Chemistry 5.50 Site Directed Mutagenesis Methods.

Site directed mutagenesis is a standard method to replace a natural amino acid with any other natural amino acid. The method was developed by Zoller and Smith. Smith won the Nobel Prize in Chemistry for this work.

References: Methods in Enzymology 100, 468-500 (1983) describes the use of M13 vectors. Methods in Enzymology 154, 329-50. These references and additional references within the volumes describe the original procedures for making mutants. If you know nothing about cloning of genes, use of M13 phage and their life cycle, this is a good place to start.

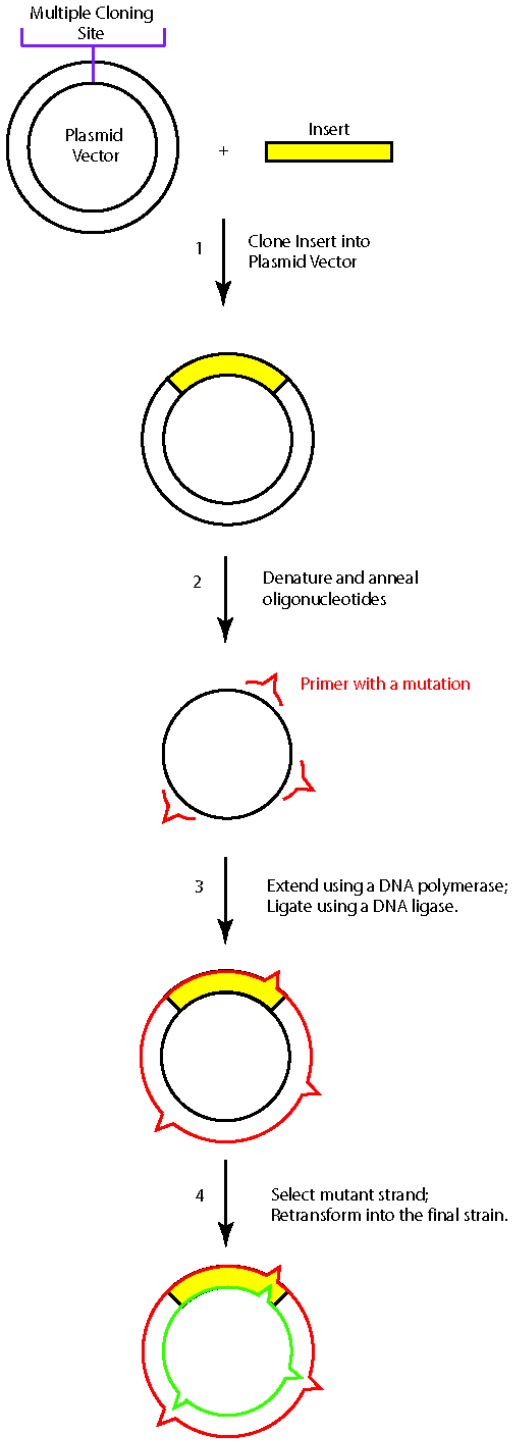
In the next generation of technology associated with making mutants, the success rate of generating the desired mutant was dramatically increased. The methods were developed by Kunkel and Eckstein. The Kunkel method is still used and will be described subsequently.

References: Methods in Enzymology 154, 367 (1987) for the Kunkel method. Nucleic Acids Research 13, 8764 and 14, 9679 describe the Eckstein method.

These methods have been largely replaced using PCR based methods. Two of these methods are described below. All of these methods are now available in "kit" form were the details of the biology are described. A generic overview of the method is described in Figure 1. This figure was redrawn based on the figure from Cosby and Lesley (1997) Promega Notes Magazine Number 61, 12. In the figure the multiple cloning site is a region of the plasmid DNA (can also use genetically manipulated phage M13 DNA) generated to have a large number of restriction sites so that you can take a piece of DNA containing your favorite gene and paste it into this region. One can then transform (transfect in the case of the phage) the duplex DNA and make lots of the plasmid that contains your gene. There are kits that now allow you to easily isolate plasmids. For mutageneis you need to make a synthetic oligonucleotide with the desired mutation. You need to use the genetic code table to make a minimal number of changes to convert the triplet codon coding for the amino acid normally present in the DNA to the triple codon for the amino acid of interest. You will then synthesize an oligonucleotide that contains the desired nucleotide changes, a 15-mer to a 20-mer with the mutation in the middle of the oligonucleotide. One needs to have a sufficient number of bases on either side of the changed bases to ensure unique hybridization to your gene. One then hybridizes the oligonucleotide to the denatured DNA containing your gene and uses a DNA polymerase to make the intact plasmid. The ligase is then used to convert the nicked DNA to closed circular duplex DNA. DNA in this form is most efficiently transformed into *E. coli*. The methods to make mutants differ in their approach to remove the wt or non-mutated strand from the strand that is mutated in the duplex DNA. Once the non-mutated stand is removed the DNA is replicated and gives predominantly DNA with the desired mutation.

Kunkel method (Figure 2): The Kunkel method takes advantage of the abililty of DNA from bacteriophage such as M13 to be isolated in both ss and ds forms. The phage DNA is generated in an *E. coli* that lacks two genes: *dut* and *ung*. The dut gene codes for uracil glycosylase that removes uracil from deoxyuridine that is incorporated into

Figure 1


Multiple Cloning Site
Plasmid Vector
+
Insert
1
Clone Insert into Plasmid Vector
2
Denature and anneal oligonucleotides
Primer with a mutation
3
Extend using a DNA polymerase; Ligate using a DNA ligase.
4
Select mutant strand; Retransform into the final strain.

DNA. It is a DNA repair enzyme. The ung gene codes for an enzyme that converts dUTP to dUMP and PPi and thus prevents dUTP from accumulating and being incorporated into DNA. The ss-DNA with the deoxyuridine will be the strand of the duplex DNA that can be removed by ultimately taking the mutated duplex DNA and transforming it into *E. coli* that now contains uracil glycosylase the dUTP pyrophosphorylase. The undesired wt-strand of DNA is now degraded, leaving only the mutated strand.

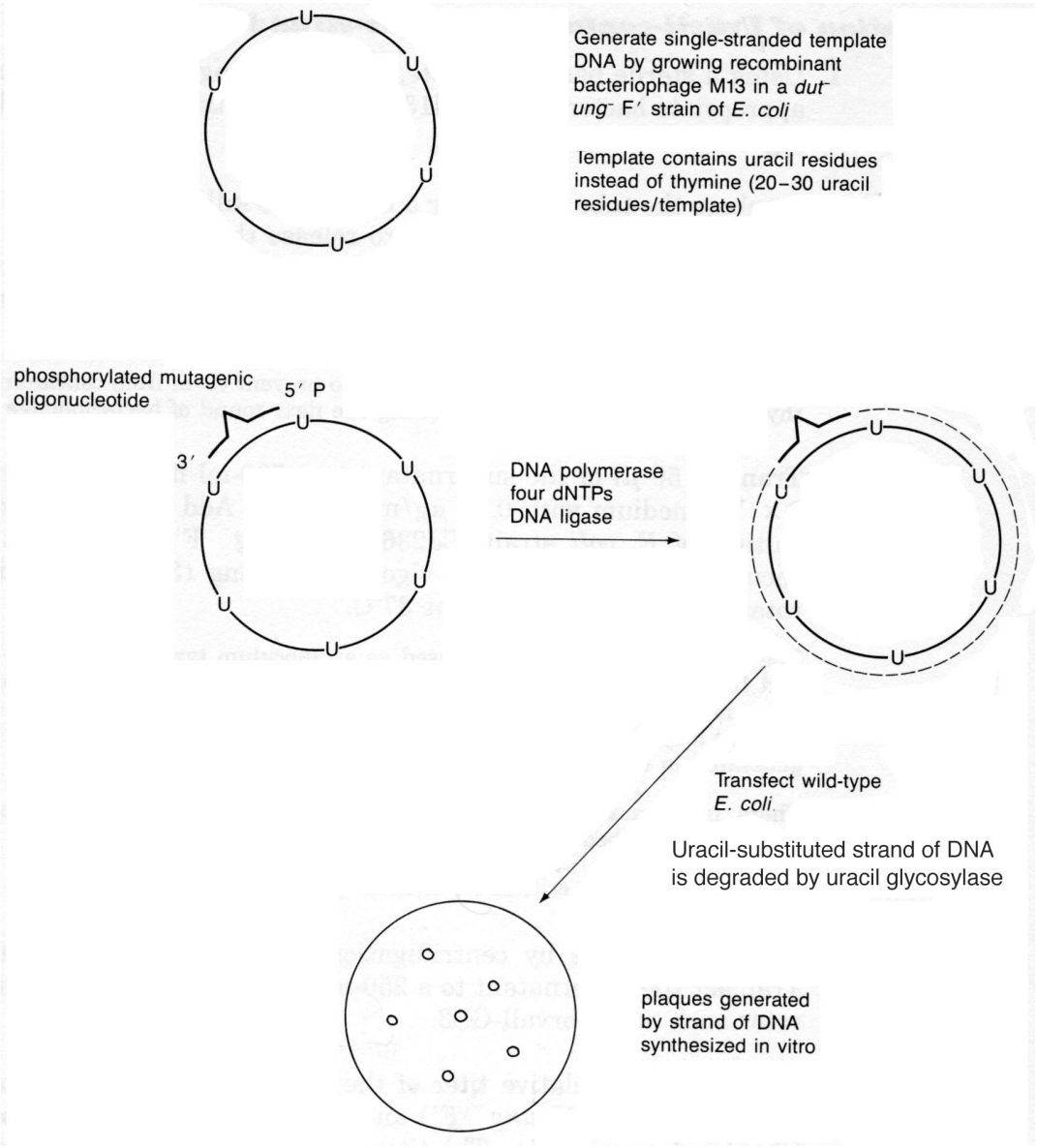


Figure 2: Oligonucleotide site directed mutagenesis using the Kunkel method.

Picture taken from Molecular Cloning: A Laboratory Manual, 2nd edition. Edited by Sambrook, Fritsch, and Maniatis. Cold Spring Harbor Laboratory Press, 1989.

Two PCR based methods are now routinely used to make SDMs. One is available from Stratagene and is called a "Quick-change Kit". This method is shown in Figure 3, taken from the Statagene catalogue. In this case two oligonulceotides containing the mutation of interest, complementary to the two DNA stands in the plasmid, are made synthetically. Removal of the unwanted wt DNA is based on the fact that DNA isolated from *E. coli* is methylated, while the new DNA generated from the oligonucleotides is not. Treatment of duplex DNA with Dpn1, an enzyme that digests methylated DNA, leaves the strands with the desired mutation.

A PCR Based Method for Mutagenesis - Stratagene's "Quick-change" Kit.
The basis for selection is that the parental DNA isolated from E. coli is methylated and sensitive to digestion with the enzyme DpnI. The mutated DNA made by PCR is not methylated and therefore stable to DpnI.

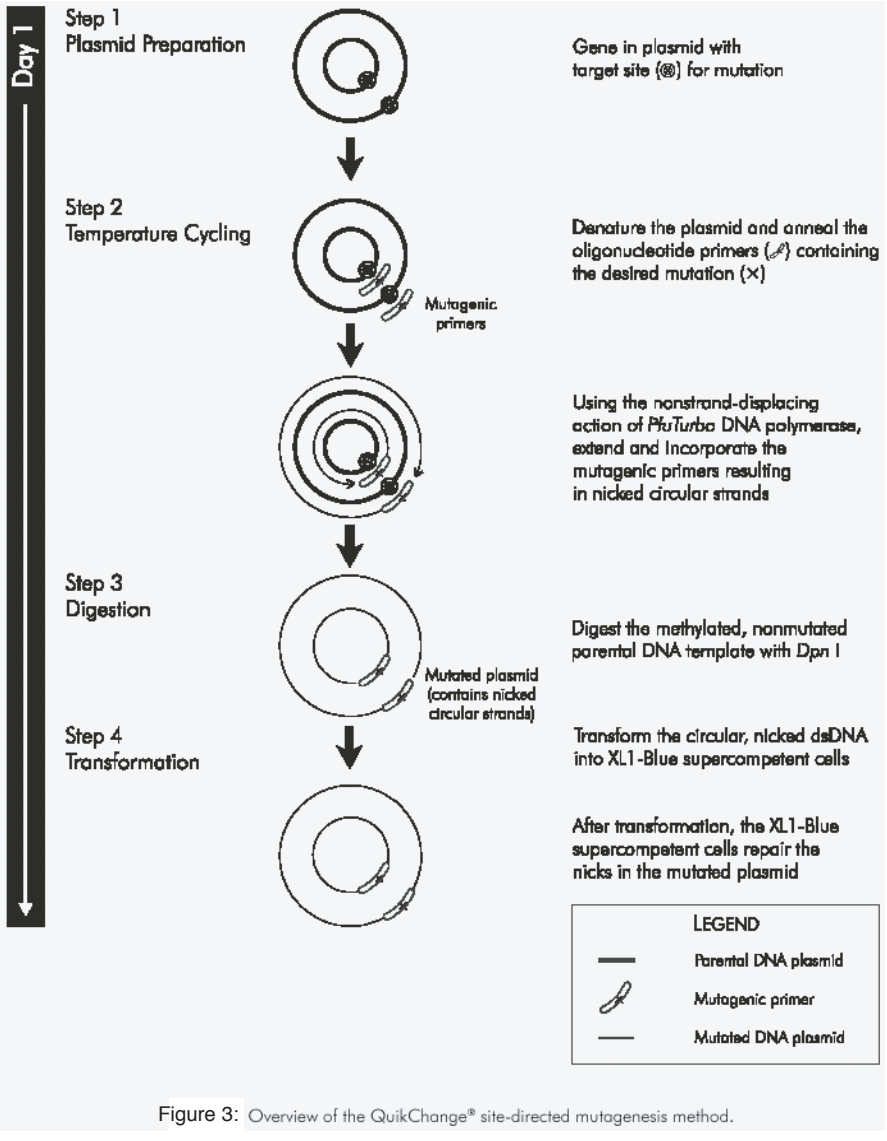
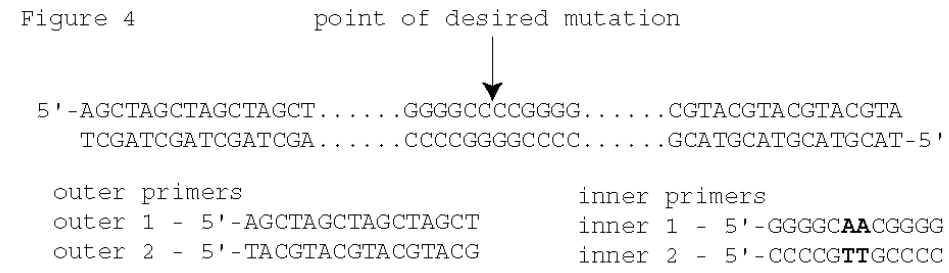


Figure 3: Overview of the QuikChange® site-directed mutagenesis method.

The second PCR based method is outlined in Figure 4. In all of the methods, at the completion of the process, one needs to sequence the DNA to insure that it contains the mutation of interest and no additional mutations.



1) design two sets of primers, outer to amplify entire DNA sequence and inner primers to introducethe desired mutation.

2) do two separate PCR reactions. one with outer primer 1 and inner primer 2 and the other with outer primer 2 and inner primer 1. the resulting products are shown below.

```
5'-AGCTAGCTAGCTAGCT......GGGGCAACGGGG
   TCGATCGATCGATCGA......CCCCGTTGCCCC

                        GGGGCAACGGGG......CGTACGTACGTACGTA
                        CCCCGTTGCCCC......GCATGCATGCATGCAT-5'
```

3) perform a third PCR reaction containing the products from the previous steps, each serving as a template in the final PCR reaction.

```
5'-AGCTAGCTAGCTAGCT......GGGGCAACGGGG  ---DNA synth.--->
<---New DNA synth.---  GGGGCAACGGGG......CGTACGTACGTACGTA
```

4) the final product now contains the entire DNA sequence between the two selected outer primers with the desired mutation introduced at with the inner mutagenic primers.