Site Directed Mutagenesis effect on the coding sequence of Red Fluorescent Protein expression through a P1-RFP plasmid

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Abstract: Recombinant DNA and expressing desired traits through gene expression has become a desired field. The ability to measure success of manipulation required the need of a marker, Red Fluorescent Protein(RFP). RFP is a reporter gene[3] that fluoresces so that one can analyze intensity of a gene it is binding onto[4]. Our experiment is to introduce the RFP gene into a plasmid with a conserved promoter and ribosome binding site and mutate the RFP coding region to measure its altered fluorescence. Sub experiments along the way test Magnesium concentrations role in Polymerase chain reaction and suffer and molar ratios test ligation and its efficacy for RFP and bacterial E. coli DH5 alpha cells. Our results showed that the absence of stuffer aided ligation of RFP into the promoter and ribosome binding site plasmid, while the molar ratio had no statistical effect. The stuffer and molar ratio for transformation efficacy did not display any statistical advantage. Our results also prompted us in the direction of increasing Magnesium concentration in polymerase chain reaction to yield more product while unaccounted for mutations during SDM began to uncover truths about other regions of the RFP sequence with both not having statistical power to make definitive conclusions. Further analysis of the polymerase chain reagents for optimization, molar ratio for ligations, and other mutation regions within the RFP gene or cis and trans locations could yield interesting results based on the intensity of RFP expression.

Introduction:
Recombinant DNA techniques have been used in recent years to improve: food and agriculture, health and disease, and the environment[1]. The ability to target and induce cis and trans-regulatory mutations allows the creation of molecular "switches" that allows for control over gene expression[2]. Red Fluorescent Protein (RFP) is a reporter gene[3] that can be analyzed through its fluorescent intensity to correlate recombinant activity[4]. Instances such as Site-Directed Mutagenesis (SDM) of Propene Monooxygenase has shown the importance of side chain volume of V188 in accordance to its stereoselectivity[5]. Optimum $MgCl_2$ concentrations is said to range from 1.5-4.5mM[6] and with ligations with no stuffer[7] and a molar ratio of 4:1 of insert: vector[8]. In our experiment, we are inserting RFP into a Biobrick plasmid with a promoter (P1) and ribosome binding site (RBS) where we will then introduce a SDM mutation to analyze the phenotypic difference in color through absorbance. We hypothesize that this substitution will result in a lower expression of RFP in the bacteria. "In order to accomplish this task, we begin with QIAprep Alkaline Lysis Plasmid purifications to isolate the P1 plasmid from the chromosomal DNA. In preparation for ligating RFP in an equal and increased molar ratio we PCR the RFP sequence using primers to produce tons of copies of RFP which we clean from the PCR reagents. Adding specific type II restriction enzymes Spe1 and Pst1 to P1 and Pst1 and Xba1 to RFP we create sticky ends on the plasmid and RFP sequence that are specific for one another and allows the Biobrick plasmid to anneal together following the removal of the stuffer region from the P1 plasmid so that the RFP has a greater chance at annealing with the P1 plasmid without the stuffer re-annealing back in. Once ligated, confirmation of the ligation can be seen through the properties of the lacZ gene with alpha complementation giving a visual cue as to if the ligation occurred with stuffer, or RFP. Once the P1-RFP plasmid has been made designing primers with a substitution through NEBaseChanger is utilized to create a substitution that will alter the coding sequence of RFP through PCR where linear double stranded RFP will be made to be treated with kinase, ligase, Dpn1 (KLD) to attain a plasmid form of the RFP. Once the treatment has worked and has been confirmed on a gel as supercoiled, one can sequence the RFP and see if the mutations took place while the RFP can be streaked to observe the phenotypic color intensity."[15] Two side experiments were run in the process of attaining the P1-RFP bacteria. The first experiment was to test increasing concentration of Magnesium (Mg^{2+}) and its effects on PCR yield. The second experiment was to test the the presence of stuffer and/or the molar ratio in regards to ligation efficiency. We hypothesize that increasing the Mg^{2+} concentration will not increase PCR yield. From the ligations we hypothesize that no stuffer will yield higher ligation efficiency, and increased molar ratio of RFP: P1/RBS will also yield a higher ligation efficiency.

Materials and Methods:
Testing PCR amplification of RFP for insertion into P1/RBS plasmid with increased magnesium concentration:

- Go Taq Green Master Mix
- Forward and reverse primer
- Plasmid DNA
- DiH_2O
- $MgCl_2$

Add 25µl of 2x Go Taq master mix, 2µl of 5µM forward and reverse primer, 5µl of 2ng/µl plasmid DNA with 18µl of di-H_2O to have 50µl of our positive control for 1.5mM Mg^{2+}. The negative control had no DNA added (5µl di-H_2O added). Our 2Mm Mg^{2+} experiment had 2.5µl of 10 $MgCl_2$ with 15.5µl di-H_2O and our 3mM Mg^{2+} experiment had 7.5µl of $MgCl_2$ with 10.5µl di-H_2O with all the other conditions remaining the same. PCR the samples in the PCR machine: 1x: 94˚C for 3 min, 35x: 94˚C for 30 seconds, 55˚C for 30 seconds, and 72˚C for 90 seconds, and finally 1x 72˚C for 7 min to complete one PCR cycle. Run the cycle 30 times.

SDM treatment:
Table 1. Forward and reverse primer sequence as given by NEBaseChanger when given a substitution of the RFP gene at bp 219 to substitute GAA---AAA (59˚C)

Primer	Sequence
Forward	5'aggtagcGTTAATGGTCATGAATTTGAAATTG3'
Reverse	5'ttcatacgACCTTGAAGCGCATAAATTC3'

Create a forward primer that has the substitution that we wanted to input into the DNA in the sequence for the primer. Create a reverse primer that goes in the opposite direction of the forward primer beginning one nucleotide before the forward primer. Use NEBaseChanger to confirm forward and reverse primer or use their primer design and buy primers. Run PCR of with SDM primers using the NEB Q5 SDM manual.

KLD treatment:

- SDM PCR product
- KLD reaction buffer
- KLD enzyme mix
- Nuclease-free water
- Ice cold dilution buffer
- P1-RFP plasmid
- LB/Ampicillin plates

Mix 1µl of SDM PCR, 5µl of 2X KLD reaction buffer, 1µl of 10X KLD enzyme mix, and 3µl of Nuclease-free water. Transform competent cells by adding 1.2ml of ice cold dilution buffer to competent cells. Use 95µl for each of 7 samples. Dilute or concentrate P1-RFP plasmids to 20ng/µl. Spread transformations of 2 plates of 5µl KLD of experimental SDM PCR, 2 plates of 5µl KLD of control SDM PCR, 2 plates of 5µl of 20ng/µl P1-RFP, and 1 plate of 5µl of H_2O onto plates with LB/Ampicillin. After incubation use satellite colony.

Results:
Table 2. Nanodrop reading of 2µl of RFP, P1 #1, and P1 #2 following blanking with warm EB buffer.

Plasmid	Concentration(ng/µl)	A260/A280
RFP	61.0	1.72
P1 #1	76.1	1.74
P1 #2	44.8	1.86

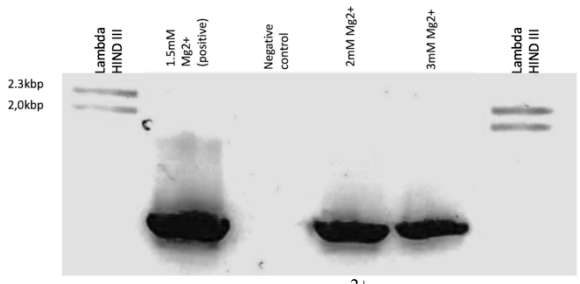

Fig 1. Gel electrophoresis of Mg^{2+} on a 0.8% agarose gel run at 150V. Lane 1 and 6 were loaded with 10µl of lambda HIND III. Lane 2 has 5µl of 1.5mM Mg^{2+}, lane 3 has no Mg^{2+}, lane 4 has 5µl of 2mM Mg^{2+}, and lane 2 has 5µl of 3mM Mg^{2+}.

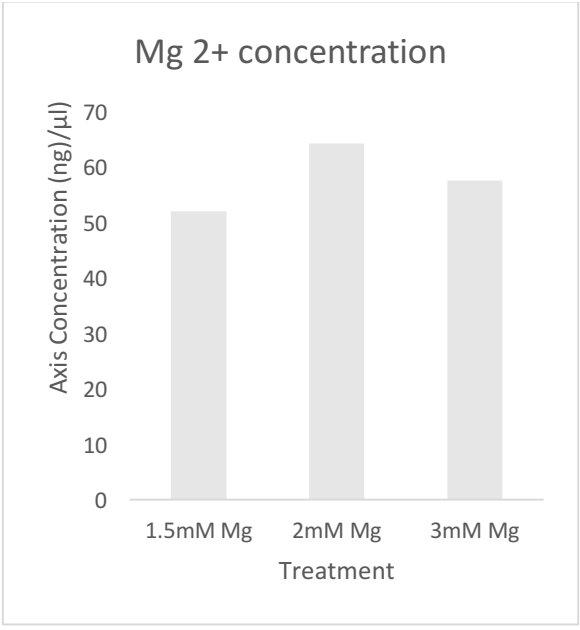

Fig 2. Image studio lite analysis of Fig 1 bands with respect to the intensity of the band per treatment without the smearing being accounted for.

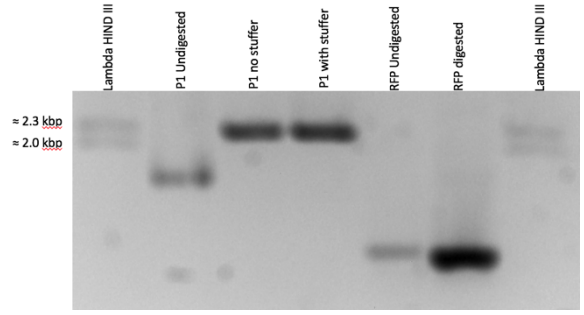

Fig 3. Gel electrophoresis of digested and undigested P1 and RFP with and without the presence of stuffer run on a 0.8% agarose gel at 150V. Lane 1 and 7 have 10µl of lambda HIND III, lane 2-6 have 6µl of P1 undigested, P1 no stuffer, P1 with stuffer, RFP undigested, and RFP digested respectively.

Table 3. Our blue, red, and white colonies were counted using a counter from plates incubated with the various treatments. (N=1)

Condition	Red Colonies (%)	Transformation efficacy(%)
Positive control	0	100
Negative control	0	0
1:1 with stuffer	0	41.18
2:1 with stuffer	0	30.07
1:1 no stuffer	0	22.88
2:1 no stuffer	0	35.95

Table 4. (N=5) Average percent of red colonies and percent transformation efficacy from 5 groups.

Condition	Red Colonies (%)	Transformation efficacy(%)
Positive control	0	100
Negative control	0	0
1:1 with stuffer	3.95	51.37
2:1 with stuffer	2.95	31.80
1:1 no stuffer	60.22	25.64
2:1 no stuffer	59.15	32.62

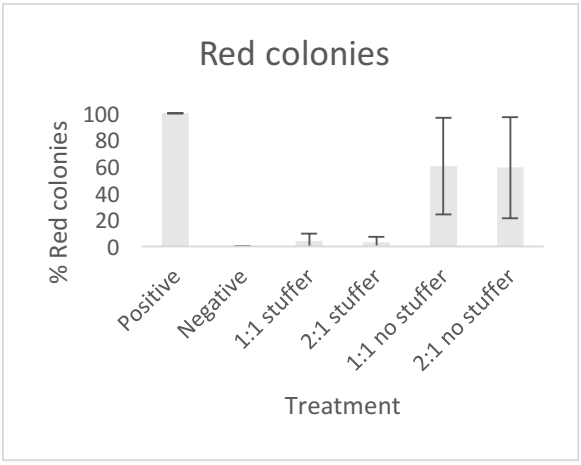

Fig 4. (N=5) One way Anova testing of molar ratio yielded F=0.0007 P=0.9816, there is not sufficient evidence at the alpha=0.05 level to show that molar ratio affects the production of red colonies. One way Anova testing of stuffer yielded F=5897.4782 P=0.0002, there is sufficient evidence at the alpha=0.05 level to show that the presence of stuffer affects the production of red colonies.

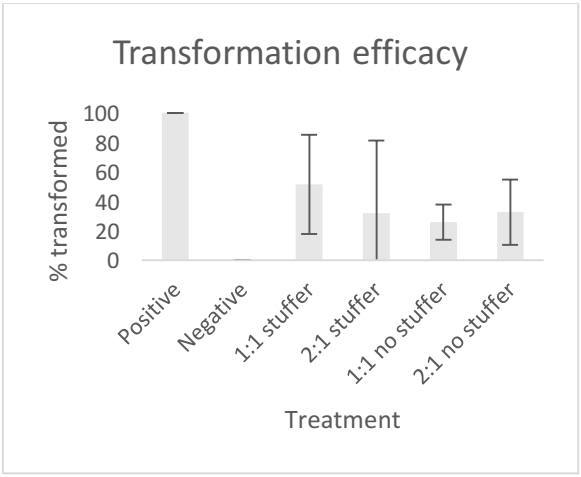

Fig 5. (N=5) One way Anova testing of molar ratio yielded F=0.0392 P=0.6732, there is not sufficient evidence at the alpha=0.05 level to show that molar ratio affects the transformation efficacy. One way Anova testing of stuffer yielded F=1.4373 P=0.3534, there is not sufficient evidence at the alpha=0.05 level to show that the presence of stuffer affects the transformation efficacy.

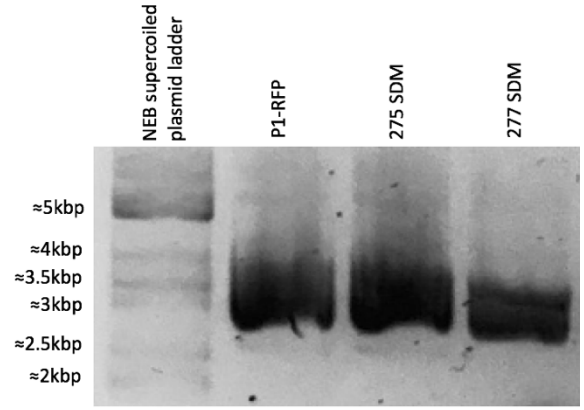

Fig 6. Gel electrophoresis of P1-RFP and SDM mutated plasmids to confirm purity on a 0.8% agarose gel run at 150V. Lane 1 contains 10µl of NEB supercoiled plasmid ladder, lane 2-4 have 12µl of P1-RFP, 275 SDM (not plasmid of interest), and 277 SDM plasmids respectively.

Table 5. Nanodrop reading of 2µl of P1-RFP and 277 SDM following blanking with warm EB buffer.

Plasmid	Concentration(ng/µl)	A260/A280
P1-RFP	118.0	1.79
277 SDM	79.5	1.82

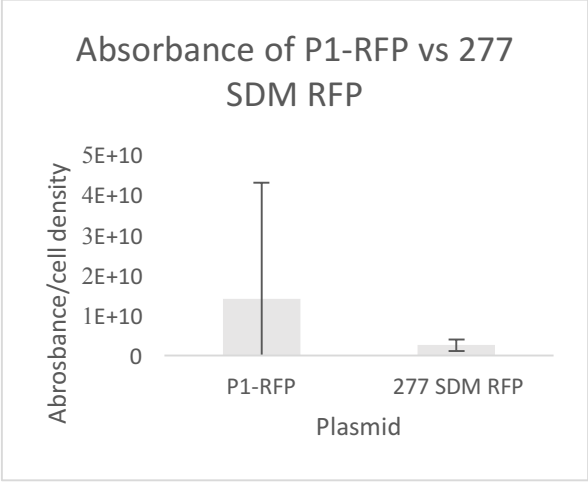

Fig 7. (N=2) Statistics can not be run on the 277 SDM with the P1-RFP.

Table 6. Highlighted sequence shows 3 point mutation substitutions that were made through SDM.

Location	sequence	277 SDM	Amino acid
160bp	5' ATC 3'	result	stop
129bp	5' ATA 3'	expected	tyrosine
225bp	5' AGT 3'	result	serine
194bp	5' AAT 3'	expected	leucine
-10	5' TATCAT 3'	result	-10 site
-10	5' TATAAT 3'	expected	-10 site

Discussion: Although statistical analysis could not be taken into account for our absorbance data, Fig 7 shows a trend that supports our hypothesis that we would see a lower expression of RFP once our SDM mutation was induced. While a trend of lower expression is seen, Table 6 shows that 3 alternative substitution mutations are the reason for the altered expression. As the -10 consensus region was altered it could have produced a araBAD promoter as seen as araXc effects araBAD as it goes from a AT to GC[9]. In addition, leucine to serine would have affects on the alpha sheets and corresponding polarity and hydrogen bonds[10]. And finally stop codons stop translation once noticed by the ribosome[11], but the stop codon does not necessarily always mean that translation will stop[12]. Thus the trend shows that none of the mutations that occurred show hard evidence of a loss of function of the RFP yet do all show changes that would result in under expression of RFP. Our hypothesis shows strong signs of yielding true but can not be confirmed due to the lack of statistical data that our 277 SDM mutation holds. The Mg^{2+} PCR conditions in fig 2. show that instead of Mg^{2+} creating small fragments by increased Taq polymerase fidelity to Magnesium[13], that 2mM of Mg^{2+} is about the optimum concentration to yield the most PCR product. The most conclusive result being the ligations in fig 4. showed statistical evidence that the greatest number of red colonies are produced without the presence of stuffer in the P1 plasmid. The molar ratio provided no statistical evidence to improve ligation of RFP into the P1 plasmid. Fig 5 does not show a statistical data to show that stuffer or molar ratio aid in transforming the plasmid. This confirms part of our hypothesis such that in the red colony portion of the ligations, no stuffer was indeed a method that effectively ligates RFP into P1 yet the molar ratio that was used was not sufficient to produce the results we hypothesized. The results attained prove that recombinant DNA can be synthesized and manipulated to produce proteins to express genes. The other factors that were altered in this experiment allow for new factors to test to

optimize the recombinant DNA process to yield more accurate results and cleaner products. Further experiments can further test Mg^{2+} concentrations within the range of 2mM to 3mM to find the optimum concentration. Other factors and reagents in PCR can also be manipulated for optimization of products. Ligations can also be manipulated further by playing with molar ratio's as insert to vector ratio's of 4:1[8] or 3:1 give the highest recombination frequency[14], and beyond. And finally SDM primers can be manipulated in a more precise way as our NEBaseChanger primers when analyzed nucleotide by nucleotide did not match our RFP sequence and as a result direct substitution of the nucleotide of interest was possibly missed as a result. The designing of primers was possibly the greatest factor for why our target sequence was not altered which also goes to show how cis mutations affect the expression and a further exploration into other cis elements as well as trans elements can introduce new findings through RFP expression. 3 other substitution mutations were present through our treatments. Through the many manipulations, we ultimately expressed the RFP gene through minimal expression. Our hypothesis for the various experiments turned out to direct us in various patterns to further explore Mg^{2+} concentration, ligations with variable molar ratios of insert to vector, and various other regions in the RFP gene other than the coding sequence to alter to test gene expression. And one hypothesis of the absence of stuffer proved sufficient to say that no stuffer in the P1 plasmid during ligation helps RFP insert in. Future experiments can go beyond the scope of this lab and optimize various other plasmids with fluorescent gene tagging to produce various health and environmental benefits for humanity through the process of recombinant DNA techniques. Through limitation of the gene's given to us we can see possibilities of recombinant DNA and how it affects our lives today in the form of disease treatment and therapy to how we can potentially design treatments based on a persons' genomic sequence to personalize their treatment in the near future through concepts utilized in this experiment.

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