Site Directed Mutagenesis (QuickChange Method) Cornell iGEM 2012 Protocol

Source: Dylan Webster (Adapted from QuickChange II XL Site-Directed Mutagenesis Kit Protocol)
http://www.chem.agilent.com/Library/usermanuals/Public/200521.pdf
http://www.neb.com/nebecomm/products/protocolProductE0553.asp

Mutagenic Primer Design:
Use the Agilent webpage to help you design mutagenic primers: www.agilent.com/genomics/qcpd
In general, primer pairs should anneal to the same sequence of opposite strands, contain the desired mutation flanked by 10-15 bases of correct sequence on both sides, and have a melting temperature ≥ 78˚C.

Mutant Strand Synthesis Reaction:
** Ensure that miniprepped plasmid came from a* dam+ *strain (DH5α is good).*
Prepare the following 50 µL reaction **on ice**:

- 10 µL of 5X Phusion HF or GC Buffer (Try HF first).
- 1 µL 10 mM dNTPs.
- 2.5 µL sense primer.
- 2.5 µL antisense primer.
- 1.5 µL DMSO.
- X µL template DNA (Try 10ng first).
- X µL ddH_20 (Up to 50 µL)
- 0.5 µL Phusion DNA polymerase (1.0 unit, **ADD LAST**).

Gently flick the PCR tube to mix the reaction mixture, and perform a quick spin in the Bio-Rad microcentrifuge. Put the PCR tube back on ice after spinning down, and preheat the thermal cycler to 98˚C before transferring the PCR tube. Cycle the reaction according to the following parameters:

Segment	Cycles	Temperature	Used:	Time	Used:
1	1	98 ˚C		1 minute	
2	18	98 ˚C		30 seconds	
		*60-65 ˚C		50 seconds	
		72 ˚C		15-30 sec/kb	
3	1	72 ˚C		10 minutes	
4	1	4 ˚C		Indefinite	

* This is experimental, since the polymerase that the QuickChange kit uses tends to need a lower annealing temperature than our Phusion does. The QuickChange protocol calls for a 60 ˚C annealing temperature. We may need to optimize this empirically.

Make sure the PCR reaction has been at 4˚C for at least 5 minutes before proceeding. Also, make sure to have a 37˚C incubator ready to go!

Optional: 10 µL of PCR reaction can be checked on a gel. QuickChange protocol says to not worry about it if bands aren't visualized at this stage, and to proceed with DpnI digestion in either case.

DpnI Digestion of the PCR Products

- Add 1 µL DpnI directly to the PCR reaction in an ice bucket.
- Pipette up and down and flick to mix the reaction, and then spin down the mixture in the Bio-Rad microcentrifuge.
- As quickly as possible, transfer the reaction to a 37˚C incubator (either the thermal cycler, the water bath, or the heat block).
- Heat inactivate DpnI by incubating at 80˚C for 20 minutes. (The easiest way to do this is to set up a program on the thermal cycler for both incubations.) [*This step is not necessary if DNA is to be spin-column purified prior to ligation.*]

Electroporation of Mutated Plasmid into DH5α

- Prior to electroporation, it is necessary to desalt the reaction mixture. This can be done either via membrane dialysis or via spin-column purification. If the spin-column method is used, I (Dylan) think that DpnI is more likely to be excluded from the desalted plasmid. I'm not sure if this will greatly enhance transformation efficiency, but it's possible.
- ***Possibility to loop back up to another mutant strand synthesis reaction from here to perform multiple mutations on the same template. I'd quantify the cleaned-up DNA before doing another PCR. There'd also be enough DNA to loop back AND proceed with a transformation just in case the loop method doesn't work.***
- Thaw cells on ice while chilling electroporation cuvettes.
- Add 2 µL desalted DNA to thawed cells, gently mix, and keep on ice for a few minutes.
- Transfer mixture to the gap of a chilled cuvette.
- Pulse at 1.8 kV, etc.
- Immediately add 950 mL sterile SOB or SOC (heated to 37˚C) to cuvette and resuspend cells.
- Transfer to a culture tube and recover in the shake incubator for 1 hour.
- Plate on antibiotic plate and incubate for about 16 hours.