Mutagenesis

Mutagenesis PCR I (Multiple Site Directed Mutagenesis)

Mixture

- 25µl total reaction volume :

1. 2.5 µl of 10X Taq lligase buffer (need the NAD for Taq ligase)
2. 0.5 µl 100mM ATP
3. X µl (50-100 ng) of dsDNA template
4. X µl of each oligonucleotide primer (check primer concentration with Nanodrop)
    a. For 1-3 primers, add 100 ng each primer. For 4-5 primers, add 50 ng each primer.
    b. If primers are greater than 20% different in length, scale the amount of primer added so that primer is added in approximately equimolar amounts. See Stratagene QuikChange Multi Site-Directed Mutagenesis manual for details.
5. 3µl of dNTP mix (100mM total dNTP mix with 25 mM each individual dNTP)
6. ddH_2O to a final volume of 22 µl

- Then add :

1. 1 µl of Phusion DNA polymerase
2. 1 µl of Taq Ligase
3. 1 µl of T4 PNK

Procedure

This procedure is primarily derived from the Stratagene QuikChange Multi Site-Directed Mutagenesis manual with some modifications based on past experience.

1. Design mutagenesis primers.

- The primer should be designed so that the desired mutation occurs at the exact center of the primer with 10-15bp of matching sequence on each side.
- Primers should be 25-45bp in length with a melting temp of >=75°C. Stratagene recommends not using primers greater than 45bp in order to avoid formation of secondary structure. Primers should have comparable melting temperatures.
- See the Stratagene manual for more detailed information. In particular, adhere to their formula for calculating the melting temperature of your primers and design your primers to have a melting temperature >=75°C.
- Primers should have at least 40% GC content and terminate in one or more C or G bases at the 3' end.
- PAGE purification of primers may improve mutagenesis efficiency

2. Purify template plasmid from a dam$^+$ *E. coli* strain via miniprep.
3. Set up mutagenesis PCR mix as described above.
4. Run Reaction
    1. 37°C for 30 min (T4 PNK step)
    2. 95°C for 3 min
    3. 95°C for 1 min
    4. 55°C for 1 min
    5. 65°C for 30 sec/kb of plasmid length minimum (is optimal temperature for Taq ligase)
    6. Run reaction for 30 cycles.
    - Stratagene recommends using a PCR machine with heated lid or overlaying the reaction with mineral oil.
5. Cool the reaction to <=37°C (set storage temperature at 37°C)
6. Add 1µl DpnI restriction enzyme to the PCR tube directly. (Purification is not necessary at this stage).
7. Incubate 1 hour at 37°C (as Stratagene manual recommends).
8. Transform purified DNA into highly competent cells.
9. Screen the transformants for the desired mutation using colony PCR, restriction digest or sequencing as appropriate.

Primers phosphorylation of 5' (optional)

1. Mix :
    - 3µl 100µM sense oligo (final concentration 10µM)
    - 3µl 100µM anti-sense oligo (final concentration 10µM)
    - 3µl 10X T4 DNA ligase buffer
    - 2µl T4 Polynucleotide kinase (PNK)
    - 19µl double distilled water

   (Total volume 30 µL)
2. Incubate at 37°C for 1.5 hours.
3. Heat kill PNK by 10 minutes at 70 °C

Mutagenesis PCR II (Single Site Directed Mutagenesis)

This protocol in based in a routine Phusion® PCR by New England BioLabs
These guidelines cover a site direct mutagenesis.
Note the difference in the steps and program than a routine PCR.

Reaction Setup: We recommend assembling all reaction components on ice and quickly transferring the reactions to a thermocycler preheated to the denaturation temperature (98°C). All components should be mixed and centrifuged prior to use. It is important to add Phusion DNA Polymerase last in order to prevent any primer degradation caused by the 3´→ 5´ exonuclease activity.

Procedure

1. PCR recipe

Component	20 µl Reaction	50 µl Reaction	Final Concentration
Nuclease-free	to 20 µl	to 50 µl	

water			
5X Phusion HF or GC Buffer	4 µl	10 µl	1X
10 mM dNTPs	0.4 µl	1 µl	200 µM
10 µM Forward Primer	1 µl	2.5 µl	0.5 µM
Template DNA	100-500ng	100-500ng	variable
DMSO (optional)	(0.6 µl)	(1.5 µl)	3%
Phusion DNA Polymerase	0.2 µl	0.5 µl	1.0 units/50 µl PCR

2. Transfer PCR tubes from ice to a PCR machine with the block preheated to 98°C and begin thermocycling

3. PCR Program

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
16-20 Cycles*	98°C 55°C 72°C	5-10 seconds 10-30 seconds 15-30 seconds per kb
Final Extension	72°C	5-10 minutes
Hold	37°C**	1-2 Hrs***

*After complete the first half of the 8-10 cycles, stop the machine, let the reaction mix gradually cold, open the tube and add the following

** Set storage temperature at 37°C, another option is place the cooled tube inside a 37°C incubator.

4.

Component	**20 µl Reaction**	**50 µl Reaction**	**Final Concentration**
10 µM Reverse Primer	1 µl	2.5 µl	<0.5 µM

5. After adding the primers, resume the remaining cycles.
6. Once that the program is complete with the two primers, in the holding step add 1 µl DpnI restriction enzyme to the PCR tube directly. Only digestiong the PCR products for no more that 2 hrs. (Purification is not necessary at this stage).
7. Transform purified DNA into highly competent cells.
8. Screen the transformants for the desired mutation using colony PCR, restriction digest or sequencing as appropriate.

Primers phosphorylation of 5’ (optional)

1. Mix:
   - 3µl 100µM sense oligo (final concentration 10µM)
   - 3µl 100µM anti-sense oligo (final concentration 10µM)
   - 3µl 10X T4 DNA ligase buffer
   - 2µl T4 Polynucleotide kinase (PNK)
   - 19µl double distilled water

   (Total volume 30 µl)
2. Incubate at 37°C for 1.5 hours.
3. Heat kill PNK by 10 minutes at 70°C.

Mutagenesis PCR III (Single Site Directed Mutagenesis)

This protocol in based in a routine Phusion® PCR by New England BioLabs
These guidelines cover a site direct mutagenesis.

Reaction Setup: We recommend assembling all reaction components on ice and quickly transferring the reactions to a thermocycler preheated to the denaturation temperature (98°C). All components should be mixed and centrifuged prior to use. It is important to add Phusion DNA Polymerase last in order to prevent any primer degradation caused by the 3′→ 5′ exonuclease activity.

Procedure

1.

Component	20 µl Reaction	50 µl Reaction	Final Concentration
Nuclease-free water	to 20 µl	to 50 µl	
5X Phusion HF or GC Buffer	4 µl	10 µl	1X
10 mM dNTPs	0.4 µl	1 µl	200 µM
10 µM Forward Primer	0.2µl	0.5 µl	0.25 µM
10 µM Reverse Primer	0.2µl	0.5 µl	0.25 µM
Template DNA	100-500ng	100-500ng	variable
DMSO (optional)	(0.6 µl)	(1.5 µl)	3%
Phusion DNA Polymerase	0.2 µl	0.5 µl	1.0 units/50 µl PCR

2. Transfer PCR tubes from ice to a PCR machine with the block preheated to 98°C and begin thermocycling

3. PCR Program

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
16-20 Cycles	98°C 55°C 72°C	5-10 seconds 10-30 seconds 15-30 seconds per kb
Final Extension	72°C	5-10 minutes
Hold	37°C*	1-2 Hrs**

*Set storage temperature at 37°C, another option is place the cooled tube inside a 37°C incubator.

4. Once that the program is complete with the two primers, in the holding step add 1 µl DpnI restriction enzyme to the PCR tube directly. Only digesting the PCR products for no more that 2 hrs. (Purification is not necessary at this stage).
5. Transform purified DNA into highly competent cells.
6. Screen the transformants for the desired mutation using colony PCR, restriction digest or sequencing as appropriate.

Primers phosphorylation of 5' (optional)

1. Mix:

- 3µl 100µM sense oligo (final concentration 10µM)
- 3µl 100µM anti-sense oligo (final concentration 10µM)
- 3µl 10X T4 DNA ligase buffer
- 2µl T4 Polynucleotide kinase (PNK)
- 19µl double distilled water
  (Total volume 30 µL)

2. Incubate at 37°C for 1.5Hrs
3. Heat kill PNK by 10 min at 70°C