the complementary strand

1. Prepare the following reagents to a total volume of 20 μl.

Template DNA	50 fmol
Selection primer (5 pmol/μl)	1 μl
Mutagenic oligonucleotide	50 pmol
Annealing buffer	2 μl
Sterilized distilled water	X μl
Total	Total

2. Heat at 100℃ for 3 min, and chill in an ice water bath for 5 min. ＊
3. Add 3 μl of Extension buffer, 1 μl of T4 DNA Ligase, 1 μl of T4 DNA polymerase and 5 μl of sterilized distilled water.
4. Incubate at 25℃ for 2 hours (or 37℃ for 1.5 hours).
5. Add 3 μl of 0.2 M EDTA, pH8.0, and incubate at 65℃for 5 min. (When consecutive transformation is not performed, store the solution at -20℃ . Avoid repeated freeze-thaw cycles.)

＊ Note : 1) Ice water bath must be used for chilling.
2) The annealing temperature may be modified depending on the base composition and length of synthetic oligonucleotides. It is important to optimize the annealing temperature and time when synthetic oligonucleotides contains many mismatches.

C. Transformation (1) - fixation of mutation

1. Add 10 μl of the synthesized DNA solution from the above section to 100 μl of *E. coli* BMH71-18 *mut*S competent cells and mix.
2. Incubate at 0℃ for 30 min., 42℃ for 45 sec., and then at 0℃ for 1 - 2 min.
3. Add LB medium (or SOC medium) prewarmed at 37℃ to a total volume of 1 ml, and incubate at 37℃ with shaking for 1 hour.
4. Add 2 ml of LB medium (or SOC medium), and kanamycin to the final concentration of 100 μg/ml.
5. Incubate overnight.

D. Purification of DNA (Minipreps of DNA)

Purify DNA from the culture by minipreps; Alkaline Lysis Miniprep, Boiling Miniprep, or Lithium Miniprep.
Miniprep DNA Purification Kit (Cat. #9085) is recommended.

E. Transformation (2) - selection of mutagenized DNA

1. Add 1 - 10 μl ＊1 of the purified DNA to 100 μl of *E. coli* MV1184 Competent cells ＊2, and mix. Carry out transformation by following the same protocol described in C. Transformation (1)'.
2. Add LB medium (or SOC medium) prewarmed at 37℃ to a total volume of 1 ml and incubate with shaking at 37℃ for 1 hour. ＊3
3. Spread a suitable volume of the culture onto LB plate containing 100 ＊4 μg/ml of kanamycin and incubate at 37℃ overnight.
4. Select a few colonies and prepare DNA for sequencing from them. Confirm the mutants by DNA sequence analysis.

＊1 : Care should be taken not to add the excess amount of purified DNA solution. A host cell might transform more unmutagenized plasmids along with mutagenized one.
＊2 : Electro cells can also be used instead of competent cells.
＊3 : When using a low efficiency competent cell, it is recommended to extend the incubation time to 2 - 3 hours.
＊4 : If the concentration of Kanamycin is low, unmutagenized colonies might appear.

Fig. 4 Flowchart of Protocol

< Day 1 >

Reaction Mixture

Total volume : 20 μl

Template DNA	50 fmol
Selection primer	1 μl
Mutagenic oligonucleotide	50 pmol
Annealing buffer	2 μl
Sterilzed distilled water	up to 20 μl

- Incubate at 100℃ for 3 min.
- Chill in an ice water bath for 5 min.

annealing - Extension

- Add 3 μl Extension buffer
- Add 1 μl T4 DNA ligase (60 U)
- Add 1 μl T4 DNA polymerase (1 U)
- Add 5 μl Sterilized distilled water

Total volume : 30 μl

- Incubate at 25℃ for 2 hr (or at 37℃ for 1.5 hr)
- Add 3 μl 0.2M EDTA, pH8.0
- Incubate at 65℃ for 5 min.

Sample DNA solution

Take 10 μl of sample DNA solution

Transformation (1) - fixation of mutation

- Mix with 100 μl of *E. coli* BMH71-18 *mut*S Competent cell
- Incubate at
  - 0℃ for 30 min.
  - 42℃ for 45 sec.
  - 0℃ for 1 - 2 min.
- Add 890 μl of LB medium (or SOC medium)
- Incubate with shaking at 37℃ for 1 hr.
- Add 2 ml LB medium (the final concentration of Kanamycin : 100 μg/ml)
- Incubate with shaking at 37℃ overnight.

< Day 2 >

Miniprep of DNA

Purified DNA solution

Take 1 - 10 μl of purified DNA solution

Transformation (2) - selection of mutagenized DNA

- Mix with 100 μl of *E. coli* MV1184 Competent cell
- Incubate at
  - 0℃ for 30 min.
  - 42℃ for 45 sec.
  - 0℃ for 1 - 2 min.
- Add 900 μl of SOC medium
- Incubate with shaking at 37℃ for 1 hr.

Spread an appropriate amount of the culture on the LB plate containing Km

- Incubate at 37℃ overnight.

< Day 3 >

Growth of colonies

DNA sequence analysis ——— **Confirmation of mutants**

10. Control experiment using pKF19kM

A. Principle

Since pKF19kM contains an amber mutation (TAG) on codon 16 of the lacZ α gene, it does not express α-complementation and forms white colonies on a plate containing X-Gal/IPTG. By introducing a mutation to change this amber codon to tryptophan codon (TGG), the mutagenized DNA will form blue colonies. Mutagenesis efficiency can be calculated as the percentage of blue colonies to the total number of colonies.

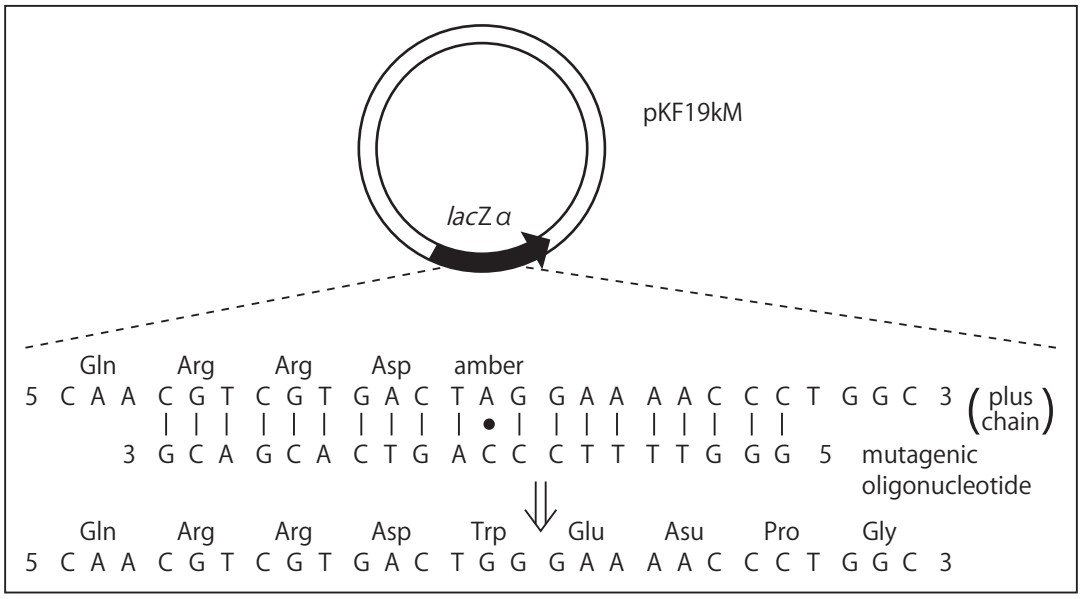


Fig. 5 Principle of control experiments using pKF19kM

B. Protocol

1. Prepare the following reagents.

Control dsDNA solution	1 μl
Selection primer	1 μl
Control synthetic oligonucleotide	1 μl
Annealing buffer	2 μl
Sterilized distilled water	15 μl

2. Carry out transformations by following the protocol given at '9. Protocol for mutagenesis reaction A - D'. At the final step of forming colonies, prepare LB plate containing 20 μg/ml X-Gal and 0.2 mM IPTG. More than 70% of blue colonies are obtained.

11. Mutagenesis reaction using LA PCR ＊2

PCR (Polymerase Chain Reaction) ＊1 can be employed when conducting a mutagenesis reaction of low efficiency. Through PCR using mutagenic oligonucleotides and selection primers, mutagenic-selection DNA are obtained as shown in Fig. 6. Carry out mutagenesis with this DNA by following the '9. Protocol for mutagenesis reaction'. In this case, the annealing temperatures after heat treatment and immediate cooling are important. It may be necessary to adjust those temperatures for better yield of transformants. When amplifying the region between the mutagenic oligonucleotide and the selection primer with *Taq* DNA polymerase, mutations may be introduced in the regions that are not desired. To avoid these unexpected mutations, it is advisable to proceed with the following treatments; increase the amount of template DNA, or reduce the cycles of temperature cycling. However, since these treatments may not yield the adequate mutagenic-selection DNA, it is recommended to carry out *TaKaRa LA Taq*™ (Cat. #RR002A/B), *TaKaRa LA Taq*™ Hot Start Version (Cat. #RR042A/B) and TaKaRa LA PCR Kit Ver.2.1 (Cat. #RR013A/B).

＊1 : The polymerase chain reaction (PCR) process is covered by patent owned by Hoffmann-La Roche.
＊2 : LA technology is licensed under U.S.patent

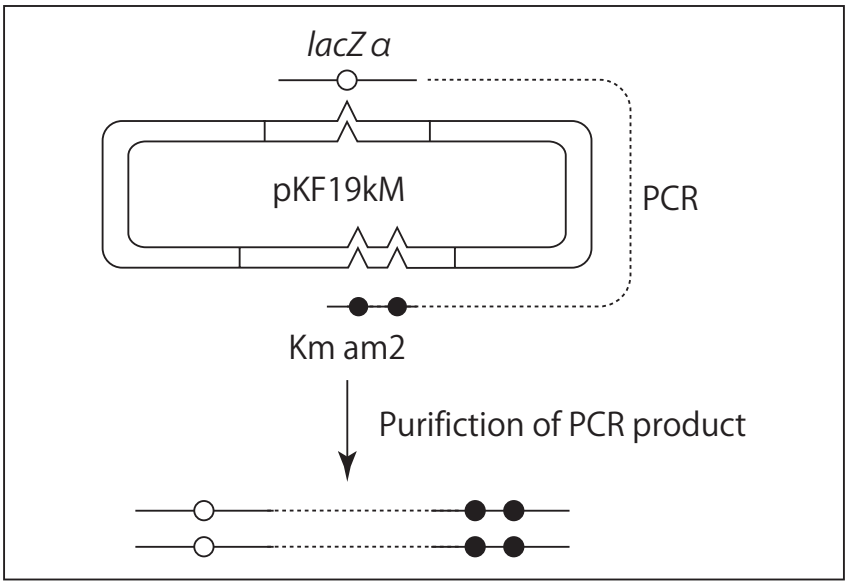


Fig. 6 Preparation of mutagenic-selection DNA

Note : 1) In this reaction, synthetic mutagenic oligonucleotides should be designed to anneal to the opposite chain, minus chain ＊, to the one selection primer does.
＊ : Please refer to the Fig.5 for minus chain.
2) PCR products should be blunt-ended before used with this kit.

12. Q & A

Q1 : Mutagenized colonies can not be yielded at last stage of protocol?

A1 : (1) Change extension condition to 1.5 hours at 37℃
(2) Decrease the amount of selection primer to 0.5 μl
(3) Increase the amount of mutagenic oligonucleotide to around 100 - 200 pmol
(4) Decrease the amount of template DNA to around half.
When the above method does not work and the mutagenized colonies cannot still be yielded, transform pKF18k-2 DNA (or pKF19k-2 DNA)which is subcloned with a target DNA fragment in MV1184 as a host. Confirm that no colonies are formed on LB plate containing 100 μg/ml of Kanamycin.

Q2 : Is it possible that a plural point of mutation can be introduced at the same time?

A2 : It depends on the design of mutagenic oligonucleotide. If a primer includes 2, 3 points of mismatch, it can be possible. In this case, it is necessary to modify a protocol, e.g. to use excessive amount of primer. Using two kinds of mutagenic oligonucleotide is not recommended, because more unmutagenized colonies can be yielded.

Q3 : What kind of strain should be used as a host, when inserting the target DNA fragment into pKF18k-2 DNA or pKF19k-2 DNA.

A3 : *E. coli* JM109 (*sup*E44) is recommended.

Q4 : Notes for sequencing

A4 : Use sequencing primers for pUC vectors. But RV primer cannot be used, because pKF 18k-2 DNA (pKF 19k-2 DNA) includes *Nde* I site, please use RV-N primer.

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