LAB 15: *IN VITRO* SITE-DIRECTED MUTAGENESIS STRATEGIES AND VERIFICATION

DEVELOPED AND WRITTEN BY

Andre P. Walther
Cedar Crest College
awalther@cedarcrest.edu

Stuart J. Haring
North Dakota State University
stuart.haring@ndsu.edu

Purpose

In this lab module, two strategies for mutagenizing (editing) plasmid DNA are presented. One utilizes PCR amplification and ligation, and the other is ligation-free and has the potential to incorporate multiple mutations simultaneously.

Overview

The power of reverse genetics lies in the ability to change (mutate) the sequence of a DNA at any location while controlling specifically what that sequence change is. This ability to alter the DNA sequence on a plasmid in this manner is generally referred to as *in vitro* site-directed mutagenesis (*iv*SDM) and is based on the fact that an oligonucleotide (primer) used for *in vitro* DNA synthesis becomes a permanent fixture of the DNA. Hence, if one engineers a mutation (base change or changes) into an oligonucleotide, this change becomes part of the final synthesized DNA product.

There are multiple methods for using *in vitro* DNA synthesis to achieve site-directed mutagenesis. Each has its advantages and disadvantages. In this exercise, two different ways to achieve *iv*SDM are described. The first method, called “Round-the-Horn" mutagenesis, uses two primers, with one (mutagenic) primer containing the desired mutation near its 5’ end, to exponentially amplify the entire plasmid. Following exponential amplification, DNA ligase joins the ends of the amplified DNA together. The second method is based on the QuikChange Site-Directed Mutagenesis Kit (Agilent), with the distinction being that only a single primer annealing to one strand is used to synthesize the DNA. While the DNA amplification is not exponential, one can use multiple mutagenic primers on the same DNA strand in the same reaction to generate multiple mutations on the same plasmid simultaneously. The use of any *iv*SDM method is typically application-specific and/or based on the researcher’s previous success with that method. While the two methods presented have fundamental differences, the overall goal is the same—to mutagenize the DNA sequence exactly where and as the researcher desires.

Student Learning Objectives

Upon completion of this module, a student will have

- Designed an oligonucleotide(s) to generate a mutation(s) in plasmid DNA;
- Gained knowledge about *in vitro* DNA synthesis and how plasmid mutagenesis protocols generally work;
- Gained an understanding of the advantages/disadvantages of different *ivSDM* protocols and how these factor into the choice of the type of mutagenesis to be used;
- Performed *iv*SDM to generate their designed mutation(s) in plasmid DNA; and
- Gained experience in confirming the engineered changes in the plasmid DNA.

Safety Precautions

The activities in this module involve the use of non-pathogenic bacteria, reagents, and equipment to manipulate and analyze DNA, and the generation of novel DNA molecules.

- Basic precautions for handling bacteria (e.g., sterile technique, hand washing) should be followed.
- Equipment such as microcentrifuges, which contain motors that spin at up to 14,000 rpm, can be hazardous if the rotor is not seated properly or samples are not balanced.
- Power supplies and electrophoresis boxes pose a high-voltage danger and should always be examined for loose or exposed wires and closed completely to prevent access to the tank while they are running.
- Staining of agarose gels often involves the use of ethidium bromide (EtBr), and gloves (and possibly a lab coat) should be used when touching anything that could have residual EtBr (including the gel itself). Alternatively, a "safe" DNA stain can be used to observe DNA in the gel.
- Analysis of stained gels involves ultraviolet (UV) light, and exposure to this should be minimized through the use of containment in a box (gel documentation station) or through the use of a UV face shield.
- Other safety precautions should be carefully considered by the instructor and tailored to the method by which the exercises in this module are executed.

Introduction

The amino acid sequence of a protein ultimately determines its three-dimensional structure and its function. Changes within the amino acid sequence often lead to changes in protein structure, changes in sites of post-translational modifications, or changes in regulatory or catalytic residues, and as a consequence, lead to alterations in the functional characteristics of that protein. The ability to perform reverse genetics studies through directed mutagenesis of genes has revolutionized our ability to study amino acid contributions to protein function.

In this lab we will be examining two different site-directed mutagenesis strategies. The first will utilize mutagenesis to alter the functional activity of the green fluorescent protein (GFP), which was first isolated from the crystal jellyfish, *Aequorea victoria,* by Osamu Shimomura (1962). GFP is composed of an ordered array of 11 beta sheets that form a structure called a beta-barrel (Figure 1). The beta sheets position important amino acid side chains that form an active site, called a chromophore, that fluoresces green in the presence of ultraviolet light (Tsien, 1998). Mutations in the amino acids that make up the active site can lead to changes in the fluorescence profile of GFP, which can be seen as a change in the color.

The second mutagenesis strategy is one in which multiple mutations in different regions of a gene can be generated at the same time using multiple primers that anneal to the same strand of DNA. For example, even though two sites one desires to mutate lie almost 360 bp (120 aa) from each other, a single mutagenesis reaction can be used to create either a single mutation or a double mutation within the same mutagenesis reaction. This can be a powerful tool when one wishes to mutate multiple sites and/or combinations of sites. In this lab, a gene (*RFA2*) is being mutated at codons for two Ser–Gln (SQ) sites in distant regions of the protein. The primers are designed not only to include missense mutations changing the codons for serine to a codon for alanine at each site, but also to contain DNA sequence changes that do not alter the amino acid coding sequence (silent mutations). This makes it possible to identify that a mutation(s) has occurred through diagnostic restriction digestion and is especially useful when there is not an obvious phenotype (or you need to investigate to identify a phenotype) associated with the change.

Both strategies involve mutation incorporation into the primer(s) used for mutagenesis, but differ in their fundamental approach to mutagenizing DNA sequences on a plasmid, and the use of each will depend on the goals set by the researcher.

References

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Methods

Method 1: "Round-the-Horn" Mutagenesis

This experiment is based on a site-directed mutagenesis study published by Girón and Salto (2011); however, they used a non-PCR (nonexponential) amplification site-directed mutagenesis strategy. In this exercise, their study has been adapted to use an *in vitro* site-directed mutagenesis strategy that involves PCR amplification followed by ligation to make changes in the primary sequence of GFP. Following mutagenesis using this technique, we will examine the phenotypic changes that result from these nucleotide (and ultimately amino acid) changes. To change the GFP protein sequence, we will mutate specific codons in the GFP expression plasmid pGLO (Bio-Rad).

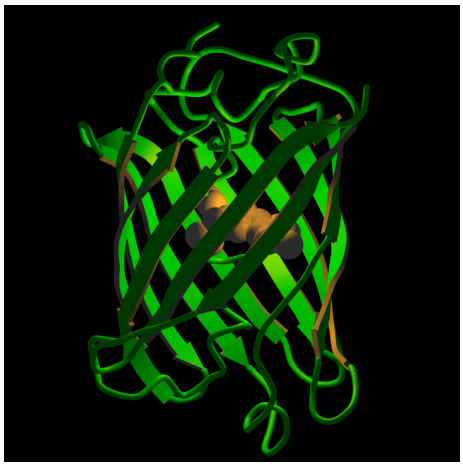

Figure 1: Structure of green fluorescent protein (GFP; Tsien Lab).

There are many ways to incorporate mutations into plasmids, but we will be using a system based on a technique called "Round-the-Horn" mutagenesis. This has been commercialized as the *Q5* Site-Directed Mutagenesis Kit (New England Biolabs). The Q5 site-directed mutagenesis method is performed using thermostable *Q5* DNA polymerase capable of synthesizing large

DNA products by polymerase chain reaction (PCR). The basic procedure utilizes a supercoiled double-stranded DNA (dsDNA) vector and two synthetic oligonucleotide primers containing the desired mutations (Figure 2). The oligonucleotide primers, each complementary to an opposite strand of the vector, are extended during temperature cycling by *Q5* DNA polymerase. Incorporation of the oligonucleotide primers into the newly synthesized DNA molecule leads to linear molecules that are identical to the original plasmid except for the mutation(s) located in the primers. The ends of the linear DNA can then be "glued" together (ligated) to reform a circular plasmid by DNA ligase, and the synthesized vector DNA containing the desired mutations is transformed into ultracompetent bacterial cells. The small amount of starting DNA template required to perform this method, the high fidelity of the *Q5* DNA polymerase, and the small number of thermal cycles all contribute to the high mutation efficiency and decreased potential for generating unwanted random mutations during the reaction.

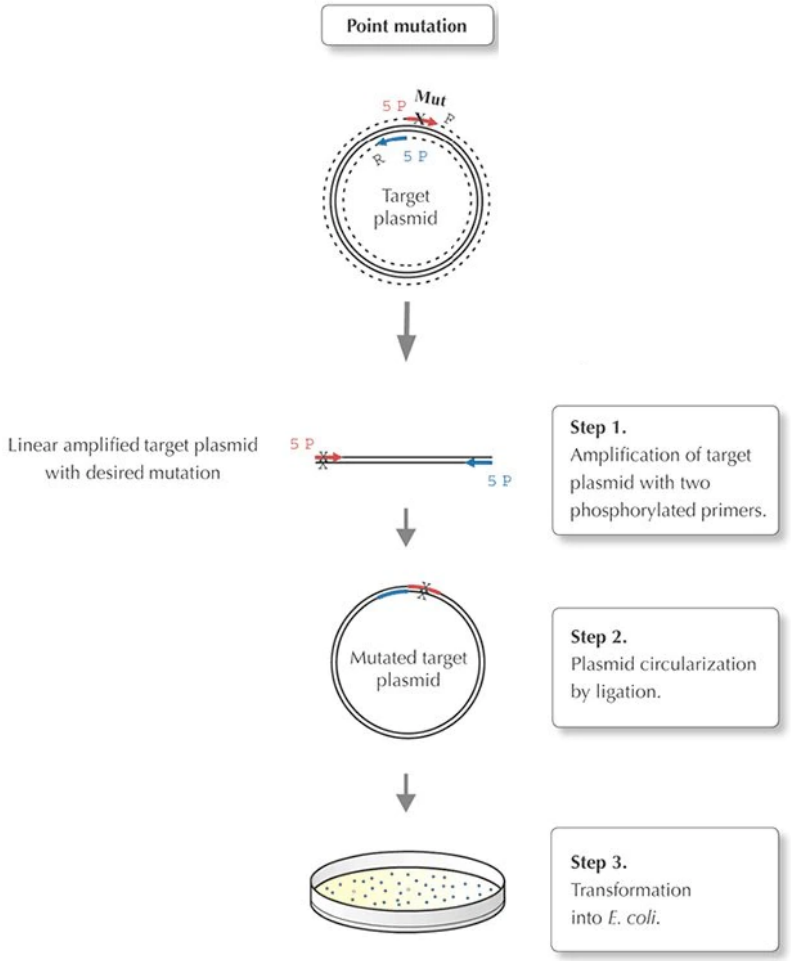

Figure 2: "Round-the-Horn" site-directed mutagenesis. This flow chart depicts the generation of a point (or short DNA sequence) mutation, where the mutation is incorporated into one of the flanking primers used for PCR around the entire plasmid. "5 P" indicates that the primers must be phosphorylated on their 5' ends. This figure has been modified from https://www.thermofisher.com/order/catalog/product/F541#/F541.

Primer Design

The pGLO plasmid (or already altered derivatives) contains the sequence encoding for GFP (or YFP, BFP, or already altered GFP) driven from the bacterial promoter for the arabinose operon (P_{BAD}), and this plasmid DNA will be provided for the student or isolated by the student from bacteria.

Amino acids 66, 145, and 203 of GFP (Figure 3) are found located in a region within the central chromophore. Therefore, mutating these residues (or residues nearby) changes the fluorescent properties of GFP.

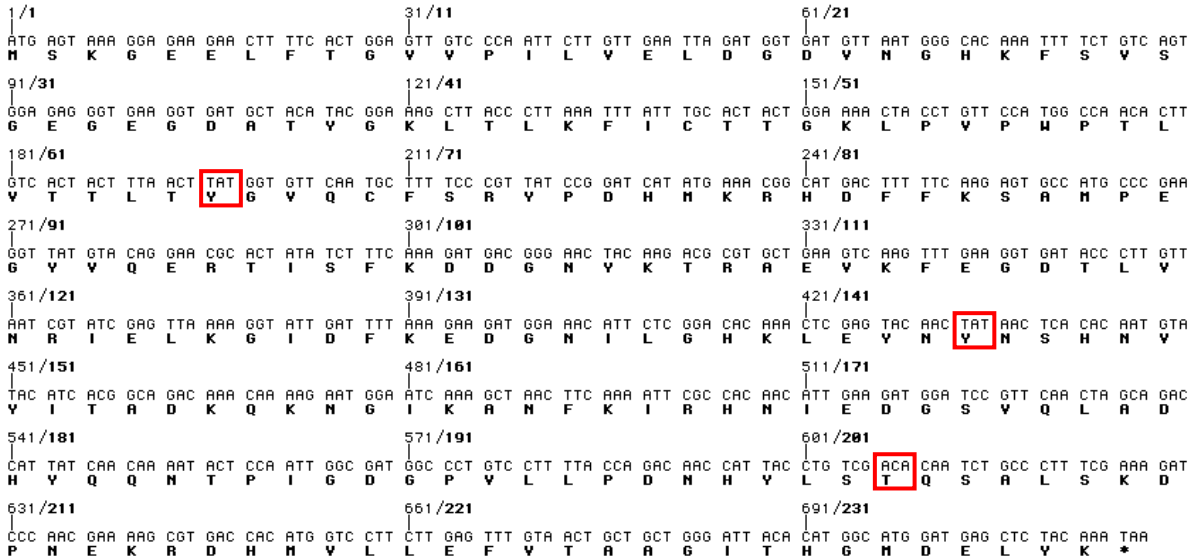

Figure 3: Coding sequence for green fluorescent protein. The primary amino acid sequence of GFP is shown. Red boxes denote amino acids in the central chromophore.

In this exercise, primers are designed to mutate the boxed residues (or residues nearby). Table 1 shows the starting plasmids, the mutation being added by the primer set, and the GFP variant that will result.

Table 1: Primers to generate GFP variants

Starting Plasmid	Mutation Added	Primer Sets	Primer Sequence (5'→3')	Resulting Variant
YFP	Y203T (TAT→ACA)	915	GacaCAATCTGCCCTTTCGAAAGATCC	GFP
		914	GACAGGTAATGGTTGTCTGGTAAAA	
GFP	T203Y (ACA→TAT)	913	GtatCAATCTGCCCTTTCGAAAGATCC	YFP
		914	GACAGGTAATGGTTGTCTGGTAAAA	
GFP (Y145F)	Y66H (TAT→CAT)	910	aaCTcATGGTGTTCAATGCTTTTC	BFP*
		911	AAAGTAGTGACAAGTGTTGGC	
GFP	Y66H (TAT→CAT)	910	aaCTcATGGTGTTCAATGCTTTTC	BFP
		911	AAAGTAGTGACAAGTGTTGGC	
BFP	H66Y (CAT→TAT)	911	AAAGTAGTGACAAGTGTTGGC	GFP
		912	aaCTtATGGTGTTCAATGCTTTTC	

Note: Primers 911 and 914 are nonmutagenic primers used as one of the flanking primers for DNA synthesis around the plasmid template.
* Y145F Y66H results in brighter BFP than Y66H alone.

Mutagenic Primer Design

Since this technique is based on PCR amplification of the template plasmid, the general rules of PCR primer design are in place. The two primers will abut on their respective 5’ ends, but will face in opposite directions, with one being complementary to the Crick strand, and the other complementary to the Watson strand. The mutations will be included in the 5’ end of one of the primers (mutagenic primer), and the second adjacent primer is usually perfectly complementary to the template. The addition of a diagnostic restriction site can be helpful if the expected mutation does not generate an obvious phenotypic change. Generally the mutagenic primer should

- Include 18–21 nucleotides (nt) on the 3’ end with perfect complementarity to template sequence (i.e., plasmid);
- Have the desired mutation(s) located on the 5’ end of the of the oligonucleotide beyond the region with perfect complementarity;
- Have at least 1–2 G or C on the 3’ end of the oligonucleotide, and if possible, have a relatively high GC content (>50%); and
- Generate a base change(s) that adds or removes a restriction site (even if this is through making a silent mutation adjacent to the desired mutation) that can easily be detected by diagnostic restriction digestion and gel electrophoresis.

1. The PCR (i.e., mutagenesis) reaction is set up in a 0.2-mL thin-walled PCR tube as shown in Table 2.

Table 2: Round-the-Horn DNA synthesis reaction

Volume	Component
9 µL	ddH_2O
12.5 µL	2× Q5 Master Mix
1 µL	10 ng/µL Plasmid Template DNA
2.5 µL	10× Primer Set
25 µL	Total volume

2. The tube is then incubated in a thermal cycler using the program in Table 3.

Table 3: Thermal cycler settings for Round-the-Horn DNA synthesis

# cycles	1	30			1
Temperature	98°C	98°C	60°C	72°C	4°C
Time	60 s	20 s	30 s	40 s/kbp	∞

Phosphorylation and Ligation of the "Round-the-Horn" Amplification Products

Once amplified, the products need to be ligated together. If the primers were already ordered with the 5' ends phosphorylated, you only need to set up a ligation reaction. However, if the primers had not been previously phosphorylated, then the following reaction allows both the addition of phosphate to the 5' ends of the amplified product and subsequent ligation of the phosphorylated ends.

3. Set up the reaction as shown in Table 4.

Table 4: Round-the-Horn DNA synthesis reaction

Volume	Component
8 µL	"Round-the-Horn" DNA product
1 µL	T4 polynucleotide kinase (PNK)
10 µL	2x Quick (T4) ligase buffer
1 µL	Quick (T4) ligase
20 µL	Total volume

4. Gently and thoroughly mix each reaction mixture by pipetting the solution up and down several times.
5. Spin down the reaction mixtures in a microfuge by pulsing for 3–5 s (do not go above 10k rpm).
6. Immediately incubate each reaction at 30°C for 30 min to phosphorylate and ligate the plasmid. (The optimum temperature for T4 DNA ligase is 25°C and that for T4 PNK is

37°C, so an intermediate temperature is used to perform both reactions simultaneously.)

Transformation of NEB Ultracompetent Cells

7. Gently thaw the NEB 10-beta Competent *Escherichia coli* cells on ice. For each PCR reaction to be transformed, aliquot 40 µL of the competent cells to a *prechilled* 14-mL BD Falcon polypropylene round-bottomed tube. For the control plasmid (original, unmutated plasmid) to be transformed, aliquot 10 µL of the competent cells to a *prechilled* 14-mL BD Falcon polypropylene round-bottomed tube.
8. Transfer 5 µL of the ligated DNA from each sample reaction to the 40-µL aliquot of the ultracompetent cells. Transfer 1 µL of positive control plasmid DNA to the 10-µL aliquot of the ultracompetent cells. Carefully flick the tube four or five times to mix cells and DNA. *Note*: Do not vortex the tubes.
9. Place the mixture on ice for 30 min. Do not mix.
10. Heat pulse the transformation reactions for 30 s at 42°C.
11. Place the reactions on ice for 5 min. Do not mix
12. Add 450 µL of SOC broth and incubate the transformation reactions at 37°C for at least 1 h with shaking at 225–250 rpm.
13. For the PCR reaction, plate 250 µL of transformation reaction on two LB+Amp+0.2% Arabinose plates and spread the cells with glass beads. For the plasmid reaction, plate 100 µL of the transformation reaction on LB+Amp+0.2% Arabinose and spread the cells with glass beads.
14. Discard the glass beads in a beaker at the front of the classroom and place the plates in a 37°C incubator (cover side down).
15. Let the cells grow overnight (O/N) for 16–20 h.

Phenotypic Analysis (Verification)

To determine the success of the experiment, observe the transformants with a handheld black light that emits in the 395–nm range, and compare changes in the color of the mutated plasmid versus the original plasmid.

Method 2: Same-Strand Primer Extension Site-Directed Mutagenesis

Plasmid DNA Isolation and Quantitation

As with most molecular biology protocols involving recombinant DNA techniques, it is important to have isolated the DNA (genomic or plasmid) that will be used. In this case, plasmid DNA of the vector to be mutagenized must be isolated (e.g., mini-prepped) from bacterial cells and should be quantitated via a standard DNA quantitation method (e.g., spectrophotometry, fluorimetry, or qPCR).

For this particular lab, the vector to be used is pRS315-RFA2 (Figure 4), and we will be attempting to generate up to two mutations simultaneously in the *RFA2* (YNL312W) coding sequence contained within this plasmid. It should be noted that the following protocol can also be used to generate each mutation individually, and even in the context of attempting to mutate two locations at the same time, all combinations of mutations can (and will) be recovered. The concepts of this mutagenesis method are the same, regardless of the number of mutagenic primers used, and the number of primers used in the single mutagenesis reaction really depends on the amount of effort the researcher wishes to invest to confirm each mutation.

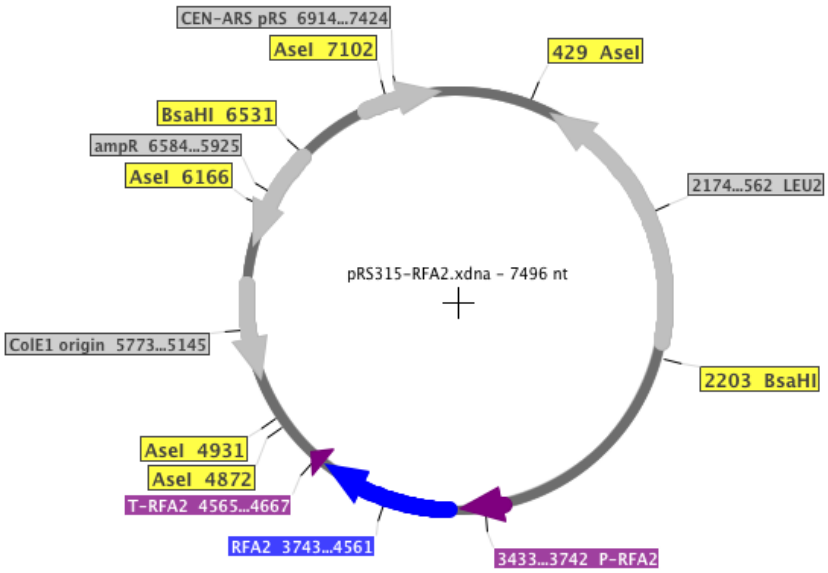

Figure 4: Vector map of pRS315-RFA2 (graphic map generated via *Serial Cloner*). The locations of basic plasmid features are denoted in gray with numbers denoting the start and end positions of every feature. All promoters for expression in yeast are denoted in purple. The coding sequence of the *RFA2* gene is denoted in blue. Each missense mutation generated is accompanied by a silent mutation that generates a new *Bsa*HI or *Ase*I restriction site (i.e., restriction fragment length polymorphism; RFLP) that can be used to identify whether mutagenesis was successful at that site in the *RFA2* gene. All mutations can be confirmed by standard genotyping methods (e.g., sequencing of PCR encompassing the sequence change).

Mutagenic Primer Design

While it is popular to design primers using commercially available and/or web-based oligonucleotide design tools, these are often insufficient for generating primers with desired specific base change(s). Therefore, it is important to understand that there are some standard features of oligonucleotides that are helpful for maximizing specificity and annealing. While it can be argued which are important and which are not, this protocol for primer design uses the following guidelines for the manual design of mutagenic primers. Primers used for *iv*SDM should

- Be at least 35 nucleotides (nt) in length;
- Generate a base change(s) that adds or removes a restriction site (even if this is through making a silent mutation adjacent to the desired mutation) that can easily be detected by diagnostic restriction digestion and gel electrophoresis;
- Have the desired mutation(s) located in the middle of the oligonucleotide, with at least 15 nt of sequence on each side perfectly complementary to the template sequence (i.e., plasmid);
- Have a relatively high melting temperature (>72°C);
- Have at least one or two G or C at the 3' end of the oligonucleotide; and if possible,
- Have a relatively high GC content (>50%).

Note: The above are guidelines, and you can apply other oligonucleotide guidelines as you see fit.

For the DNA template shown in Figure 5, the researcher wishes to mutate the serines (S) at amino acid (aa) positions 122, 238, and 240 to alanine (A). All the desired changes do not fit within the span of one oligonucleotide; however, aa 238 and 240 are close enough for their nucleotide sequence to easily fit with one oligonucleotide. Therefore, two oligonucleotides would be needed to mutate all three sites. We will focus on mutating one site (S122), as primer design guidelines here apply to the design of the other oligonucleotide.

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ATG GCA AGT TAT CAA CCA TAT AAC GAA TAT TCA TCA GTA ACG GGC GGT GGC TTT GAG AAC  < 60
M   A   S   Y   Q   P   Y   N   E   Y   S   S   V   T   G   G   G   F   E   N
            10          20          30          40          50

TCT GAG TCC CGC CCA GGT AGT GGG GAG TCG GAA ACT AAC ACT AGA GTT AAC ACC TTG ACA  < 120
S   E   S   R   P   G   S   G   E   S   E   T   N   T   R   V   N   T   L   T
            70          80          90          100         110

CCT GTG ACG ATC AAA CAA ATT CTA GAG TCC AAA CAG GAT ATT CAG GAC GGC CCC TTC GTT  < 180
P   V   T   I   K   Q   I   L   E   S   K   Q   D   I   Q   D   G   P   F   V
            130         140         150         160         170

TCG CAT AAC CAA GAA CTT CAT CAC GTT TGT TTT GTA GGT GTG GTG AGA AAC ATT ACA GAC  < 240
S   H   N   Q   E   L   H   H   V   C   F   V   G   V   V   R   N   I   T   D
              190         200         210         220         230

CAT ACT GCA AAT ATT TTT TTA ACT ATT GAG GAT GGA ACT GGT CAA ATA GAA GTG AGA AAA  < 300
H   T   A   N   I   F   L   T   I   E   D   G   T   G   Q   I   E   V   R   K
            250         260         270         280         290

TGG AGC GAA GAT GCA AAT GAC TTG GCT GCC GGT AAC GAT GAC TCT TCT GGT AAA GGT TAT  < 360
W   S   E   D   A   N   D   L   A   A   G   N   D   D   S   S   G   K   G   Y
            310         320         330         340         350

GGT TCG CAA GTC GCC CAA CAA TTT GAA ATT GGC GGT TAC GTA AAA GTT TTT GGT GCT TTG  < 420
G   S   Q   V   A   Q   Q   F   E   I   G   G   Y   V   K   V   F   G   A   L
            370         380         390         400         410

AAA GAG TTT GGT GGT AAG AAA AAT ATA CAG TAT GCG GTG ATT AAG CCC ATA GAT TCA TTC  < 480
K   E   F   G   G   K   K   N   I   Q   Y   A   V   I   K   P   I   D   S   F
            430         440         450         460         470

AAT GAA GTG TTG ACG CAT CAC TTG GAA GTC ATC AAA TGT CAT TCC ATA GCC AGT GGA ATG  < 540
N   E   V   L   T   H   H   L   E   V   I   K   C   H   S   I   A   S   G   M
            490         500         510         520         530

ATG AAA CAA CCT TTG GAG AGT GCA TCC AAC AAC AAT GGG CAA TCA TTA TTT GTC AAG GAT  < 600
M   K   Q   P   L   E   S   A   S   N   N   N   G   Q   S   L   F   V   K   D
            550         560         570         580         590

GAT AAC GAT ACA TCT TCC GGC TCC AGT CCG TTA CAA AGA ATT CTA GAA TTT TGT AAG AAG  < 660
D   N   D   T   S   S   G   S   S   P   L   Q   R   I   L   E   F   C   K   K
            610         620         630         640         650

CAA TGT GAG GGC AAA GAC GCT AAT TCA TTC GCT GTT CCC ATT CCA TTG ATC TCG CAA TCC  < 720
Q   C   E   G   K   D   A   N   S   F   A   V   P   I   P   L   I   S   Q   S
            670         680         690         700         710

TTG AAT TTG GAT GAA ACT ACC GTC AGA AAC TGC TGT ACG ACC TTG ACT GAC CAG GGT TTT  < 780
L   N   L   D   E   T   T   V   R   N   C   C   T   T   L   T   D   Q   G   F
            730         740         750         760         770

ATC TAC CCA ACT TTT GAT GAC AAT AAC TTC TTT GCC CTA TGA  < 822
I   Y   P   T   F   D   D   N   N   F   F   A   L   *
            790         800         810         820
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Figure 5: Sequence of the *RFA2* coding region (sequence map generated via *Serial Cloner*). The nucleotide sequence is shown as codons, with the amino acid encoded for shown below each codon. Numbers indicate base position within the 822 base pair (bp) coding sequence of the *RFA2* gene. Sequences highlighted in green are those mutated using *iv*SDM to generate desired missense mutations. Sequences highlighted in magenta are silent mutations used to generate a RFLP.

To mutate serine 122 to alanine (S122A), one must alter the codon (5'-TCG-3') at nucleotide position 364 to an alanine codon (5'-GCN-3'). Unfortunately, this will not create a RFLP. However, altering a nucleotide just one position upstream of the serine codon, and in combination with the S122A change, will allow the generation of a silent mutation (Figure 6) that generates a new *Bsa*HI site.

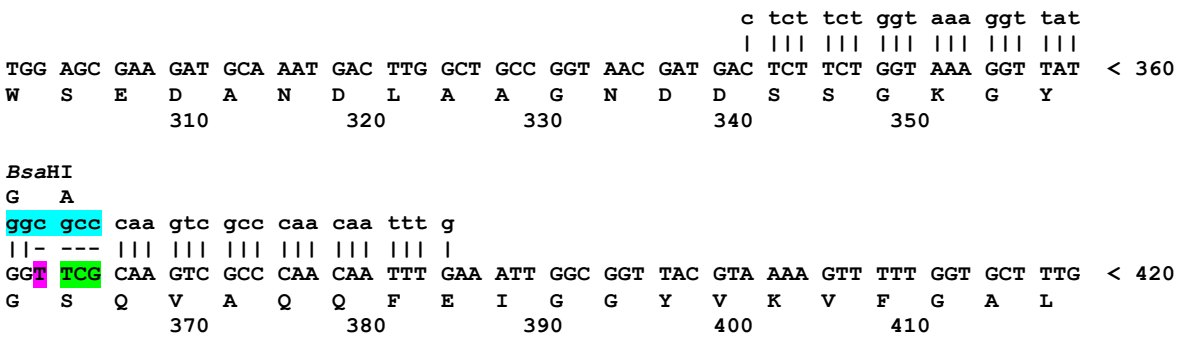

Figure 6: Primer designed to generate the S122A mutation in the *RFA2* gene. A portion of the nucleotide sequence from Figure 2 is shown. Above the *RFA2* coding region in lowercase letters is the oligonucleotide designed to make the S122A mutation. The sequence highlighted in light blue contains a missense mutation (5'-TCG-3' mutated to 5'-GCC-3'; S122A) and a silent mutation (5'-GGT-3' mutated to 5'-GGC-3'; G121G) to generate a novel *Bsa*HI site (5'-GRCGYC-3'; R = purine and Y = pyrimidine). This primer is 44 nt in length, contains the desired mutation in the middle with >15 nt of perfectly complementary sequence on both the 5' and 3' ends, has a high predicted melting temperature (80.8°C), and has a G on the 3' end.

This same sort of rational design can be used at S238 and S240 to generate missense mutations at these sites, as well as a diagnostic RFLP for mutation identification. Of course, once mutations are identified, sequencing analysis is used to verify proper changes in the DNA sequence.

Mutagenesis Protocol

The protocol in Figure 7 is essentially a primer extension reaction on a plasmid template. Multiple primers (must be on the same DNA strand) can be annealed to the same DNA template, and *in vitro* DNA synthesis performed using a high-fidelity DNA polymerase. This protocol does not require the primers to be phosphorylated, as modification (phosphorylation) and ligation occur within the bacterial cell. Furthermore, multiple primers can be used, because Q5 (or Phusion) DNA polymerase (New England Biolabs) does not have nick-translation activity or 5'→3' exonuclease (primer removal) activity. Therefore, the DNA polymerase synthesizing DNA from one primer will not peel off or chew up DNA synthesized from another primer.

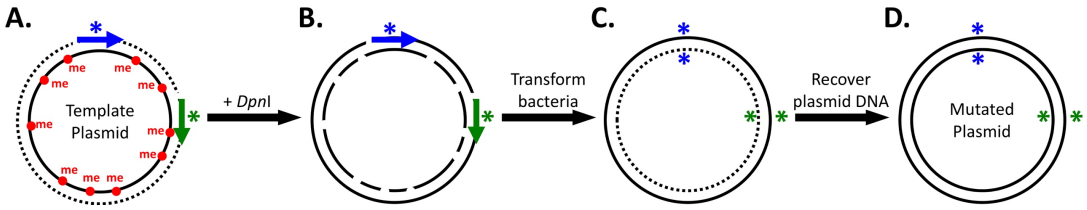

Figure 7: *In vitro* site-directed mutagenesis (*iv*SDM) using primer extension of multiple mutagenic primers. (A) Template plasmid DNA is isolated from bacterial cells. This DNA was methylated by bacterial deoxyadenosine methylase (red circles labeled "me"). The isolated plasmid DNA is denatured by heat (only one strand shown; solid black line), one (or more) mutagenic primers (blue and green arrows; mutations denoted by asterisks) anneal to the template DNA, and DNA synthesis occurs (black dotted line). (B) Following 30 cycles of the primer extension reaction, the restriction enzyme *Dpn*I is added to cleave the methylated template DNA strand (dashed line indicates cleavage by *Dpn*I). Note that two nicks remain on the synthesized strand in (A) (i.e., no DNA ligase was added); however, this DNA strand is impervious to *Dpn*I digestion, since it is unmethylated. (C) This "double-stranded DNA," which is nicked on the synthesized strand with mutations and multiply-cleaved on the template DNA strand, is transformed into bacterial cells. Using cellular enzymes, the synthesized DNA in (A) is now modified and ligated, and the original template strand, being broken on average every 256 bp, is restored via DNA synthesis (black dotted line) using the *mutagenized* DNA strand as the template. (D) The final result is a fully mutated plasmid that is recovered with high efficiency. NOTE: Although the figure shows both mutations happening on the same DNA, this requires that both primers anneal to the same single DNA strand. In reality, one will recover all combinations of mutations; in this case, plasmids containing the single blue mutation, the single green mutation, or the double mutation. Further analysis is needed to identify single/double mutant plasmids.

Additionally, this protocol takes advantage of DNA methylation of 5'-GATC-3' by deoxyadenosine methylase (dam). Since this activity is only in the bacterial cell from which the plasmid DNA was isolated and not in the *in vitro* reaction, the newly synthesized DNA will not be methylated. Subsequent restriction digestion with *Dpn*I (5'-GA^{me}TC) cleaves only the DNA template and not the mutagenized strand, maximizing avoidance of the bacterial mismatch repair system and increasing efficiency of mutated plasmid recovery.

The protocol is as follows:

1. Plasmid DNA (pRS315-RFA2) is isolated and quantitated. This DNA is diluted to a concentration of 100 ng/µL.
2. The mutagenic primers are diluted to a working concentration of 5 µM.

    a. Mutagenic primer 1: rfa2-S122A-BsaHI (5'-C TCT TCT GGT AAA GGT TAT GGc gCc CAA GTC GCC CAA CAA TTT G-3')
    b. Mutagenic primer 2: rfa2-S238A,S240A-AseI-F (5'-AAT TCA TTC GCT GTT CCC ATT CCA TTa ATC gCG CAA gCC TTG AAT TTG GAT GAA ACT ACC-3')

3. The first (DNA synthesis) reaction is set up as shown in Table 5 in a 0.2-mL thin-walled PCR tube.

Table 5: The *in vitro* DNA synthesis reaction for *iv*SDM

Volume (µL)	Component
X	ddH_2O
10	5× Q5 HF buffer
3	25 mM $MgCl_2$
2	10 mM dNTPs
10	5 µM Mutagenic Primer 1
(10)	(5 µM Mutagenic Primer 2+)
5	100 ng/µL plasmid template DNA
1.5	100% DMSO
0.5	2 U/µL Q5 HF DNA polymerase
50	Total volume

4. Vortex and spin-down (gently; under 10k rpm for 3–5 s) contents in tube.
5. Place tubes in the thermal cycler and program as shown in Table 6.

Table 6: Thermal cycler settings for *in vitro* DNA synthesis reaction

# cycles	1	30			1	
Temperature	95°C	95°C	55°C	72°C	72°C	4°C
Time	3 min	1 min	1 min	1 min/kbp	5 min	∞

6. Upon completion of the DNA synthesis reaction, add 1 µL of *Dpn*I to the tube.
7. Vortex and spin down (gently; under 10k rpm for 3–5 s) contents in tube.
8. Incubate the tube at 37°C for 1 h.
9. Transform the reaction mixture into competent bacterial cells and selected-for transformants using LB+Amp plates.

    a. If doing transformation of bacterial cells by electroporation (recommended), 0.5 µL of the mutagenesis reaction should be transformed.
    b. If doing chemical transformation of bacterial cells, transformation of 5–10 µL of the reaction is recommended.
    c. A positive (+) control (pRS315-RFA2) for transformation should also be used.

Diagnostic Restriction Digestion and Gel Electrophoresis to Identify Mutagenesis Candidates

Once colonies (transformants) are observed, one can utilize colony cracking (DNA Technology I: Generating a Vector through Golden Gate Assembly for CRISPR-Cas9 Gene Editing) followed by diagnostic restriction digestion and gel electrophoresis to identify RFLPs indicative of successful mutagenesis. Included in each primer named above is the restriction enzyme that should be used to identify the RFLP for each mutation. A 1.0% agarose gel is recommended to identify changes in the restriction digestion pattern, and these changes should be compared with unmutagenized pRS315-RFA2 digested with these same restriction enzymes. Furthermore, Serial Cloner can be used to virtually predict or confirm that the changes in restriction digestion

pattern are those that should be generated upon changes in the base sequence of the plasmid DNA (i.e., mutagenesis). Ultimately, DNA sequencing should also be performed (the process for doing this type of analysis is described in Module B).

Student Assessment

Assessments for this lab focus on understanding how site-directed mutagenesis works and that there are multiple methods for performing mutagenesis with common features and differing features that make each advantageous and disadvantageous. This exercise is also useful if a working knowledge of Serial Cloner has been acquired (Module C). Some suggested questions or topics are as follows:

- Serial Cloner manipulation of DNA
    - Get the plasmid sequences for pGLO and pRS315-RFA2, enter them into *S*erial Cloner, scan for features, and save (and submit) the plasmid sequences as .xdna files.
    - Use "Align" in Serial Cloner to locate the sequences where the mutagenic primers anneal with their appropriate plasmid.
    - Manually search for the sequence that each primer anneals to and manually edit the sequence to represent the potentially-mutated vector. Save (and submit) these plasmid sequences as .xdna files.
- DNA analysis by sequence alignment, virtual diagnostic restriction digestion, and virtual gel electrophoresis
    - Align the mutated plasmid vector with the mutagenic primer. What do you see (and submit your results)?
    - Align the mutated plasmid vector with the original plasmid. What do you see (and submit your results)?
    - Does a mutation generate a difference in restriction enzymes that cut in that region? Submit your results and if a difference is observed, highlight the sequence where a restriction site is gained/lost.
    - For mutated plasmids that show a restriction site gain/loss, run a virtual for both the original and mutated plasmid with that enzyme and submit your results (with virtual gel also included).
- "Round-the-Horn" mutagenesis
    - What is a potential advantage of this procedure and why might this increase mutation frequency?
    - What is a potential disadvantage of this procedure and explain why this is a disadvantage?
    - Within the mutagenic primer, where can base sequence changes absolutely *not* be engineered and why?
    - Why do the 5' ends of the primers, or after the mutagenesis reaction the 5' ends of the amplified DNA product, need to have phosphates added?

- In some other mutagenesis strategies, the enzyme *Dpn*I is added to help increase mutagenesis efficiency. Why is there no need to add *Dpn*I in "Round-the-Horn" mutagenesis?
- For mutation of GFP specifically, why does the medium the cells are plated on after transformation contain arabinose?

- "Same-strand" mutagenesis

  - What is a potential advantage of this procedure? Explain why this is an advantage.
  - What is a potential disadvantage of this procedure and why is it a disadvantage?
  - Within the mutagenic primer, where can base sequence changes absolutely *not* be engineered and why?
  - Why do the 5' ends of the primers *not* need to have phosphates added?
  - In this mutagenesis strategy, the enzyme *Dpn*I is added to help increase mutagenesis efficiency. How does this increase mutagenesis frequency?
  - If three mutagenic primers are used on a plasmid vector in the same reaction, what are the possible outcomes for recovery of mutant plasmids?

Instructor's Notes

Laboratory Preparation

Equipment

- Microfuge
- Vortexer
- P-10, P-100, and P-1000 (or similar volume range) variable volume micropipettors
- Spectrophotometer/Nano-Drop
- Thermal cycler
- Electroporator (only necessary if transforming DNA by electroporation)
- 37°C incubator
- Gel box and casting tray
- Power supply
- Gel documentation system

Reagents

- Bacterial cells containing pGLO or pRS315-RFA2 (or the purified plasmid DNAs of each)
- Components/kit for mini-preps (if having students isolate DNA)
- Components for PCR (*in vitro* DNA synthesis)
- Q5 DNA polymerase and 5× buffer
- 2× Q5 Master Mix
- T4 polynucleotide kinase (PNK)
- Quick (T4) ligase and 2× buffer
- 0.2-mL thin-walled tubes for thermal cycler
- Competent bacterial cells
- 0.1-cm electroporation cuvettes (if using electroporation)
- LB+Amp plates
- LB+Amp+0.2% arabinose plates
- Gel loading dye
- Agarose
- 1× TBE or TAE
- 1 mg/mL ethidium bromide (or alternative DNA staining solution)
- Lysozyme
- Sucrose
- 1 M Tris-HCl (pH 8.0)
- 0.5 M EDTA (pH 8.0)
- 10 mg/mL RNase A (DNase-free)
- *Ase*I and *Bsa*HI and 10× buffer (or other restriction enzymes you choose for diagnostic digestion)

Connections to Other Lab Exercises

Knowledge of how to use Serial Cloner and how to apply it to recombinant DNA experimental design (described in Module C) is particularly useful for planning mutations, designing mutagenic primers, and virtual verification of wet lab results.

Recommended Schedule for the Lab Exercise

This lab exercise is scheduled to be performed over the course of four to six ~1- to 2-h lab periods. This spacing is recommended, as the product from (completion of) one lab is often required for the next.

- Period 1: Setting up mutagenesis reaction (either type).
- Period 2: T4 PNK/ligase reaction or *Dpn*I digestion (depending on type of mutagenesis reaction), followed by bacterial transformation/electroporation.
- Period 3: Colony recovery and growth of cells.
- Period 4: Phenotypic analysis (GFP change) or colony cracking and restriction digestion of crude plasmid DNA.
- Period 5: Gel electrophoresis and RFLP analysis of digested plasmid DNA to confirm mutagenesis.
- Period 6: Sequencing analysis (although not described in this module, the procedure for doing this is described in Module B).