KAPA HiFi™ DNA Polymerase

Site-directed Mutagenesis

1. Introduction

Site-directed mutagenesis is a powerful tool for protein engineering and the study of protein structure and function. Numerous methods of site-directed mutagenesis that exploit the primer extension technique have been developed (1-2). These methods generate heteroduplex species containing one mutated and one non-mutated strand; subsequent replication and segregation of these molecules inevitably results in a population heavily contaminated with wild-type DNA. Various methods that circumvent the replication of non-mutated DNA or that separate the two species have been developed, but these methods tend to be more difficult and lengthy (3-5). The site-directed mutagenesis protocol described here enables a fast, simple, and efficient system for the introduction of specific mutations, insertions and/or deletions into DNA cloned in a double-stranded plasmid.

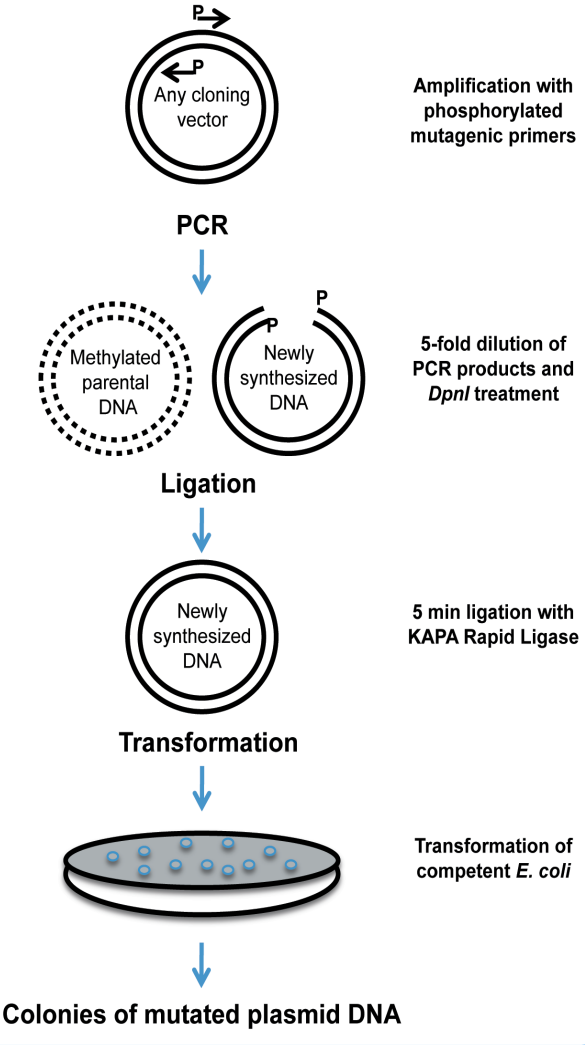
The KAPAHiFi™ site-directed mutagenesis protocol requires the use of one or two phosphorylated primers designed with their 5' ends adjacent to each other. Only one of the primers carries the required mutation. The two primers anneal to opposite strands on the target vector and KAPAHiFi™ DNA Polymerase is then used to amplify the entire vector. The PCR amplification generates large numbers of mutated linear duplex molecules, which significantly outnumber the original wild-type template. DpnI restriction endonuclease is used to digest the residual methylated template and any hemi-methylated DNA. KAPA Rapid Ligase is then used to circularize the linear mutated DNA, after which *E. coli* is transformed with the ligation mix.

2. Materials

- KAPAHiFi™ or KAPAHiFi™ HotStart DNA Polymerase and 5x Fidelity Buffer (KK2101, KK2501)
- DpnI restriction endonuclease and buffer
- KAPA Rapid Ligase 2x Rapid Buffer (KK6101)
- Mutagenic primers (see below)
- PNK and buffer (if primers are not already phosphorylated)
- Plasmid template DNA (containing gene of interest)
- Competent cells

Application Summary

KAPAHiFi™ DNA Polymerase can be used in oligonucleotide-directed mutagenesis protocols. Tail-to-tail phosphorylated oligonucleotides designed to carry the mutation and reduce primer-dimer formation, are used. Exponential amplification creates mutated DNA far in excess of the starting wild-type DNA in the reaction. The remaining wild-type DNA is further selected against by restriction digestion using DpnI restriction endonuclease. PCR amplification with KAPAHiFi™ DNA Polymerase rarely introduces extraneous mutations. This procedure enables the creation of mutated plasmid DNA in a single day and with efficiency greater than 80%.



3. Primer design

For every mutation, two oligonucleotides that anneal to complementary strands of the template should be designed. The required mutation of interest is introduced at the 5' end of only one of the primers by substitution of the nucleotide sequence (Figure 1A). The oligonucleotides are located directly adjacent to each other (on separate strands) in order to amplify the whole plasmid. In the case of deletion mutagenesis, the primers are designed with a gap between them, corresponding to the region to be deleted (Figure 1B). In the case of insertion, the additional nucleotide(s) are added at the 5' end of one or both primers (Figure 1C). One or both oligonucleotides in each reaction are phosphorylated; this is required for ligation of the PCR products.

Oligonucleotides are either phosphorylated using Polynucleotide Kinase (PNK) (for phosphorylation protocol, refer to Appendix) or phosphorylated by the oligo supplier. Oligonucleotides may be HPLC or PAGE purified to ensure that they are full-length. Further requirements concerning primer design includes having at least 17 nucleotides, corresponding exactly to the DNA sequence and ending with G or C at the terminal 3' position. Silent restriction sites (mutations which incorporate a restriction site without changing the amino acid sequence) may also be included to facilitate analysis of the resulting clones. When additional "silent" mutations are included in the primers, a minimum of 8 correct nucleotides should be present between the "silent" mutation and the 3' end of the primers.

Figure 1. Example of primer design scheme. (**A**) A mutation (red) is inserted in the DNA sequence (grey), using oligonucleotides (black). (**B**) A deletion in the DNA sequence (blue) is introduced by designing the primers with a gap between them, corresponding to the region to be deleted. (**C**) An insertion is made by including the additional nucleotides (red) at the 5' end of the mutagenic primer. Primers are 5' phosphorylated to enable ligation of PCR products following amplification.

4. Mutagenesis PCR

PCR amplification is carried out in 50µL reactions, using KAPAHiFi™ DNA Polymerase with 1 ng template DNA and 1U of KAPAHiFi™ HotStart or KAPAHiFi™ in 1x KAPAHiFi™ Fidelity Buffer. Each primer is used at a concentration of 0.3 µM and dNTPs at a concentration of 0.3mM. A negative control without KAPAHiFi™ DNA Polymerase should always be included.

Recommended cycling protocol:

Initial Denaturation:	95 °C	2 min	
Denaturation:	98 °C	20 sec	16 cycles**
Annealing:	Tm*	15 sec	
Extension:	72 °C	30 sec/kb	
Final Extension:	72 °C	1 - 5 min	

* The annealing temperature depends on the primers. Generally, this temperature is set to the lowest Tm of the two primers. However, optimizing the annealing temperature for the specific primers used may be beneficial.

** For higher PCR yields, the number of cycles can be increased to 20 or 25 cycles, however, this may result in the introduction of superfluous secondary mutations.

5. DpnI digestion and ligation

Once successful amplification has been confirmed by agarose gel electrophoresis, treat the PCR products (and control) with DpnI to digest methylated and hemi-methylated DNA; this requires dilution of the PCR mix five-fold in 1x DpnI buffer. For a 50µL DpnI reaction: 10 µL PCR reaction, 5 µL of 10x DpnI buffer, 1 µL (10U) DpnI and 34 µL nuclease-free water are mixed in a PCR tube. The reaction is then incubated at 37 ºC for 1 hour.

The DpnI enzyme is subsequently heat inactivated at 80 ºC for 20 minutes. The DpnI-treated products can be directly used in rapid ligation reactions, without any purification. A typical ligation mix consists of 9 µL DpnI-treated product, 10 µL 2x KAPA Rapid Ligase buffer and 1 µL KAPA Rapid Ligase. The ligations are incubated at ambient temperature for 5 minutes and then placed on ice.

6. Transformation and analysis

An aliquot (5 µL) of the ligation mix and control is used to transform competent *E. coli* cells, using a standard protocol (for transformation protocols see Appendix). Transformants are selected by their ability to propagate on antibiotic-containing medium. Resulting mutants can be analyzed by PCR (using KAPA2G™ Robust DNA Polymerase) and subsequent restriction digest analysis using the "silent" sites included in the primers, if desired. Plasmid DNA can be extracted using a method of choice and DNA sequencing may be done to verify that the desired mutations are successfully introduced and that no other mutations are found in the rest of the gene.

7. Efficiency of mutagenesis

The efficiency of this site-directed mutagenesis protocol was assessed. The yield of PCR amplification products may vary widely with different primers and at different annealing temperatures as exemplified in Figure 2. The mutagenic efficiency obtained using this method is consistently higher than 80%.

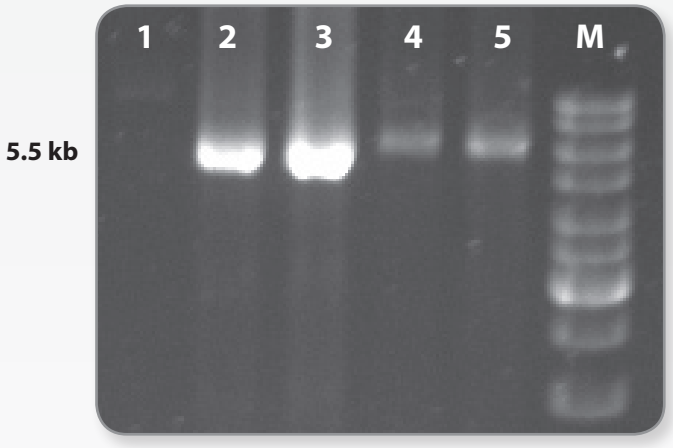


Figure 2. Mutagenesis PCR products. Ethidium bromide stained gel. Negative control consisting of PCR reation without polymerase (lane 1), PCR products in which 3 nucleotides were substituted (lanes 2 and 3, annealing temperatures of 50 ºC and 60 ºC respectively), PCR products using a different primer set to introduce a mutation in a different amino acid of the same gene (lanes 4 and 5, annealing temperatures of 50 ºC and 60 ºC respectively), DNA ladder (lane M). 5.5 kb linear product in 0.8% agarose gel electrophoresis.

8. Conclusions

Several protocols have been developed for oligonucleotide-mediated site-directed mutagenesis each with advantages and disadvantages (6, 7). The KAPAHiFi™ site-directed mutagenesis application note provides a simple and highly efficient mutagenesis method, for use with KAPAHiFi™ HotStart and KAPAHiFi™ DNA Polymerases. This protocol allows a variety of modifications to be made in a gene sequence, using any cloning vector containing an antibiotic resistance gene. In addition, the use of KAPAHiFi™ Hotstart or KAPAHiFi™ DNA Polymerases ensures the high fidelity replication of the plasmid, thereby minimizing polymerase-induced errors.

9. Troubleshooting

1. I have very little or no PCR products when visualized on an agarose gel.

First make sure that the template DNA is not degraded by using a fresh prep and running an aliquot on a gel. If the plasmid DNA is intact, there should be a brighter high molecular weight band representing the supercoiled DNA topoform, very little of the open circular (nicked) and none of the linear conformation. Attempt the PCR using a range of DNA concentrations.
Then check the design, purity and concentration of the primers. If PNK was used to phosphorylate the primers, try purifying the primers using a spin column before using them in PCR. Finally, perform an annealing temperature gradient to find the optimal annealing temperature of the primers.

2. There are only a couple or no bacterial colonies on the plate.

Check the transformation efficiency of the cells by transforming a supercoiled plasmid. Make sure the PCR was successful evidenced by a single band of correct size on agarose gel analysis. Increasing the amount of ligated products used in the transformation may be beneficial.

3. The resulting clones do not have the mutation.

Include a DpnI efficiency control by treating the no polymerase control with DpnI (in a similar manner to the reactions). The control transformation should yield no (or very few) colonies. If a substantial number of colonies appears, add more DpnI or perform two sequential DpnI digests.

10. References

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11. Appendix

Oligonucleotide phosphorylation using T4 Polynucleotide Kinase (PNK)

1. Combine the following:

Deionized water up to 20 µl	x µl
Oligonucleotide (100 µM stock, final concentration 2.5 µM)	0.5 µl
10x T4 PNK Reaction Buffer	2 µl
10 mM ATP Solution (final concentration 1 mM)	2 µl
T4 Polynucleotide Kinase (10 U/µl, final concentration 0.5 U)	1 µl
Total reaction volume	20 µl

2. Incubate at 37 ºC for 30 minutes.

3. Heat inactivate the PNK at 75 ºC for 10 minutes.

Transformation protocol (for chemically competent cells)

1. Thaw competent cells on ice
2. Chill ~ 5µL ligation mix on ice in sterile microcentrifuge tube.
3. Add 50 µL thawed, mixed competent cells to DNA and gently mix by flicking tubes. Return to ice for 30 min.
4. Heat shock tubes 45-50 sec at 42 ºC and return immediately to ice for 2-5 min.
5. Add 950 µl cold SOC* medium to cells, mix gently.
6. Incubate at 37 ºC for 60 min (preferably while shaking).
7. Spread 100 µl onto desired plate medium (e.g. LB Amp plates).

Transformation protocol (for electro-competent cells)

1. Thaw electrocompetent cells on ice. Also place electroporation cuvettes on ice and set electroporator power source to capacitance of 25 µFD, resistance of 200 Ω and voltage to 1.8kV for 0.1cm gap cuvettes or 2.5kV for 0.2cm gap cuvettes.
2. Transfer ~ 1µL** ligation mix to about 40 µL cells on ice.
3. Pipette the DNA/cell mixture into a chilled cuvette, place the cap on the cuvette and flick to settle the mixture to the bottom of the cuvette.
4. Dry off any moisture on the outside of the cuvette before transferring the cuvette to an electroporator cuvette holder.
5. Having 1ml SOC ready in a pipette, deliver a single pulse to the cells (should produce a time constant between 4.0 and 5.0 msec) then immediately add the SOC to cells and mix.
6. Transfer the mixture to a sterile 15ml tube and Incubate at 37 ºC for 1 hour (preferably while shaking).
7. Plate cells on appropriate antibiotic medium.

* SOC medium: 2% Bactotryptone, 0.5% yeast extract, 2.5mM KCl, 10mM NaCl, 10mM $MgSO_4$, 10mM $MgCl_2$ and 20mM glucose.

** Adding more than 2 µL of the ligation increases the salt content which may cause sparking. Alternatively, the DNA may be purified using a spin column and resuspended in Tris-HCl or H_2O.