Mutagenesis PCR mix

25µL total reaction volume:

2.5 µL of 10X Taq ligase buffer (need the NAD for Taq ligase)
0.5 µL 100mM ATP
X µL (50-100 ng) of dsDNA template
X µL of each oligonucleotide primer (check primer concentration with Nanodrop)
For 1-3 primers, add 100 ng each primer. For 4-5 primers, add 50 ng each primer.
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.
3µL of dNTP mix (100mM total dNTP mix with 25 mM each individual dNTP)
ddH_2O to a final volume of 22 µL

Then add

1 µL of Phusion DNA polymerase
1 µL of Taq Ligase
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.

Extra

Primers phosphorylation of 5’

1. Mix:
2. 3μ 100μM sense oligo (final concentration 10μM)
3. 3μ 100μM anti-sense oligo (final concentration 10μM)
4. 3μ 10X T4 DNA ligase buffer
5. 2μ T4 Polynucleotide kinase (PNK)
6. 19μ double distilled water
   (Total volume 30 μL)

7. Incubate at 37^{o}C for 1.5Hrs
8. Heat kill PNK by 10 min at 70^{o}C