How the mutagenesis system works.

Mutant Form

aacactagtcactactctgtgctatgGTGTTCAATGCTTTTCAAGATAGCCAGATCTTATGAAACGGCATGACTTTTTCaagagtgccatgcccgaa
ttgtgatcagtgatgagacacgatacCACAAGTTACGAAAAGTTCTATCGGTCTAGAATACTTTGCCGTACTGAAAAAGttctcacggtacgggctt

The bases that are underined are the ones we want to change.

First cycle

Separate (heat-denature), cool and anneal primers:

aacactagtcactactctgtgctatgGTGTTCAATGCTTTTCAAGATAGCCAGATCTTATGAAACGGCATGACTTTTTCaagagtgccatgcccgaa
CACAAGTTACGAAAAGTTCTatgggc5'

Notice the "G-G" and "A-C" mismatch near the 5′ end of the primer (underlined). Those are the bases we are changing.

5'gatcaTATGAAACGGCATGACTTTTTC
ttgtgatcagtgatgagacacgatacCACAAGTTACGAAAAGTTCTATCGGTCTAGAATACTTTGCCGTACTGAAAAAGttctcacggtacgggctt

Notice the "A-A" mismatch

Extend all the way around the circle

aacactagtcactactctgtgctatgGTGTTCAATGCTTTTCAAGATAGCCA-GATCTTATGAAACGGCATGACTTTTTCaagagtgccatgcccgaa
ttgtgatcagtgatgagacacgatacCACAAGTTACGAAAAGTTCTatgggc CTAGAATACTTTGCCGTACTGAAAAAGttctcacggtacgggctt

Top: original Template
Bottom, new strand is linear and has desired change at 5' end (from primer), but not at 3'

aacactagtcactactctgtgctatgGTGTTCAATGCTTTTCAAGATAGCCA gatcaTATGAAACGGCATGACTTTTTCaagagtgccatgcccgaa
ttgtgatcagtgatgagacacgatacCACAAGTTACGAAAAGTTCTATCGGT-CTAGAATACTTTGCCGTACTGAAAAAGttctcacggtacgggctt

Top: new strand is linear and has desired change at 5' end (from primer), but not at 3' end
Bottom:original Template

Second Cycle:
Heat to denature product from above generating new linear DNA

Only showing sequence at ends. Also, let's only consider new (half-fixed) red strand. A similar fate would go to the half-fixed purple strand.

5′gatcaTATGAAACGGCATGACTTTTTC————————————//————————————GTGTTCAATGCTTTTCAAGATAGCCA3′

Note has altered sequence at 5′ end correcting gatct to gatca (from the primer), mutating second half of the BglII site. But, it still has the stop codon (TAG) and the first "A" of the BglII site at the 3′end.

Anneal primer (purple one)

This primer has the fix to the stop codon and the switch the first "A" of the BglII site

5′gatcaTATGAAACGGCATGACTTTTTC————————————//————————————GTGTTCAATGCTTTTCAAGATAGCCA3′
CACAAGTTACGAAAAGTTCTatgggc5′

Extend all along the linear

5′gatcaTATGAAACGGCATGACTTTTTC————————————//————————————GTGTTCAATGCTTTTCAAGATAGCCA3′
3′CTAGTATACTTTGCCGTACTGAAAAAC————————————//————————————CACAAGTTACGAAAAGTTCTatgggc5′

The new purple strand now has all the changes we want: It has the change to the stop codon and the "beginning" of the BglII site because of the purple primer. It has the other mutations because the template red strand already had that mutation…it just copied it.

Go to third cycle below.

Third Cycle

Heat Denature

Let's look at the purple strand, which has all of our changes.

3'CTAGTATACTTTGCCGTACTGAAAAAC——————————————//——————————————CACAAGTTACGAAAAGTTCTatgggc5'

Anneal Red primer

5'gatcaTATGAAACGGCATGACTTTTTC
3'CTAGTATACTTTGCCGTACTGAAAAAC——————————————//——————————————CACAAGTTACGAAAAGTTCTatgggc5'

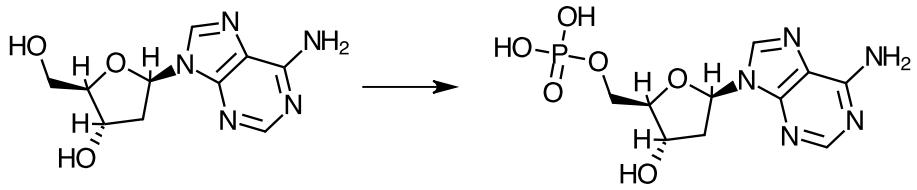
Extend along linear

5'gatcaTATGAAACGGCATGACTTTTTC——————————————-//——————————————-GTGTTCAATGCTTTTCAAGATACCCG3'
3'CTAGTATACTTTGCCGTACTGAAAAAC——————————————//——————————————CACAAGTTACGAAAAGTTCTatgggc5'

This new duplex DNA is completely changed to the form we want. After another 22 cycles, this completely changed linear form will dominate the population.

KLD (Kinase, Ligase, DPNI)

We want to convert this to a circular plasmid so we can transform it into bacteria. First, because we used synthetic DNA for the primers, they lack a phosphate on their 5' end. Ligase, the enzyme that would normally splice two pieces of DNA together, cannot function on DNA without a phosphate on the 5' end. An enzyme called “Polynucleotide Kinase,” or, just “kinase,” does that. It adds a phosphate to the OH at the 5’ carbon.



after that, Ligase can link the two ends of the linear molecule we made into a circle.

The next step is to get rid of as much of the original template DNA as possible, since it does not have the changes we want. We use an enzyme that cuts DNA at the sequence GATC. There are 17 occurrences of that sequence in our original plasmid, which will cut it into little pieces (if you do the math, it should occur once in every 256 bases on average—¼^4). Why won’t it also cut our newly made DNA that we want to use to transform? Because it only cuts DNA if the A in the sequence has an extra methyl group on it. Our DNA was made in our PCR machine. It has no methyl groups on it. But the original template DNA is methylated at that site. So, only our new DNA survives. We use that for transformation.