QuikChange Primer Design

By hand:
The mutagenic oligonucleotide primers for use in this protocol must be designed individually according to the desired mutation. The following considerations should be made when designing mutagenic primers:

- Both of the mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid.
- Primers should be between 25 and 45 bases in length, with a melting temperature (Tm) of ≥78°C. Primers longer than 45 bases may be used, but using longer primers increases the likelihood of secondary structure formation, which may affect the efficiency of the mutagenesis reaction.
- The following formula is commonly used for estimating the *T*m of primers:

$$T\text{m} = 81.5 + 0.41(\%\text{GC}) - (675/N) - \%\text{mismatch}$$

  For calculating *T*m:
  - *N* is the primer length in bases
  - values for **%GC** and **% mismatch** are whole numbers

  For calculating *T*m for primers intended to introduce insertions or deletions, use this modified version of the above formula:

$$T\text{m} = 81.5 + 0.41(\%\text{GC}) - (675/N)$$

  where N does not include the bases which are being inserted or deleted.

- The desired mutation (deletion or insertion) should be in the middle of the primer with ~10–15 bases of correct sequence on both sides.
- The primers optimally should have a minimum GC content of 40% and should terminate in one or more C or G bases.

By computer:

1. Use Agilent Technologies' Primer Design program: https://www.genomics.agilent.com/primerDesignProgram.jsp
2. You will need to create a free account with them
3. Paste your DNA sequence into the box
   a. click "Upload Now" if you want to control the exact nucleotide change (or if the mutation is in an untranslated region)
   b. click "Upload Translated" if you want the program to give you the amino acids at each position and it will choose the mutated codon
4. Select the type of mutation you want to do:
   a. Change nucleotides or amino acids
   b. Deletion between two bases or residues
   c. Insertion between two bases or residues
5. Click design primers
6. Check the outputted primers to make sure that the codon you or the program chose (if you "Upload Translated") is not a low usage codon
   a. http://www.kazusa.or.jp/codon/
   b. Human codon usage: http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=9606

Notes on Primer Design:

1. If your primers are >50 bp long, you should consider having them PAGE purified after synthesis. Purification will increase the likelihood that positive clones do not have additional unwanted mutations (usually deletions) in the primer region
2. For standard mutagenesis (single site), you need two perfectly complementary primers--one corresponding to the sequence of the coding strand and one to the non-coding strand
3. For multi-site protocols, you need just one primer for each site (can contain more than one mutation). All primers must correspond to the same strand (can be coding or non-coding).

Notes on SDM:

1. You can do SDM without a kit (see below), or using a kit from Aglient. It is much cheaper to do it without a kit, but some people prefer the kit and some applications work better with the kit.
2. If you do SDM without a kit, you can use various enzymes. Those used here are fairly slow, but new advances in polymerases can make the process faster.
    a. Make sure that you choose a polymerase with as high fidelity as possible.
    b. NEB Q5: 2X master mix includes dNTPs, buffer, and enzyme.
        i. Add water, template, and primers
        ii. 20-30 sec/kb elongation time
    c. NEB Phusion:
        i. 15-30 sec/kb elongation time

QuikChange Site-Directed Mutagenesis

1. Dilute primers to 1 ug/uL (use water)
2. Make 40uL of 125 ng/uL primer stocks
3. Prepare reaction mix:
    a. 5uL 10x pfuUltra buffer
    b. 1uL dNTPs
    c. 1uL fwd primer (125 ng/uL)
    d. 1uL rev primer (125 ng/uL)
    e. 5uL template DNA (10 ng/uL)
    f. 36uL water
    g. 1uL pfuUltra
4. Run PCR program
    a. 95ºC 2 min
    b. 95ºC 30 sec
    c. 55ºC 1 min
    d. 72ºC 7 min (1 min/kb)
    e. Repeat b-d 18 cycles
    f. 72ºC for 7 min
    g. Hold at 4ºC
5. Digest reaction with 2uL of DpnI for 2 hours at 37ºC
6. Transform 2uL into Z-comp cells
    a. Plate 1 drop, the rest

Multi Site-Directed Mutagenesis

1. Dilute primers to 1 ug/uL
2. Phosphorylate all of the “top” or “btm” primers
    a. 5uL 10X T4 ligase buffer
    b. 4uL oligo (1 ug/uL)
    c. 40uL water
    d. 1uL T4 PNK
    e. Incubate for 1-2 hours at 37ºC
3. Prepare reaction mix:
    a. 3uL 10X pfuUltra buffer
    b. 3uL 10X Taq ligase buffer
    c. 1uL dNTPs
    d. 1.5uL each phosphorylated oligo (use all “top” oligos or “btm” oligos for a single rxn)
    e. 5uL template DNA (10 ng/uL)
    f. 34.5uL water
    g. 1uL pfuUltra
    h. 1uL Taq ligase
4. Run PCR program
    a. 95ºC 5 min
    b. 95ºC 1 min
    c. 55ºC 1 min
    d. 72ºC 14 min (2 min/kb)
    e. Repeat b-d 30 cycles
    f. 72ºC for 14 min
    g. Hold at 4ºC
5. Digest reaction with 2uL of DpnI for 2 hours at 37ºC
6. Transform 2uL into Z-comp cells
    a. Plate 1 drop, the rest

Transformation using Z-Comp Cells

1. Prewarm plates at 37ºC for at least one hour
2. Thaw tube of Z-Comp cells on ice for ~5 minutes
    a. You can use all 50uL for one reaction or split into two 25uL aliquots
3. Add reaction or supercoiled DNA to cells and stir gently with a pipet
    a. Supercoiled DNA: 10 ng or less
    b. SDM: 2uL of reaction
    c. Gibson: 2uL of reaction
    d. Ligation: 2uL of reaction
4. Incubate for ~5 minutes on ice
5. Remove plates from incubator and label with the following information:
    a. Strain
    b. Construct and cloning technique if applicable
    c. Date
    d. Initials
6. Add ~10 sterile glass beads to each one
7. Plate all of the cells onto a single plate, or plate one drop (~10uL) onto one plate and the rest of the transformation onto a second plate if you believe you will have a lot of colonies
8. Incubate at 37ºC overnight