Optimization of Parameters for the Construction of a Sensing System to Measure the Hydrolysis of β-Lactam Antibiotics

by

Nicholas David McGuire

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Approved by:

_____
Libby G. Puckett, Ph.D., Thesis Director

_____
Matt Estep, Ph.D., Second Reader

_____
Jennifer Cecile, Ph.D., Third Reader

_____
Libby G. Puckett, Ph.D., Departmental Honors Director

_____
Leslie Sargent Jones, Ph.D., Director, Honors College

Abstract

Bacterial resistance to antibiotics is a problem that has developed over the last 75 years due to over-prescribing and improper usage of these drugs. Through these processes, bacteria have developed and spread the gene for an enzyme known as β-lactamase, which catalyzes the hydrolysis of the β-lactam ring in many penicillins, rendering them ineffective. Accordingly, the purpose of this research is to create a sensing system to measure the hydrolysis of β-lactam antibiotics using the enzyme, β-lactamase. To accomplish this, the gene for β-lactamase can be fused with the gene for a fluorescent protein known as enhanced green fluorescent protein (EGFP), which will decrease its fluorescence upon the local pH change generated by the catalysis of a β-lactam ring. While previous research in this area has been successful in the development of an *in vitro* fusion of the genes encoding for β-lactamase and EGFP, present research is focused on separately incorporating the individual genes for EGFP and β-lactamase in pFLAG-MAC expression vectors to verify the local pH theory and to create an *in vivo* protein, thus creating a whole cell sensing system.

Acknowledgements

First and foremost, I would like to thank my advisor, Dr. Libby Puckett, for providing me with the opportunity to work in her lab and for her countless hours of advice on how to better both my time spent in research and in writing this thesis. I would also like to thank my second readers, Dr. Jennifer Cecile and Dr. Matt Estep, for their willingness to take the time to read and contribute to this work. Further thanks go to the ASU Office of Student Research, the ASU University Research Council, the College of Arts and Sciences. Finally, I would like to thank the A.R. Smith Department of Chemistry for the support of this work, including Kaitlin Rzasa and Nicole Reilly who contributed to this project before me and Ashlyn Henson who taught me many of the molecular techniques used on this project.

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Introduction

β-Lactam Antibiotics

The first β-lactam antibiotics to be classified in the modern era, penicillin, was discovered by Scotsman Alexander Fleming in 1928. He noticed a bacteria free halo around a mold growing on a *Staphylococcus* culture, and he determined the bactericidal agent produced by the mold to be penicillin. β-Lactam antibiotics are a class of antibiotics characterized by a β-lactam ring. The β-lactam ring in penicillin, shown in Figure 1, is a four membered ring containing nitrogen, where the carbon atom adjacent to the nitrogen atom is a carbonyl carbon. β-lactams work as efficient antibiotics without greatly affecting humans, because they target bacterial cell wall synthesis. They interact with a bacterial protein called D-alanyl-D-alanine carboxypeptidase-transpeptidase, which is responsible for crosslinking peptidoglycan in bacterial cell walls.[1] Without the crosslinking of peptidoglycan, the rigid cell walls unravel and the cells rupture, killing the bacteria and preventing replication. These antibiotics are effective against both Gram-positive and Gram-negative bacteria in varying capacities.[2]

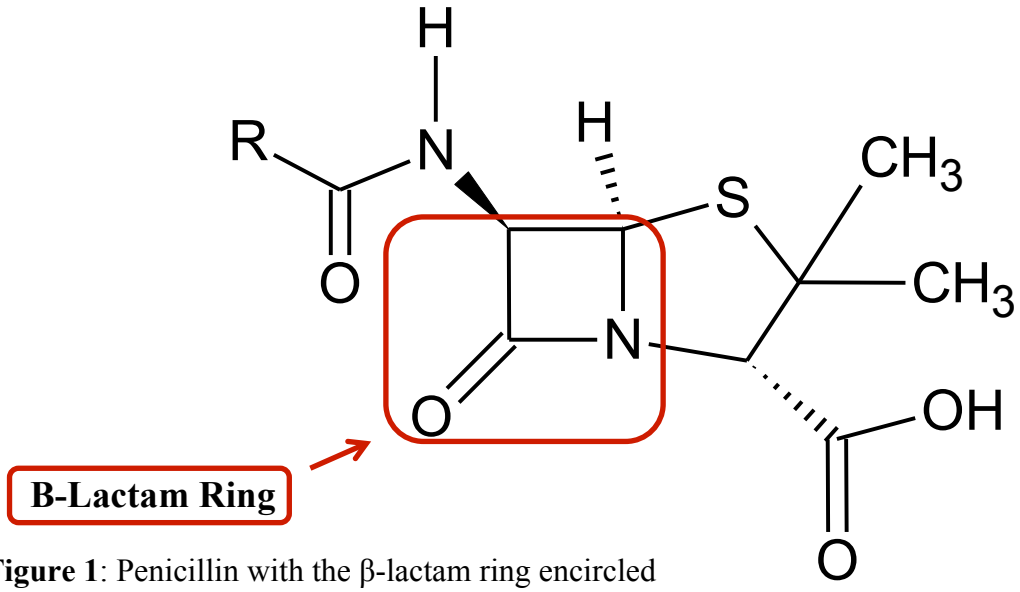


Figure 1: Penicillin with the β-lactam ring encircled

Bacteria of all kinds are either characterized as Gram-positive or Gram-negative, as seen in Figure 2, by using the Gram stain test. The Gram stain first involves staining bacteria with crystal violet dye and then washing the dye off with ethanol. Gram-positive bacteria will retain the violet color but Gram-negative bacteria will have the dye washed out. A safranin counter stain will then be added to dye the Gram-negative bacteria pink, providing a contrast to differentiate the two. Gram-positive bacteria have cell walls composed of secondary polymers like teichoic and teichuronic acids and a thick, multilayer peptidoglycan layer, no outer phospholipid membrane, and a high resistance to physical disruption from their environment. In direct contrast, Gram-negative bacteria are shielded by a thin, single layer of peptidoglycan, do possess and outer membrane of lipopolysaccharide and protein, and have a low resistance to physical disruption from their environment. Because of the additional outer membrane, Gram-negative bacteria have a greater volume of periplasm in a well-defined periplasmic space than Gram-positive bacteria.[3]

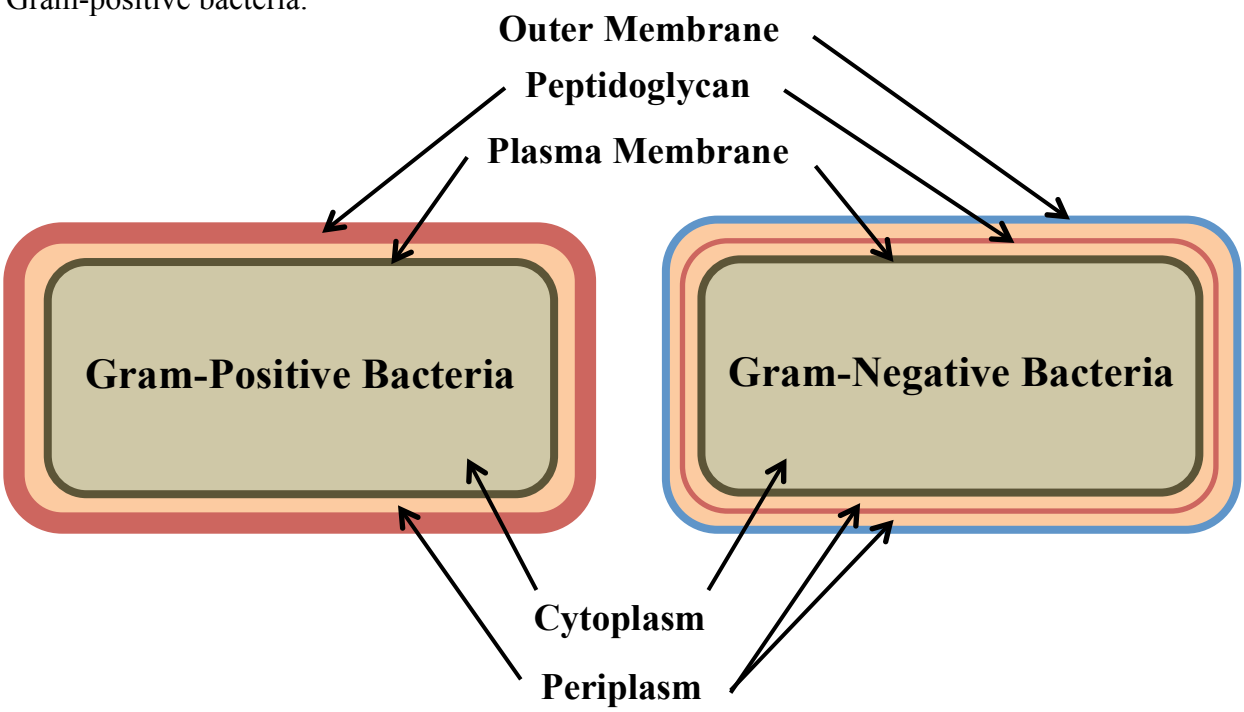
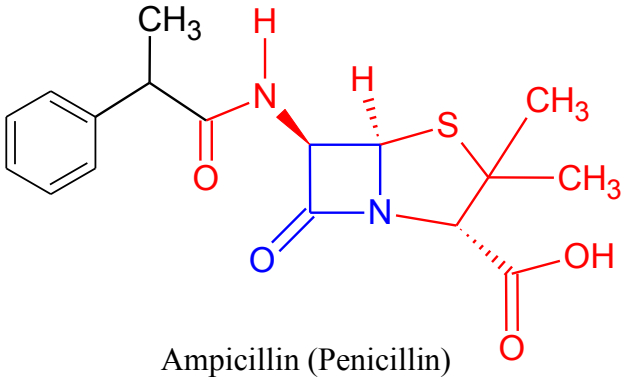
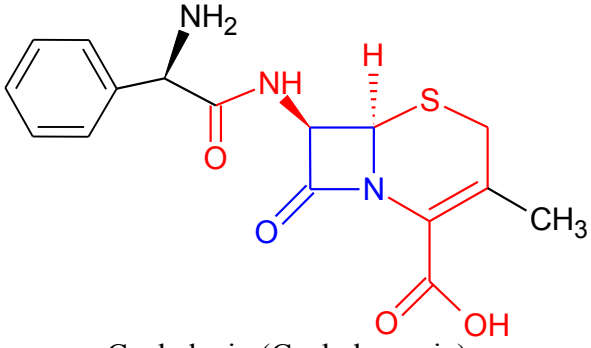


Figure 2: Comparison of Gram-positive and Gram-negative bacterial structures

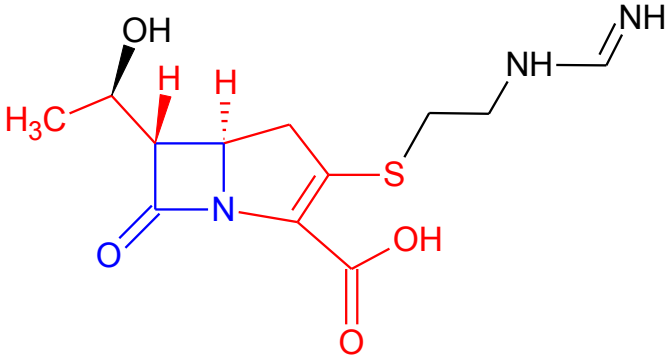
Though both Gram-positive and Gram-negative bacteria are susceptible to β-lactam antibiotics, they interact with different types of these antibiotics in different ways. β-Lactam antibiotics come in many forms and four different classes, penicillins, cephalosporins, monobactams, and carbapenems, as seen in Figure 3. Perhaps the most well-known of these, penicillins, are commonly used to treat infection by Gram-positive bacteria of the *Streptococci* and *Staphylococci* genera, but they are used to treat infections from bacteria of the *Clostridium* and *Listeria* genera as well. Penicillins are administered in unique forms through oral, intravenous, and intramuscular routes.[4]



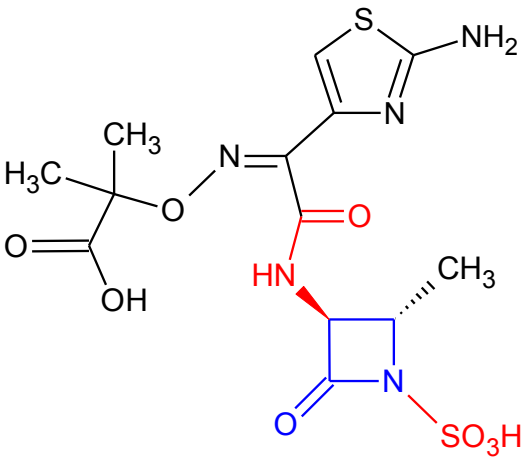
Ampicillin (Penicillin)



Cephalexin (Cephalosporin)



Imipenem (Carbapenem)



Aztreonam (Monobactam)

Figure 3: The four classes of β-lactam antibiotics. The β-lactam ring is highlighted in blue, the moieties characteristic to each β-lactam class highlighted is in red, and the R groups for each specific antibiotic are shown in black.

Currently, there are five generations of cephalosporins. First-generation cephalosporins are primarily used to combat Gram-positive bacteria such as *Streptococci* and *Staphylococci*. However, more recent generations of cephalosporins tend to demonstrate greater activity against Gram-negative bacteria and less efficacy against Gram positive bacteria.[5]

Carbapenems are useful because they generally have activity against a broader range of bacteria, including *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Bacteroides* as well as common Gram-positive bacteria, than more standard β-lactams like penicillins and cephalosporins.[6] Carbapenems are frequently used to combat multidrug resistant bacteria and are often given to patients in hospitals, particularly in Intensive Care Units, when other antibiotics are found to be ineffective. However, resistance to carbapenems is still a problem in healthcare settings.[7]

Monobactams are a group of antibiotics characterized by a β-lactam ring that is not in conjunction with another ring structure; the only commercially available drug in this class is aztreonam. While these antibiotics have been shown to be significantly less active than other β-lactams against many bacteria, they have also been shown to be significantly more stable in the presence of β-lactamases produced by both Gram-negative and Gram-positive bacteria. However, monobactams have been shown to have increased activity against some Gram-negative organisms, especially *Pseudomonas*. As such, searching for new monobactams could be in the future for the field of β-lactam research.[8]

β-Lactamase and β-Lactamase Inhibitors

β-Lactamase is a hydrolase produced by some bacteria to provide resistance to β-lactam antibiotics, seen in Figure 4. These enzymes are used by bacteria to protect themselves by

catalyzing the hydrolysis of the β-lactam ring, leaving the antibiotic completely ineffective as a bactericidal agent, as seen in Figure 5.[2] The general characteristics of the mechanism of this hydrolysis reaction by β-lactamase have been outlined from a quantum mechanics approach. First, Glu-166 acts as a general base in the acylation step of the catalysis event, and Lys-73 induces a hydrogen atom transfer (HAT) of a proton abstracted by Glu-166 via the Ser-130 hydroxyl group to the nitrogen of the β-lactam ring. The protonation of this nitrogen atom prompts an instantaneous opening of the β-lactam ring.[9]

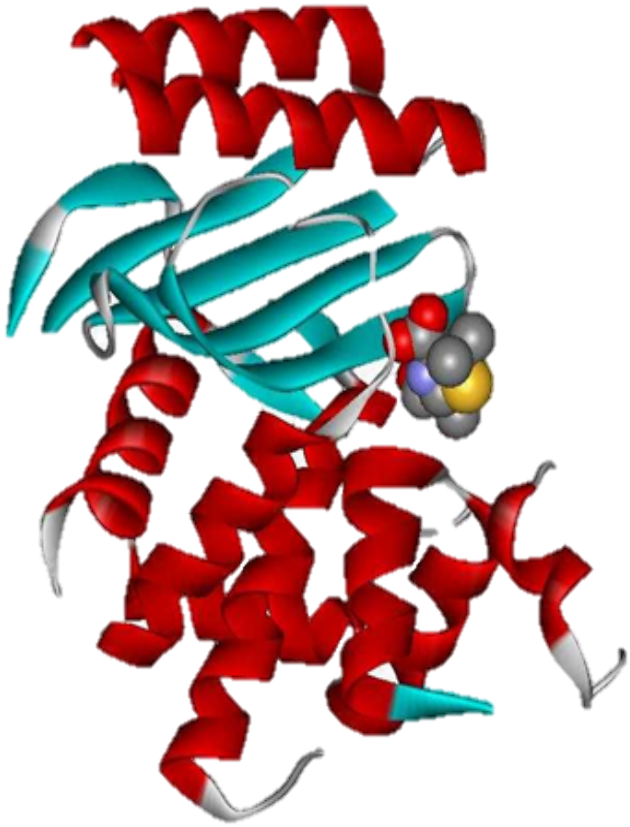


Figure 4: Model of the ribbon structure of β-lactamase

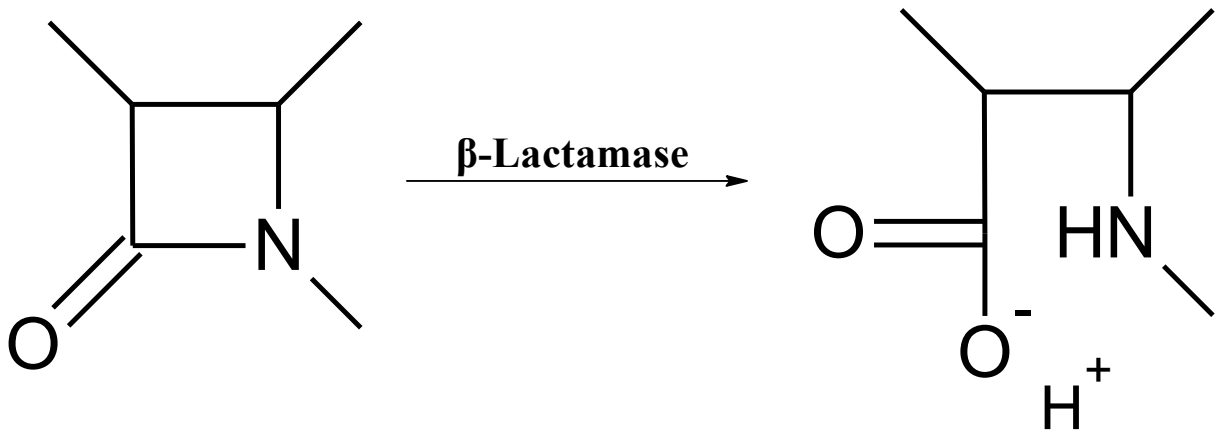


Figure 5: Cleavage of the β-lactam ring by β-lactamase

The number of bacteria producing β-lactamase has increased dramatically in the last century since the discovery of penicillin. Today, this is often attributed to antibiotics being overprescribed and the lack of completion of antibiotic regimens by patients.[1] However, it is also believed that, as β-lactamases have always been present in some percentage, the introduction of antibiotics to modern medicine has greatly increased the speed of bacterial evolution in regard to this enzyme. The ability to produce β-lactamase has become a highly favorable survival trait, so accordingly, it is seen more commonly today.[2]

In the face of the widespread abundance of β-lactamase producing bacteria, researchers have isolated and produced a series of β-lactamase inhibitors in an attempt to preserve β-lactam antibiotics as a valuable medicinal resource. These β-lactamase inhibitors prevent the hydrolysis of the β-lactam ring, allowing these antibiotics to remain effective when used in conjunction with these inhibitors.[2] Furthermore, these inhibitors can be classified into two different categories, reversible or irreversible inhibitors. Reversible inhibitors, as the name suggests, do not inactivate enzymes like β-lactamase permanently. Rather, the two sets of molecules exist with each other in a dynamic equilibrium as concentrations of either change or the inhibitor is

replaced with another molecule with higher affinity for the enzyme. Some reversible inhibitors are actually just substrates that are slowly hydrolyzed by the enzymes. Irreversible inhibitors, on the other hand, work towards a complete and permanent cessation of enzymatic activity for a particular enzyme. These inhibitors can inactivate an enzyme by forming a covalent enzyme-inhibitor complex[10].

Perhaps one of the most common of these combination drugs is known as Augmentin™, which contains both amoxicillin and the β-lactamase inhibitor, clavulanic acid, as depicted in Figure 6. Other β-lactamase inhibitors include sulbactam, which is commonly taken in combination with the antibiotic ampicillin, and tazobactam, which is frequently used in conjunction with the antibiotic piperacillin[11]. All of these β-lactamase inhibitors work as irreversible inhibitors; they bind to β-lactamase and permanently inactivate it without ever releasing. Furthermore, all three of these β-lactamase inhibitors also possess the same β-lactam ring that they prevent enzymatic cleavage of in β-lactam antibiotics, since competitive inhibitors bind to the same active site.

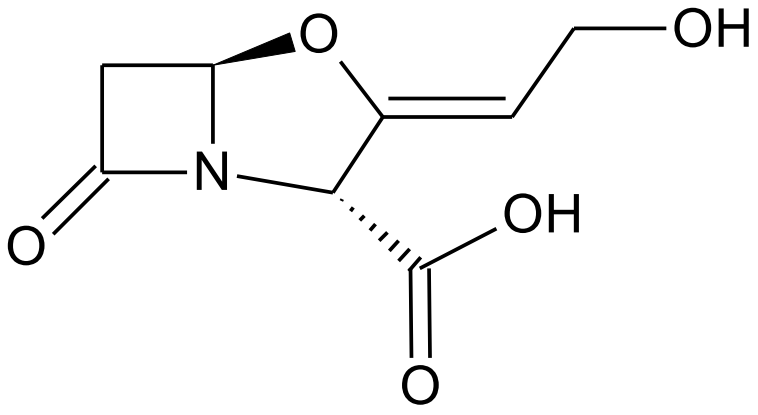


Figure 6: Clavulanic acid

Another use of β-lactamase is as a selectable marker. Selectable markers are genes introduced into a cell that can, in a bacterial system, indicate the success of a transformation of DNA. Commonly used in molecular biology as a selective marker, the gene for β-lactamase provides bacterial cells with the ability to grow on ampicillin containing agar. Accordingly, any growth at all will serve as an indicator of the success of an uptake of a vector containing the gene, which then transfers the resistance to the bacteria.

Green Fluorescent Protein

The photoprotein, green fluorescent protein, or GFP, can be used in laboratory settings as a reporter protein. This protein, isolated from the jellyfish *Aequorea victoria*, can be seen in Figure 7.[5] This protein is particularly useful in the lab setting because of its unique structure, which makes it is resistant to normal denaturing conditions, such as higher pH, higher salt content, or being in solution with organic solvents or detergents. Other fluorescent proteins have been isolated from similar sources, like aequorin also from *Aequorea victoria* or other GFP variants from the sea pansy, *Renilla reniformis*. However, though these molecules have identical chromophores, they do not have the advantage of GFP from *Aequorea victoria* of being autofluorescent. No additional cofactors or substrates are needed to induce fluorescence in this molecule.[12] GFP operates as an electron acceptor in a process known as Förster resonance energy transfer (FRET). When undergoing FRET, the photoprotein bioluminescence excited (S_1) state is coupled to the ground state (S_0) of the GFP fluorophore within a protein-protein complex. GFP has been observed to undergo this process at micromolar concentrations.[13]

Figure 7: *Aequorea victoria*, the source of isolated GFP[12]

GFP is comprised of 238 amino acid residues connected together in one polypeptide chain and is 27 kDa in size.[14] This continuous polypeptide chain is folded into 11 β-sheets that are arranged in a barrel shape, known as both a β-barrel and a β-can. This barrel structure is highly stable, and the only deviation from this cylindrical shape occurs in the form of side chain interactions between the seventh and eighth β-sheets. This has also been shown to contribute to the high level of stability in this photoprotein by assisting in tertiary structure formation. The ring of β-sheets encircles the chromophore region of GFP, which is contained in an axial helix that stretches across the inner region of the barrel, protecting it from denaturing conditions and preserving its ability to fluoresce.[15] The chromophore is formed by a cyclic tripeptide of Ser-65-Tyr-66-Gly-67, which has undergone post-translational modification as an imidazolone ring.[14] However, the β-barrel does not provide shielding against pH, and the pH sensitivity of the GFP chromophore, as well as the formation of the imidazolone ring, can be seen in Figure 8.

Cyclization is completed after the nitrogen of the glycine residue attacks the carbonyl carbon of the serine residue. Afterward, the hydroxyl group of Tyr-66 residue is susceptible to deprotonation and reprotonation, affecting the ability of the photoprotein to fluoresce.[14]

GFP has two major excitation peaks, one at 470 nm and the primary excitation peak of 395 nm (λ_{max}), and a primary emission peak at 508 nm.[16] In order to optimize both the speed and intensity of the fluorescence of GFP, mutant forms of this protein have been created. These include enhanced blue fluorescent protein (EBFP), enhanced yellow fluorescent protein (EYFP), and enhanced cyan fluorescent protein (ECFP), which all emit blue yellow, and cyan light respectively. Others include GFPuv, which fluoresces at an intensity that is eighteen times brighter than wild type GFP from *Aequorea victoria*, and enhanced green fluorescent protein (EGFP).[17]

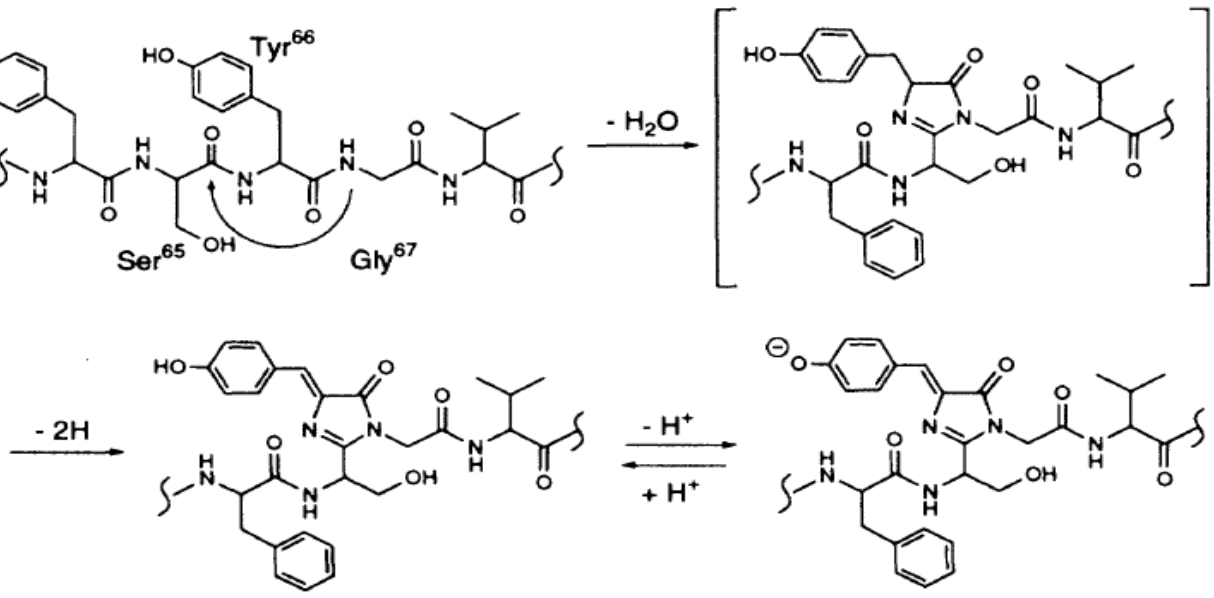


Figure 8: Formation and pH sensitivity of the GFP chromophore[14]

Enhanced Green Fluorescent Protein

The variant of GFP, EGFP, is the result of over 190 mutations and has similar properties to GFP, but it has a red shifted major excitation peak due to mutations at a couple of key amino acids near the GFP chromophore region, again comprised of the cyclic tripeptide of Ser-65-Tyr-66-Gly-67, seen in Figure 9. A red shift, or a bathochromic shift, occurs when an excitation or emission peak is changed from higher energy and shorter wavelengths to lower energy and longer wavelengths. These mutations include the replacement of Phe-64 with Leu and of Ser-65 with Thr.[17] The fluorescence of EGFP, just as in some other GFP mutants, is pH dependent. In more basic environments, the hydroxyl group of Tyr-66 in the chromophore region of EGFP is deprotonated, and the protein fluoresces intensely. However, as pH decreases, this hydroxyl group is protonated and fluorescence is measurably decreased. Furthermore, fluorescence of EGFP has been shown using fluorescence correlation spectroscopy (FCS) to flicker on a 45-300 μs time scale as a result of proton exchange between the chromophore region and the buffer solution.[18]

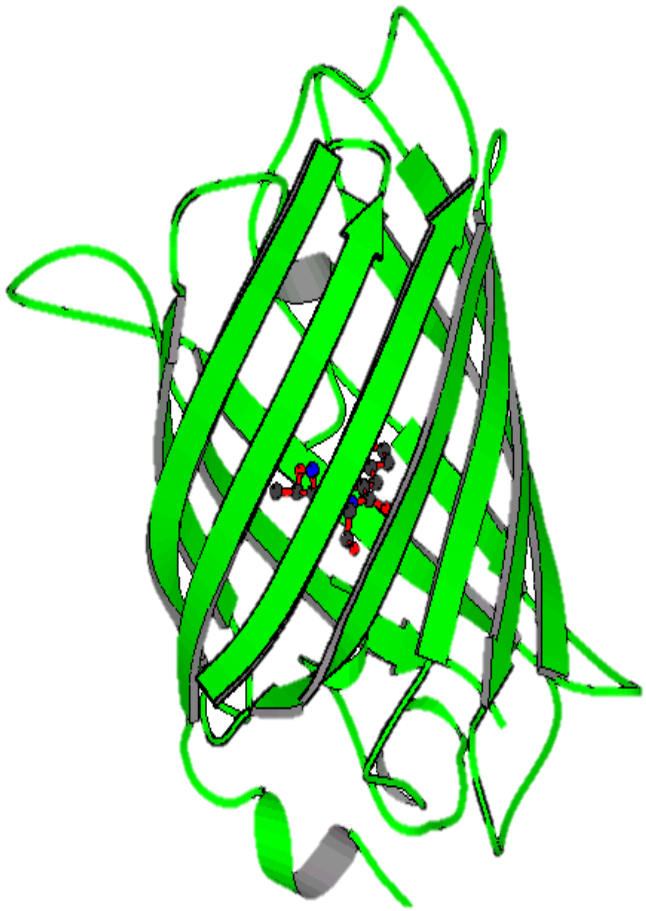


Figure 9: Ribbon structure of EGFP showing its β-can structure protecting the cyclic tripeptide of the chromophore[19]

It is EGFP, which has the red shifted λ_{max}, seen in Figure 10, that is utilized in this research.[20] Partially due to the fact EGFP has a maximum excitation shift towards longer wavelengths, EGFP has 35 times brighter fluorescence than GFP when excited at 488 nm. This is beneficial because argon lasers emit light at 488 nm, and this particular wavelength is also common in most filter sets for a wide array of analytical instruments.[17] Accordingly, the fluorescence of EGFP can be measured and quantified using a spectrofluorometer, making EGFP useful as a reporter protein.[21]

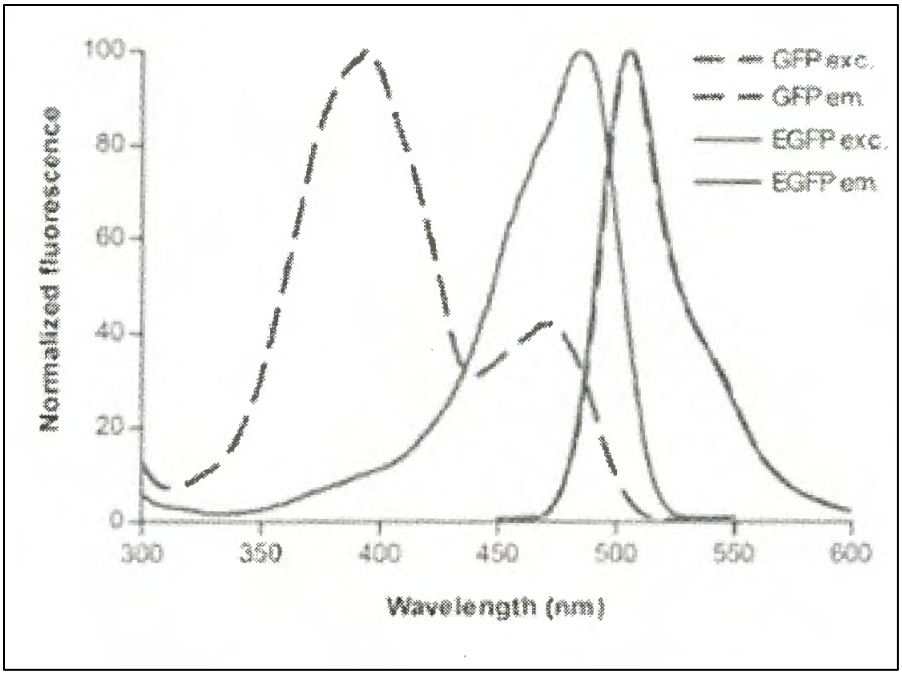


Figure 10: Excitation and emission spectra of GFP and EGFP

Existing research in this area has shown that it is possible to fuse the genes for EGFP and β-lactamase into a single protein for the monitoring of the hydrolysis mechanism.[19] It is an essential factor of this research that the pH dependence of EGFP is preserved in this fusion protein so that a fluorescence change can still be observed. This is seen in Figure 11, which shows a steady drop in the fluorescence of the fusion protein as pH changes from acidic to basic. Accordingly, a decrease in pH will cue a decrease in fluorescence at a range of wavelengths for both EGFP and the EGFP moiety of the fusion protein, including the maximum emission peak at 509 nm.

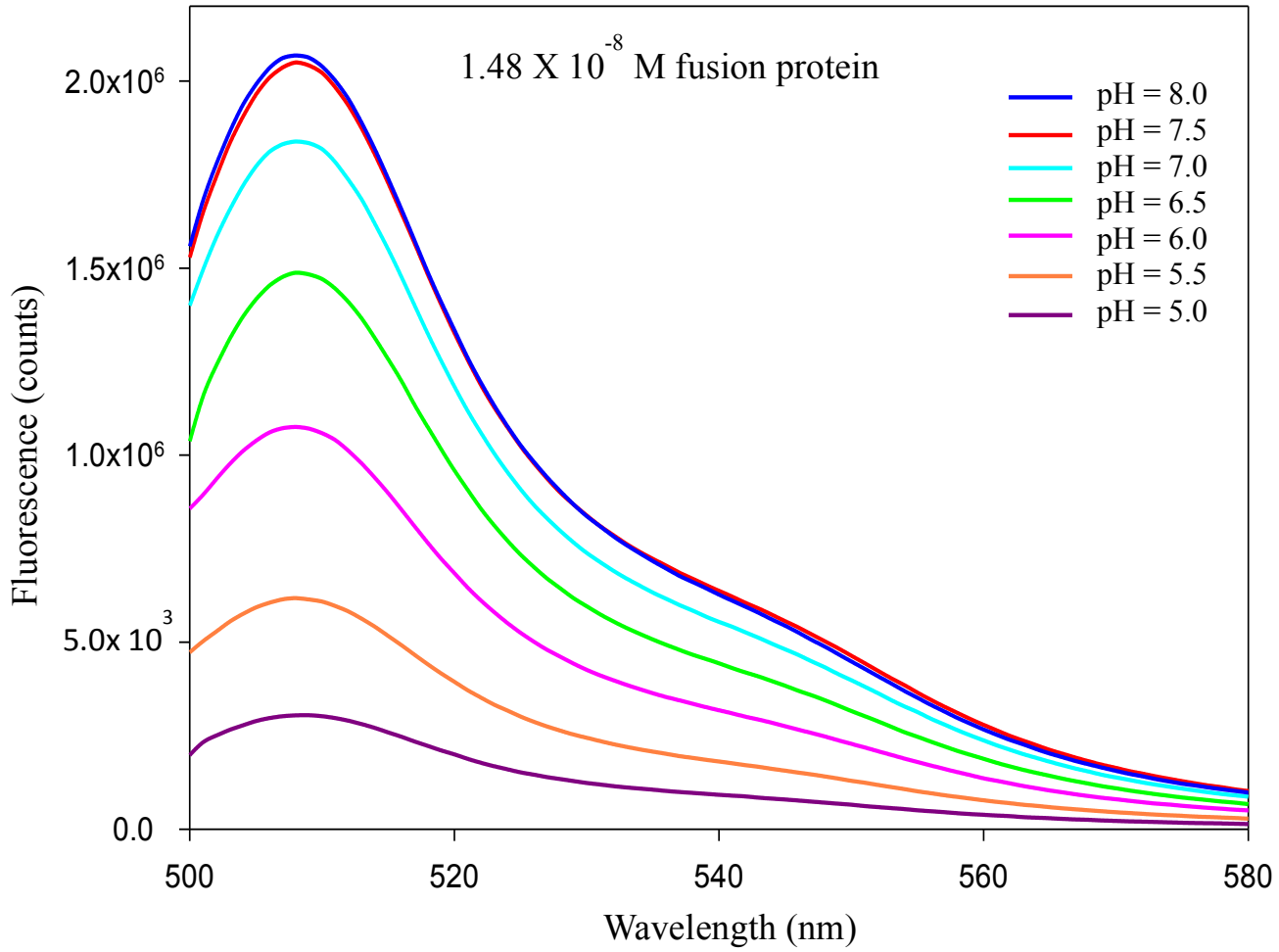


Figure 11: Response of fluorescence to pH for the fusion protein of EGFP and β-lactamase[19]

Ampicillin has been added to the presence of this fusion protein, and as was expected, the β-lactam ring was hydrolyzed by the β-lactamase portion of the molecule. However, this degradation of the β-lactam ring releases a proton that results in a local pH change. This decrease in pH was followed by a decrease in fluorescence due to the EGFP portion of the protein.[19] The change in pH as a result of β-lactamase activity can be seen in Figure 12. Accordingly, the inactivation of ampicillin by β-lactamase can be quantified by monitoring the change in fluorescence over time.[5]

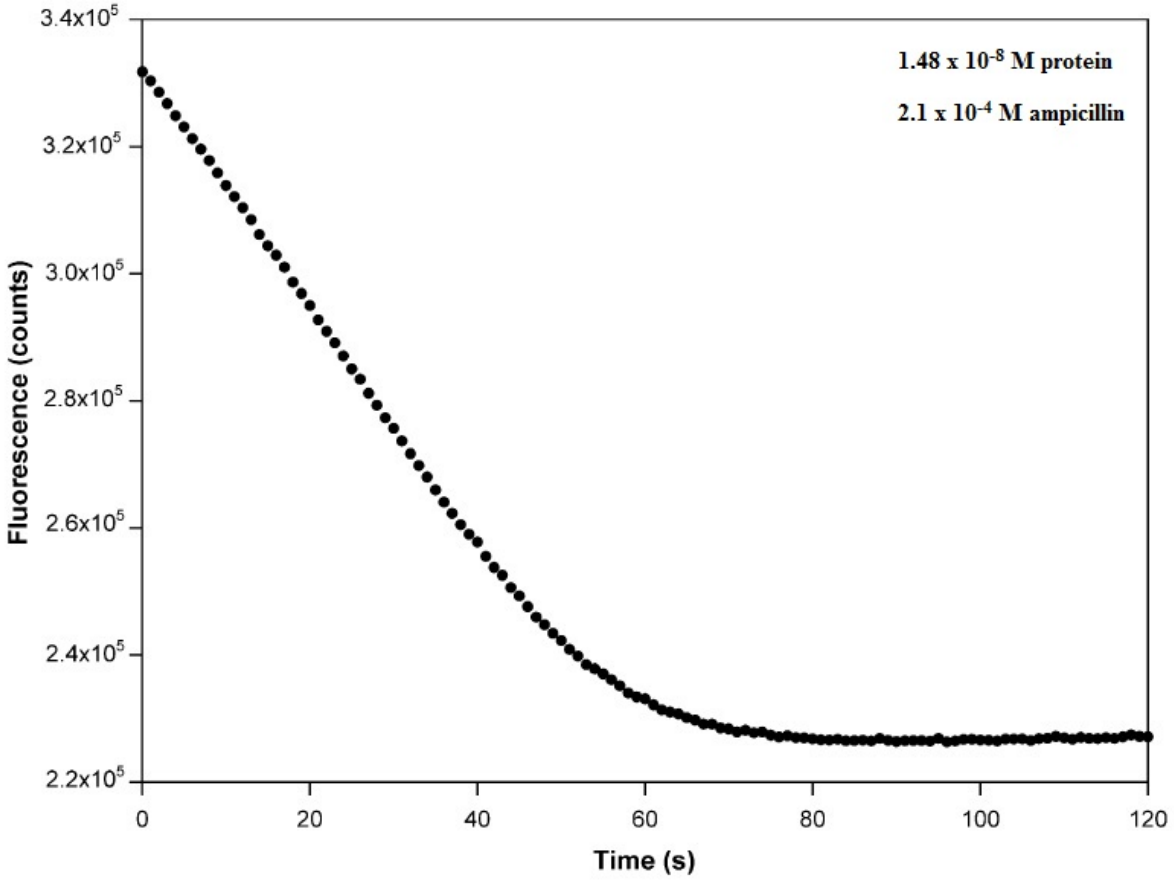


Figure 12: Change in fluorescence due to the hydrolysis of β-lactams

Biosensors

A biosensor is a chemical sensor that contains a biological component, which is used to determine the specific selectivity of the biosensor. Furthermore, biosensors are composed of a biological recognition element and a transducer working together.[22] The first biosensor to be developed was the glucose enzyme electrode developed by Clark and Lyons in 1962. This biosensor used an oxido-reductase enzyme called glucose oxidase and a platinum electrode to quantify the presence of glucose. The enzyme was put in close proximity to the platinum anode, which was polarized at + 0.6 V, and reacted with its substrate, glucose. The platinum anode responded to the peroxide produced by the enzymatic reaction by producing a quantifiable change in electrode potential, leading to the construction of sensing systems for the measurement

of glucose in blood plasma.[23] This particular research will utilize a spectrofluorometer as the transducer.

Perhaps one of the simplest examples of a biosensor that has been developed is known as the Bananatrode. The Bananatrode is a biosensor comprised of a slice of banana pulp tissue placed on a gas permeable membrane and kept in place with a dialysis membrane, graphite powder, and liquid paraffin placed into an electrode cup to measure dopamine concentration. If dopamine is in the presence of the Bananatrode, it will be oxidized from the oxygen in the air and subsequently reduced. The reduction step generates a measureable current proportional to dopamine concentration.[24] A biosensing system is an element of a biosensor, which does not have an onboard transducer, that detects the presence of an analyte in a sample. A transducer is an element of a biosensor that produces a quantifiable signal that is proportional to the amount of a target analyte in a given sample.[22]

Whole Cell Sensing Systems

A biosensing system is categorized as having either a molecular, cellular, or tissue sensing component.[25] One of the purposes of this research is to create a cellular or whole cell sensing system, which has the advantage of remaining stable and highly selective across a wider range of temperatures and pH values, including physiological pH, when compared to the other systems.[12] In comparison, a molecular based system requires the expense of isolating biological components without providing knowledge of how a particular analyte affects the cell as an entire system.[26] However, one of the greatest hurdles of utilizing a whole cell sensing system is the loss of specificity that can be introduced as a result of unforeseen interference on the molecular level from other components of the cell. These systems, which can be created from bacterial

sources like *Escherichia coli*, will respond to an environmental change, such as a pH change, and will produce a response.[27] The genetic fusion of a reporter gene, such as the gene for β-lactamase, to one of a biological recognition element, such as the gene for EGFP, can make this response visible and quantifiable.[12] In this particular case, the quantifiable response is a measurable decrease in fluorescence. These systems can be used to sense the presence of a vast array of analytes including metals, sugars, and many organic compounds.[26]

It is the goal of this research to create such a whole cell sensing system to quantitatively measure the hydrolysis of β-lactam antibiotics. This constructed sensing system will be used to find new β-lactamase inhibitors to be used in conjunction with existing β-lactam antibiotics. Once this system has been both generated and expressed, kinetic studies can be completed on it to allow for the monitoring of bioavailability of β-lactam antibiotics.

Materials and Methods

Apparati

Overnight cultures were grown on a VWR shaker table set to 200 rpm and 37 °C (Cornelius, OR). Polymerase chain reaction (PCR) was performed in an Eppendorf Mastercycler Personal Thermocycler (Hamburg, Germany). Gradient PCR was performed using a 9901 Applied Biosystems Veriti 96-Well Fast Thermal Cycler manufactured by Thermo Fisher Scientific (Singapore). Gel electrophoresis was performed using a 1% agarose gel and 1x TAE buffer in an IBI Quickscreen QS-710 electrophoresis tank (New Haven, CT) connected to a Thermo EC105 power supply (Asheville, NC). Gels were visualized using a UVP UV Transilluminator (Cornelius, OR) and a UVP BioDoc-It Imaging System (Upland, CA). Restriction digest reactions were completed in VWR Shel Lab 1211 water bath (Cornelius, OR). Gel slices were melted using a VWR Analog Heat Block (Cornelius, OR). Bacterial cultures on plates were grown in a VWR Shel Lab 1500E incubator (Cornelius, OR). Nucleic acid concentrations were determined using a ThermoScientific NanoDrop 2000C Spectrophotometer (Asheville, NC). Sterilization of plastics and agar was executed using a 3870 Tuttnauer autoclave – steam sterilizer (Beit Shemesh, Israel). Centrifugation steps were completed using a 5404 Eppendorf AG Centrifuge (Hamburg, Germany). Masses were obtained using a Mettler Toledo AL 54 analytical balance (Columbus, OH). Stock cells were stored in a Thermo Scientific -80 °C freezer (Asheville, NC).

Reagents

Luria Bertani (LB) broth and LB agar were purchased from Difco (Lawrence, KS). *Taq* PCR buffer, $MgCl_2$, dNTP mix, *Taq* polymerase, *Hin*dIII, *Eco*RI, digestion buffer M, digestion buffer H, Blue Juice gel loading buffer, DH5α cells, and SOC media were purchased from

Invitrogen (Carlsbad, LA). The *Pfu* Turbo polymerase was purchased from Agilent Technologies (Santa Clara, CA). Ethidium bromide solution and ampicillin sodium were purchased from Sigma Aldrich (St. Louis, MO). Agarose was purchased from IBI Scientific (Peosta, IA). The 1 Kb DNA ladder was purchased from Phenix (Candler, NC). DNA ligase and DNA ligase buffer were purchased from Promega (Madison, WI). Primers were purchased from Eurofins MWG Operon (Huntsville, AL). The pEGFP vector was donated by Leonidas Bachas (University of Kentucky, University of Miami) and the pFLAG-MAC vector was purchased from IBI Kodak (New Haven, CT). Tris-acetate-ethylenediaminetetraacetic acid buffer (TAE buffer) (made one liter at 25x concentrated, then diluted to 1x with DI water) was prepared in the lab using tris base (121.0 g), glacial acetic acid (28.6 mL), and EDTA (18.6 g Na_2EDTA) from EMD Chemicals (Gibbstown, NJ). Miniprep, gel extraction, and Cycle Pure purification kits were purchased from Omega (Norcross, GA). Miniprep and gel extraction kits were also purchased from QIAGEN (Valencia, CA).

Overnight Cultures

Overnight cultures of the bacterial cells containing the plasmids pEGFP and pFLAG-MAC were prepared from new stocks. To 14 mL polypropylene BD Falcon tubes, 3 mL of LB broth with 100 µg/mL ampicillin was added. To half of the tubes, a toothpick swirled through the new pEGFP stock from the -80 °C freezer was added. To the other half of the tubes, a toothpick swirled through the new pFLAG-MAC stock from the -80 °C freezer was added. This process was completed quickly to prevent the stocks from thawing and refreezing. Each tube was capped but vented and placed on the shaker table overnight at 37 °C and at 200 rpm.

Miniprep (OMEGA Protocol)

In order to isolate the DNA from the pEGFP plasmid, an OMEGA Plasmid Mini Kit 1 was used. The LB broth containing the cultured bacterial cells was added to a 1.5 mL microcentrifuge tube and was pelleted by centrifugation at 10,000 x g for 1 minute in 1.5 mL increments. Between centrifugation steps, the supernatant was decanted and disposed of so that the next 1.5 mL of culture could be added and pelleted. The resulting pellet was resuspended by adding 250 µL of Solution 1, with RNase A added, and pipetting up and down to mix and disperse the cells. To the same tube, 250 µL of Solution II was added to lyse the cells. The microcentrifuge tube was gently inverted to form a clear lysate. Within 5 minutes of this step, 350 µL of Solution III was added to neutralize the solution and immediately inverted several more times. At this point, a flocculent white precipitate could be observed. The solution was centrifuged for ten minutes at 13,000 x g. Concurrently, 100 µL of Equilibration Buffer was added to a HiBind DNA Mini Column to prepare it for the addition of the DNA. This column was placed in the centrifuge at 13,000 x g for 1 minute and the flow through was discarded. The supernatant, containing the DNA, was slowly aspirated to avoid disturbing the pelleted cellular debris and was transferred to the equilibrated column. The column was placed back into the centrifuge at 13,000 x g for 1 minute. The flow-through was discarded and the column was reused. A volume of 500 µL of Buffer HB was added to the column, which was placed in the centrifuge at 13,000 x g for 1 minute. The flow-through was discarded and the column was reused. A volume of 700 µL of DNA Wash Buffer, diluted with absolute ethanol, was added to remove residual salts, and this was also placed in the centrifuge at 13,000 x g for 1 minute. The flow-through was discarded and the column was reused. The empty column was dried for 2 minutes by centrifugation to remove all traces of ethanol. The dry column was transferred to a

clean and sterile 1.5 mL microcentrifuge tube, and 30 µL of Elution Buffer was added. This was allowed to sit for 2 minutes before the tube and column were placed back into the centrifuge for 1 minute at 13,000 x g to elute the DNA. The resulting miniprep was stored in the refrigerator.

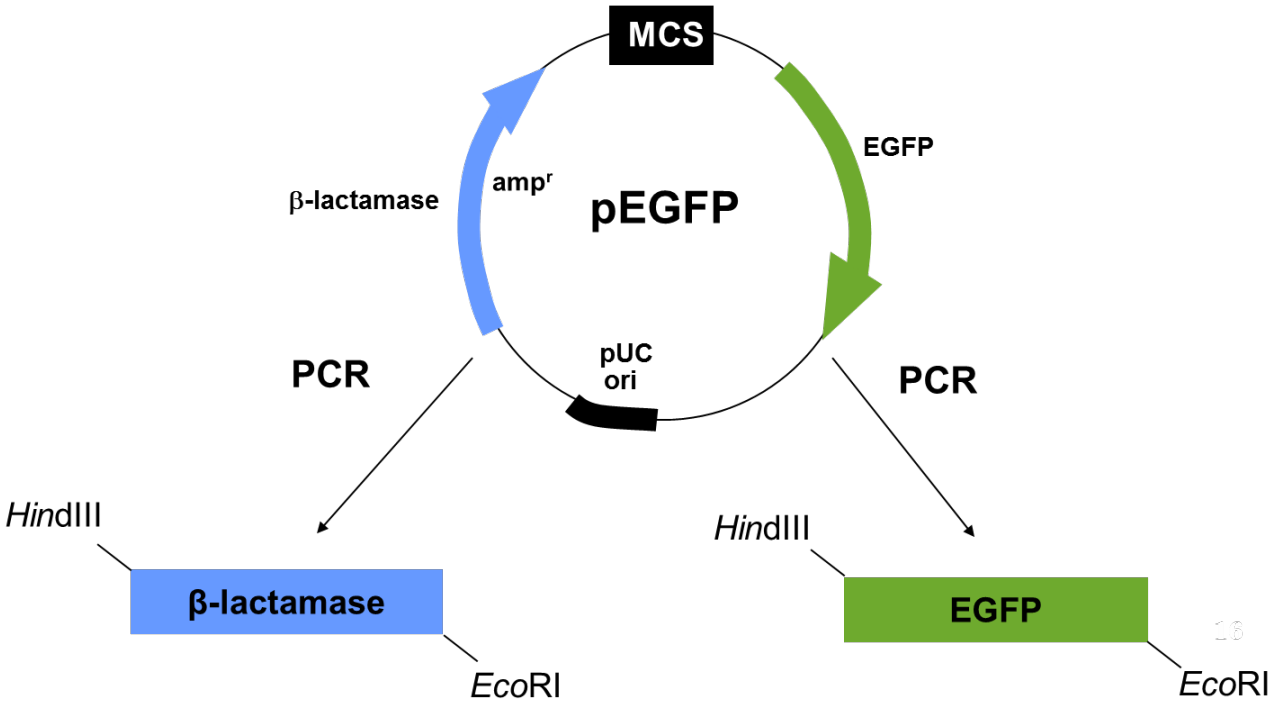
Miniprep (QIAGEN Protocol)

Another protocol used to isolate the DNA from the pEGFP plasmid was the QIAGEN protocol which used the QIAprep® Spin Miniprep Kit. The LB broth containing the cultured bacterial cells was added to a 1.5 mL microcentrifuge tube and was pelleted by centrifugation at 10,000 rpm for 1 minute in 1.5 mL increments. Between centrifugation steps, the supernatant was decanted and disposed of so that the next 1.5 mL of culture could be added and pelleted. The resulting pellet was resuspended by adding 250 µL of Buffer P1, with RNase A and LyseBlue reagent added, and pipetting up and down to mix and disperse the cells. To the same tube, 250 µL of Buffer P2 was added to lyse the cells. The microcentrifuge tube was gently inverted 4-6 times, and the solution turned blue. Immediately, 350 µL of Buffer N3 was added and inverted 4-6 more times, turning the solution colorless. The solution was centrifuged for ten minutes at 13,000 rpm to form a compact white pellet. The supernatant produced by this centrifugation step was applied to a QIAprep spin column by pipetting. The column was placed back into the centrifuge at 13,000 rpm for 1 minute, and the flow-through was discarded and the column was reused. A volume of 750 µL of Buffer PE, diluted with absolute ethanol, was added to the column, which was placed in the centrifuge at 13,000 x g for 1 minute. The flow-through was discarded and the column was reused. The empty column was dried for 1 minute by centrifugation to remove residual ethanol from the wash buffer. The dry column was transferred to a clean and sterile 1.5 mL microcentrifuge tube, and 50 µL of Buffer EB (10 mM Tris·Cl, pH 8.5) was added. This was allowed to sit for 1 minute before the tube and column were placed

back into the centrifuge for 1 minute at 13,000 rpm to elute the DNA. The resulting miniprep was stored in the refrigerator.

Polymerase Chain Reaction (Taq Protocol)

Polymerase Chain Reactions (PCRs) were conducted, with slight variations to determine optimum template DNA concentrations and temperatures by the following protocol. The purpose of PCR was to isolate the individual genes for EGFP and β-lactamase, amplify them, and use specifically designed primers to incorporate unique restriction sites. A diagram of the pEGFP plasmid from which both of these genes were isolated, with the cut sites visualized, can



be seen in Figure 13.

Figure 13: pEGFP plasmid showing the genes for EGFP and β-lactamase as well as the incorporation of unique restriction sites

The contents of each tube for PCR were the same except for the addition of the respective forward and reverse primers for EGFP and β-Lactamase, which are shown in Table 1. For both

genes, the HindIII restriction site is underlined in the forward primer and the EcoRI restriction site is underlined in the reverse primer.

Table 1: Primers used in the PCR mixture

β-Lactamase	Forward Primer	5'-ACCACCGACGTAAAGCTTATG AGTATTCAACATTTCCGTCTG-3'
	Reverse Primer	5'-GGCTGAGACTAATTCGTAAAC ATTGAATCCGTGGCACCACCA-3'
EGFP	Forward Primer	5'-ACCACCGCAGTGAAGCTT ATGGTGAGCAAGGGCGAG-3'
	Reverse Primer	5'-TACGTCCTCGACATGTTCATT GAATCCGTGACGCCACCA-3'

After thawing all reactants, except *Taq* polymerase, 60 µL sterile distilled water was added to a PCR tube. To this, 10 µL buffer (-$MgCl_2$), 10 µL of EGFP or β-lactamase reverse primer (50 pmol/mL), 10 µL of EGFP or β-lactamase forward primer (50 pmol/mL), 4 µL DNA miniprep (pEGFP), 3 µL 50mM $MgCl_2$, 2 µL dNTPs, and 1 µL Platinum *Taq* polymerase were added in this order, as depicted in Table 2. Unlike some other polymerases, *Taq* polymerase needs $MgCl_2$ to shift electron density from the α-phosphate of the incoming dNTP to aid in the nucleophilic attack of the hydroxyl group of the previously laid nucleotide. In an attempt to increase DNA concentrations, 6 µL less of water (54 µL total) was used and 6 µL more of DNA miniprep (10 µL total) were used for some reactions.

Table 2: Components of PCR with *Taq* polymerase

Reactant	Volume (µL)
Sterile Distilled Water	60
Platinum *Taq* polymerase Buffer (-$MgCl_2$)	10
Reverse Primer	10
Forward Primer	10
EGFP DNA Miniprep	4
$MgCl_2$	3
Deoxyribonucleoside triphosphate mix (dNTP)	2
Platinum *Taq* polymerase	1
Total	100

These PCR tubes were transported to a thermocycler and were run under the following protocol (Table 3): denaturing at 94.0 °C for 1 minute, annealing at 67.0 °C for 1 minute, and extending at 72.0 °C for two minutes. This cycle was repeated for a total of 50 cycles. In an attempt to increase DNA concentrations, the annealing temperature was decreased to 65.0 °C for some reactions.

Table 3: Thermocycler conditions for PCR with *Taq* polymerase

	Temperature (°C)	Time (min)
Denaturing	94.0	1
Annealing	67.0	1
Extending	72.0	2
50 Cycles		

Polymerase Chain Reaction (Pfu Turbo Protocol)

When PCRs were conducted using the enzyme *Pfu* Turbo® DNA polymerase instead of *Taq* polymerase, the following protocol was used. The contents of each tube were the same except for the addition of the respective forward and reverse primers for EGFP and β-Lactamase. After thawing all reactants, except *Pfu* Turbo, 2 µL DNA miniprep (pEGFP) was added to a PCR tube. To this, 10 µL *Pfu* Turbo buffer, 1 µL dNTP mix, 1 µL *Pfu* Turbo, 10 µL of EGFP or β-

lactamase reverse primer (50 pmol/mL), 10 μL of EGFP or β-lactamase forward primer (50 pmol/mL), and 66 μL sterile distilled water were added in this order, as depicted in Table 4. The polymerase *Pfu* Turbo does not need $MgCl_2$ to function.

Table 4: Components of PCR with *Pfu* Turbo

Reactant	Volume (μL)
EGFP DNA Miniprep	2
Pfu Turbo Buffer	10
Deoxyribonucleoside triphosphate mix (dNTP)	1
Pfu Turbo	1
Reverse Primer	10
Forward Primer	10
Sterile Distilled Water	66
Total	100

These PCR tubes were transported to a thermocycler and were run under the following protocol (Table 5): denaturing at 94.0 °C for 1 minute, annealing at 50.0 °C for 1 minute, and extending at 72.0 °C for two minutes. This cycle was repeated for a total of 30 cycles. In an attempt to increase DNA concentrations, the annealing temperature was increased to 67.0 °C for some reactions, and for some reactions the number of cycles was increased to 50 for the same purpose.

Table 5: Thermocycler conditions for PCR with *Pfu* Turbo

	Temperature (°C)	Time (min)
Denaturing	94.0	1
Annealing	50.0	1
Extending	72.0	2
30-50 Cycles		

Gradient PCR

Gradient Polymerase Chain Reactions (Gradient PCRs) were conducted to amplify DNA at a wide range of annealing temperatures to optimize the reaction. The contents of each tube, however, were the same except for the addition of the respective forward and reverse primers for

EGFP and β-Lactamase. After thawing all reactants, except *Taq* polymerase, 24 µL sterile distilled water was added to a PCR tube. To this, 4 µL buffer (-$MgCl_2$), 4 µL of EGFP or β-lactamase reverse primer (50 pmol/mL), 4 µL of EGFP or β-lactamase forward primer (50 pmol/mL), 1.5 µL DNA miniprep (pEGFP), 1.2 µL 50mM $MgCl_2$, 0.8 µL dNTPs, and 0.5 µL Platinum *Taq* polymerase were added in this order, as depicted in Table 6.

Table 6: Components of Gradient PCR

Reactant	**Volume (µL)**
Sterile Distilled Water	24
Platinum *Taq* polymerase Buffer (-$MgCl_2$)	4
Reverse Primer	4
Forward Primer	4
EGFP DNA Miniprep	1.5
$MgCl_2$	1.2
Deoxyribonucleoside triphosphate mix (dNTP)	0.8
Platinum *Taq* polymerase	0.5
Total	40

These PCR tubes were transported to a gradient thermocycler and were run under the following protocol (Table 7): denaturing at 94.0 °C for 1 minute, annealing at 6 different temperatures of 50.0 °C, 54.0 °C, 58.0 °C, 62.0 °C, 66.0 °C, and 70.0 °C for 1 minute, and extending at 72.0 °C for one minute. This cycle was repeated for a total of 50 cycles, and the reactants were held at 4 °C after completion.

Table 7: Gradient thermocycler conditions for PCR

	Temperature (°C)	**Time (min)**
Denaturing	94.0	1
Annealing	50.0, 54.0, 58.0, 62.0, 66.0, and 70.0	1
Extending	72.0	1
50 Cycles		

PCR Product Purification through Gel Electrophoresis

The products of PCR were purified by separating the components through gel electrophoresis. The agarose gel was prepared by adding approximately 0.5 g High Melting

Temp Agarose to a 125-mL Erlenmeyer flask with 50 mL 1X TAE Buffer. The mixture was heated in the microwave for 1 minute and 3 μL of ethidium bromide was added so the DNA bands could be visualized later. The contents of the flask were poured into the gel tray with the well comb added. In order to produce larger slices of gel containing DNA, two sets of three tines on each well comb were taped together. The gel was allowed to cool for 30 minutes until it solidified. Next, the gel and its casting tray were immersed in 1X TAE Buffer in the electrophoresis tank. A volume of 5 μL of Blue Juice Loading Buffer was added to and mixed into each PCR sample to load the DNA and visualize its progress through the gel. The entire contents of each PCR tube (now ~105 μL) were added to each extra wide well. To a third, normal width well, 5 μL of a 1 Kb DNA Ladder was added. A potential of 100-110 volts was applied to the gel for 30 minutes. At this point, DNA separation could be visualized using the UV transilluminator. Using the DNA ladder, the desired DNA bands could be visualized for both EGFP and β-lactamase at ~800 bp. These bands were excised using a sharp razor blade and were stored in the refrigerator for gel extraction.

BioDoc-It Gel Imager

The BioDoc-It™ Imaging System was used, alongside a standard camera, to image agarose gels after electrophoresis. After turning on the instrument, the gel was placed inside the darkroom cabinet, the cabinet door was closed, and the UV transilluminator was activated. It was verified that the overhead white light was deactivated, and the lens f-stop adjustment was rotated until the image was bright enough to observe on the LCD monitor. Next, the zoom lens adjustment was rotated until the gel image was maximized in size, and the focus adjustment was rotated until clarity of the image was maximized. Once the image is satisfactory, if the bands on the gel were dim, the "+" button on the touch pad was pressed to increase exposure time.

Conversely, if the bands were too bright, the f-stop adjustment can be rotated again. The capture button was pressed, followed by the save button to save the image on the removable USB drive for later use.

Gel Extraction (OMEGA)

Two different protocols were used to complete the gel extraction procedure, the OMEGA protocol and the QIAGEN protocol. In the OMEGA protocol, the mass of the gel slice was determined by adding the gel to a microcentrifuge tube of pre-determined mass. To this tube, 1 volume of Binding Buffer was added, assuming a density of 1 g/mL. The gel and binding buffer were incubated at approximately 60 °C for 7 minutes in a heating block until the gel was completely melted, vortexing every 2 minutes. A HiBind Mini Column was inserted into a 2 mL collection tube, and up to 700 µL of melted gel was transferred to the Mini Column. The column was placed in the centrifuge at 10,000 x g for 1 minute, and the resulting filtrate was discarded. At this point, if additional melted gel was to be added for a higher concentration of DNA, it would be pipetted in and placed back into the centrifuge under the same parameters. An additional 300 µL of Binding Buffer was added into the column, which was placed back into the centrifuge at 13,000 x g for 1 minute. The filtrate was discarded and the collection tube was reused. A volume of 700 µL of SPW Wash Buffer, diluted with absolute ethanol, was added to remove residual salts and was placed into the centrifuge at 13,000 x g for 1 minute. The filtrate was discarded and the column and collection tube were placed back into the centrifuge at 13,000 x g for 2 minutes to completely dry the column. The dry column was transferred to a clean, sterile 1.5 mL microcentrifuge tube and 30 µL of Elution Buffer was added and was allowed to sit for 2 minutes. The DNA was eluted by centrifugation at 13,000 x g for 1 minute and the DNA was stored in the refrigerator.

Gel Extraction (QIAGEN)

In the QIAGEN protocol, the mass of the gel slice was determined by adding the gel to a microcentrifuge tube of a mass previously determined using an analytical balance and subtracting this mass from the total mass with the gel slice. To this tube, 3 volumes of Buffer QG were added, assuming a density of 1 g/mL. The gel and binding buffer were incubated at 50 °C for 10 minutes until the gel was completely melted, vortexing every 2 minutes. Once the gel was completely dissolved, its color was checked. A yellow solution indicated that the procedure could proceed but an orange or violet solution indicated the need for pH adjustment. In this scenario, 10 µL of 3 M sodium acetate was added to adjust the pH. Following this, if the size of the desired DNA was less than 500 bp or greater than 4000 bp, which was only the case for pFLAG-MAC which is 5071 bp, one gel volume of isopropanol was added and mixed. A QIAquick spin column was inserted into a 2 mL collection tube, and up to 800 µL of melted gel was transferred to the spin column. The column was placed in the centrifuge at 13,000 x g for 1 minute, and the resulting filtrate was discarded. At this point, if additional melted gel was to be added for a higher concentration of DNA, it would be pipetted in and placed back into the centrifuge under the same parameters. A volume of 750 µL of Buffer PE, diluted with absolute EtOH, was added to remove residual salts and was placed into the centrifuge at 13,000 x g for 1 minute. The filtrate was discarded and the column and collection tube were placed back into the centrifuge at 13,000 x g for 2 minutes to completely dry the column. The dry column was transferred to a clean, sterile 1.5 mL microcentrifuge tube and 30 µL of Buffer EB was added and was allowed to sit for 2 minutes. The DNA was eluted by centrifugation at 13,000 x g for 1 minute and the DNA was stored in the refrigerator.

Cycle Pure Kit

After a single digestion of *Eco*RI or *Hin*dIII, the Cycle Pure Kit can be used to stop the previous digestion, wash the DNA, and prepare for the next digestion. This step can also be used after PCR to wash the DNA. To each microcentrifuge tube, five volumes of CP Buffer (500 µL based on the volume of the PCR reactions) were added and vortexed thoroughly. The mixtures were briefly centrifuged to collect all material from the lids, and the contents of each tube were transferred to a HiBind DNA Minicolumn placed in a 2 mL collection tube. Each column was placed in the centrifuge for 1 minute at 13,000 x g, and the flow through was discarded. A volume of 700 µL of Wash Buffer (diluted with absolute ethanol) was added, and each tube was placed in the centrifuge for 1 minute at 13,000 x g. After discarding the flow through, an additional 700 µL of Wash Buffer was added. Each tube was placed in the centrifuge for 1 minute at 13,000 x g, and the flow through was discarded. The column was placed back into the centrifuge for 2 minutes at 13,000 x g to completely remove any residual ethanol. The dry columns were transferred a clean 1.5 mL microcentrifuge tube, and 20 µL of Elution Buffer were added directly to each filter. After sitting for two minutes, the DNA was eluted by centrifugation at 13,000 x g for 1 minute. At this point, DNA was stored in the refrigerator to await a second digestion. The second digestion was halted by running the digested products through an agarose gel and then extracted as was described earlier.

Enzymatic Digestion

Upon the successful extraction of EGFP and β-lactamase PCR products from the agarose gel, each of these, along with the pFLAG-MAC vector that is seen in Figure 14, were sequentially digested with *Eco*RI and *Hin*dIII. For each digestion procedure, the following were added to a 1.5 mL microcentrifuge tube for EGFP, β-lactamase, and pFLAG-MAC individually:

25.5 µL DNA, 3.0 µL of digestion buffer H for *Eco*RI reactions and 3.0 µL of digestion buffer M for *Hin*dIII reactions, and 1.5 µL of either *Eco*RI or *Hin*dIII. Each tube was allowed to sit in a 37 °C warm water bath for 3 hours as digestion of the DNA completed. Between *Eco*RI and *Hin*dIII digestions, the previous digestion procedure was halted by using the Cycle Pure Kit.

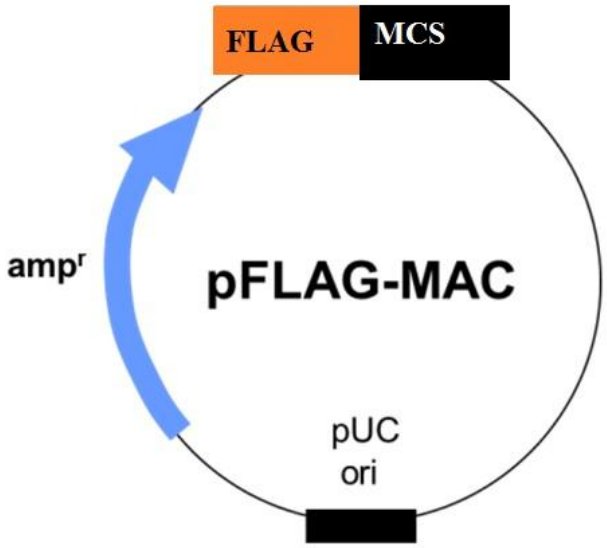


Figure 14: Plasmid map of pFLAG-MAC

NanoDrop

The NanoDrop is an instrument used to measure the concentration of the DNA after the completions of many different protocols, and the protocol for this instrument follows: to first clean the NanoDrop lens and lens table, 3 µL of ultrapure deionized water was pipetted onto the NanoDrop lens table. The lid was closed and allowed to sit for 2-3 minutes, and the water was then dabbed off both the lens and the lens table with a Kimwipe. Afterward, the arm was gently lowered back down, and the NanoDrop 2000c UV-Vis Spectrophotometer software was initiated. The Nucleic Acid program was selected and the program was allowed to proceed with wavelength verification. A volume of 2 µL of elution buffer was pipetted onto the lens table, the lid was closed and the sample was blanked. To confirm the reliability of the blank, the previous

blank sample was dabbed off and a new blank sample was measured to check if the concentration was recording at 0.0 ng/ μL. This was dabbed off with a Kimwipe and 2 μL of the DNA sample in elution buffer was added to the lens table and the "Measure" button was activated. This protocol was repeated between successive samples, and the instrument was blanked again every 2-3 measurements. The concentration of each sample was recorded along with the 260/280 and 260/230 wavelength ratio. After all samples had been measured, the lens table was cleaned with an additional 3 μL of ultrapure deionized water, allowed to sit for 2-3 minutes, and gently dabbed of with a dry Kimwipe.

LB Agar Plates with Ampicillin

LB Agar plates containing ampicillin were created for use during transformations. A volume of 400 mL of deionized water was added to a 1 L buffer bottle along with 14 g of LB agar powder and was capped with a foil lid. This mixture was placed in an autoclave bucket was autoclaved for 20 minutes at 121 °C on a liquid setting. Once the autoclave had cooled, the flask was removed and allowed to continue to cool until it reached about 50°C. At this point, 0.040 g ampicillin was added to create a 100 μg/mL solution. A volume of 20 mL of the cooled agar was poured into each of 20 sterile petri dishes. These were allowed to cool and were stored agar side up in the refrigerator for later use.

Transformation

In order to generate new stocks of pEGFP and pFLAG-MAC, old DNA minipreps of each were transformed into DH5α *E.coli* cells. To accomplish this, competent DH5α cells (stored at -80 °C) were thawed on ice alongside two 14 mL polypropylene BD Falcon tubes, which were being chilled. The thawed cells were gently mixed and were added to the Falcon tubes in 100 μL aliquots. The pEGFP and pFLAG-MAC minipreps were added to their own tube and the cells

were incubated on ice for 30 minutes. After this, the cells were heat shocked for 45 seconds in a 42 °C water bath and were placed back on ice for 2 minutes. To this mixture, 900 µL of room temperature S.O.C. Medium was added, and the Falcon tubes were placed on the shaker table for 1 hour at 37 °C and 225 rpm. A volume of 20 µL of this mixture was spread over LB agar plates made with 100 µg/mL ampicillin. The plates were incubated overnight at 37 °C. Growth was observed the next day and the cells containing pEGFP were observed glowing under UV light. These plates were stored in the refrigerator.

In order to complete the process of making new stocks, isolated colonies were picked from the fresh plates and were grown in overnight culture. A volume of 1.5mL of culture was added to a cryogenic vial and diluted with and 0.5mL of 100% sterile glycerol. The 75:25 mixture of cells was shaken thoroughly and was placed in the -80°C freezer to serve as stocks for future overnight cultures of pEGFP and pFLAG-MAC.

Results and Discussion

Generation of New Stocks

Concentrations of DNA have been consistently low throughout all portions of this research. Throughout this work, different attempts were made to try to increase these concentrations by optimizing protocols of all parts of this experiment so that concentrations of DNA can remain high enough to complete the validation of the local pH theory. In order to increase DNA concentrations, one method utilized was to generate new stocks from which overnight cultures could be grown. An old miniprep of pEGFP template DNA from previous years was transformed using the DHH5α cells, and these cells glowed green and on ampicillin containing agar, seen in Figure 15. Accordingly, it was confirmed that the cells on the plates contained the pEGFP plasmid, and all of the protocols were performed using this new set of cells.

Figure 15: Colonies of DH5α E.coli cells containing pEGFP glowing green under UV Light

Using the miniprep generated from the new stocks, PCR reactions were run and separated in an agarose gel not only to separate the genes of interest from primer dimers and other DNA, but to confirm that the genes of interest were indeed present in the gels at around 800 bp, seen in Figure 16. The band that is fourth from the bottom on the 1kb ladder marks 700 bp and the band that is fifth from the bottom marks 1000 bp. Because the bands from the PCR products are between these two reference bands, but closer to the 700 bp band, it can be validated that the genes of interest are at around 800 bp. However, these bands of both EGFP and β-lactamase were faint, and primer dimer bands at the end of the gel were still fairly bright. In an attempt to increase the concentration of the DNA found here without starting over, the gel was extracted and run through an additional round of PCR in an attempt to increase DNA concentrations, but all DNA was lost, as seen in Figure 17.

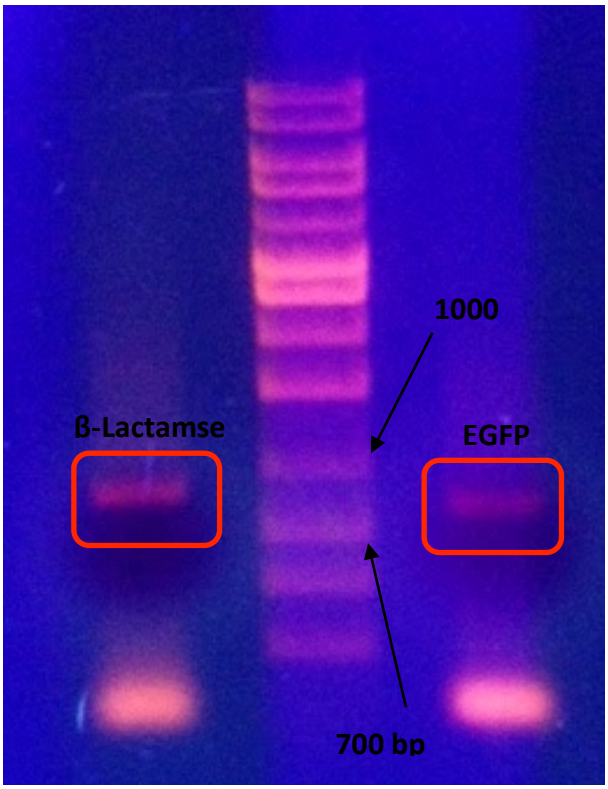


Figure 16: Faint bands of DNA present using new stocks

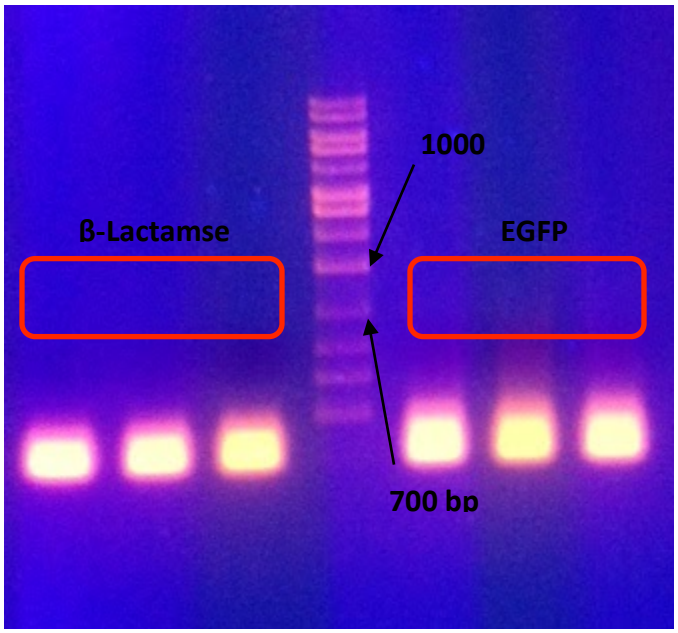


Figure 17: Gel after second round of DNA amplification

In order to determine at what stage DNA was being lost, the concentration of leftover DNA from before the second amplification procedure was checked using the NanoDrop and was found to be 0.0 ng/μL for both β-Lactamase and EGFP. Accordingly, it was suspected that a great deal of the DNA could have been lost during the gel extraction procedure. Using the same miniprep, PCR was performed at a higher annealing temperature, 67.0 °C instead of 65.0 °C, and a gel was run using wider wells. This gel exhibited much brighter bands of DNA, seen in Figure 18. The concentration of the extracts of these bands can be seen in Table 8.

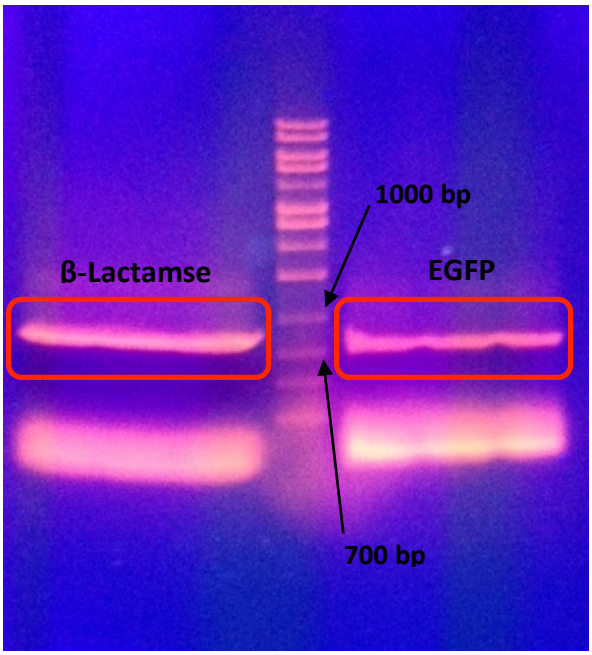


Figure 18: Separation of PCR products through gel electrophoresis

Table 8: Concentrations of β-lactamase and EGFP after amplification of new stocks

	Concentration (ng/µL)
β-Lactamase	30.6
EGFP	19.1

The DNA from these brighter bands was sequentially digested once with *Eco*RI and then again with *Hin*dIII, and another gel was run to stop the second digestion. This gel showed faint bands for EGFP and β-lactamase and a bright band for pFLAG-MAC that had been extracted from a previous gel and was digested alongside the other samples, seen in Figure 19. The concentrations of β-lactamase and EGFP, after the first and second digestions and the concentration of and pFLAG-MAC after the second digestion can be seen in Table 9.

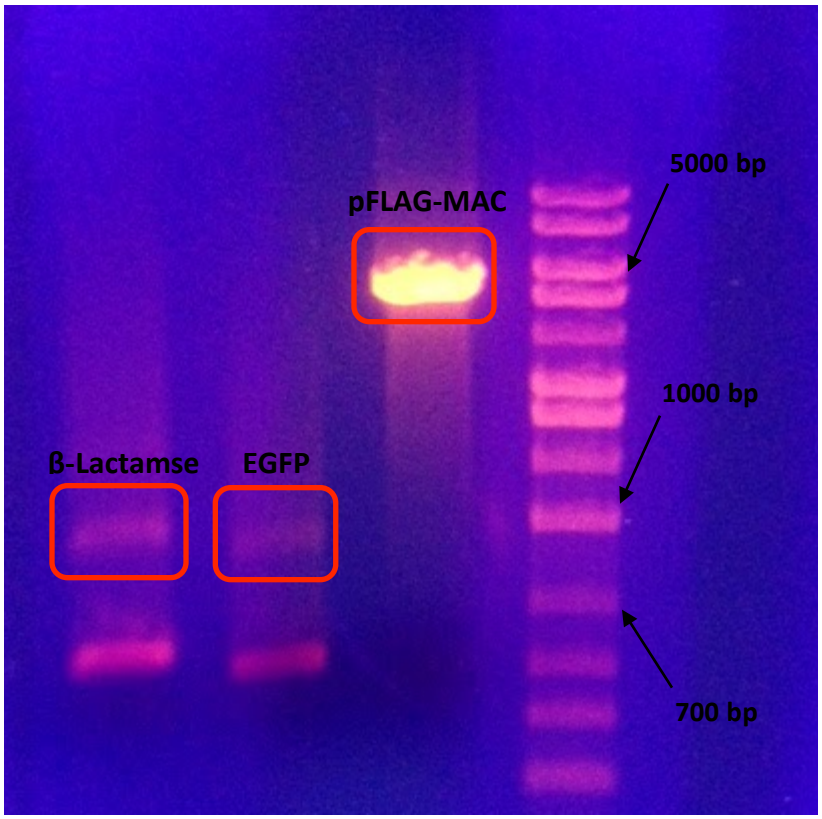


Figure 19: Post digestion products for β-lactamase, EGFP, and pFLAG-MAC

Table 9: Concentrations of digestion products after first, *Eco*RI, and second, *Hin*dIII, digestions

	Concentration (ng/µL)
β-Lactamase (First Digestion)	24.3
EGFP (First Digestion)	13.6
β-Lactamase (Second Digestion)	1.4
EGFP (Second Digestion)	0.0
pFLAG-MAC (Second Digestion)	20.8

Since concentrations of EGFP and β-Lactamase had become absent or negligible, a new PCR reaction was set up using the same miniprep generated from the new stocks and run through a gel, seen in Figure 20. However, only the β-lactamase band was present in this gel.

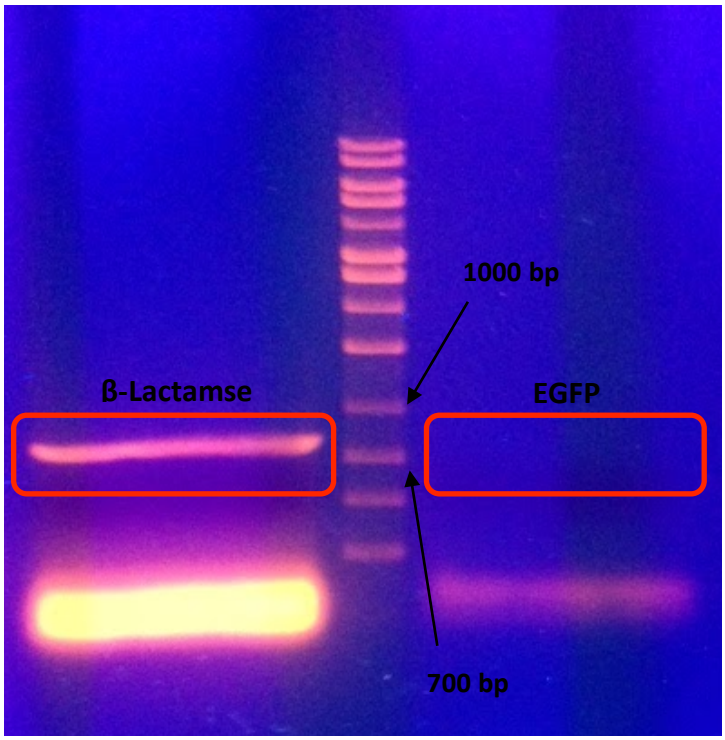


Figure 20: Gel after new PCR with no EGFP band present

In an attempt to obtain an EGFP sample to digest alongside the existing β-Lactamse sample, an attempt was made to isolate EGFP alone. Using the same miniprep for another round of PCR amplification, another gel was run using two lanes of EGFP PCR products since none was present in the previous run, seen in Figure 21.

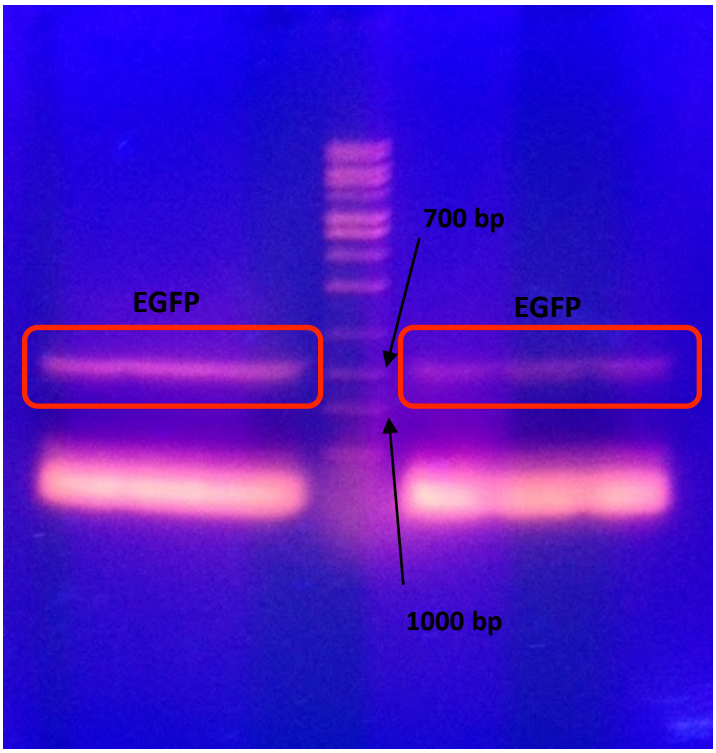


Figure 21: Gel of EGFP bands

Optimization of Gel Extraction Parameters

The bands of EGFP generated in the Figure 21 were very faint, so in an attempt to increase DNA concentrations further before proceeding, another PCR procedure was completed so that multiple slices of gel were added at once to the HiBind DNA Minicolumn during the gel extraction procedure. This additional set of PCR products to be used for this modified gel extraction protocol can be seen in Figure 22.

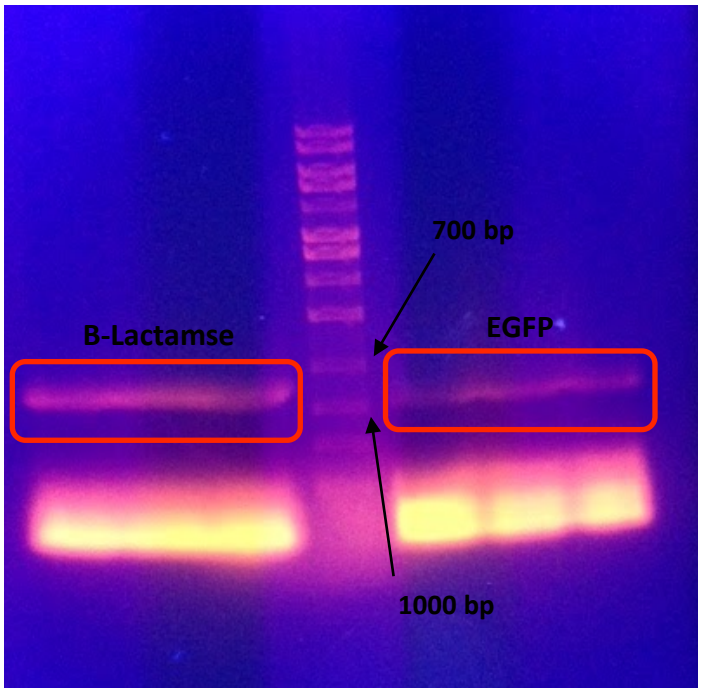


Figure 22: Gel of β-lactamase and EGFP

These gel slices, from Figure 20, Figure 21, and Figure 22, were all extracted together. The concentrations of this extraction can be seen in Table 10. However, the concentration of the DNA was still lower than would be preferred, specifically for EGFP. In order to determine if some of the DNA was being left in the column during gel extraction and test this procedure as a possible source of DNA loss, a second elution was performed on the columns.

Table 10: Concentrations of β-lactamase and EGFP after one and two elutions after gel extraction

	Concentration (ng/µL)
β-Lactamase (First Elution)	49.4
EGFP (First Elution)	10.2
β-Lactamase (Second Elution)	0.3
EGFP (Second Elution)	0.9

Accordingly, it was determined that the majority of the DNA was being eluted from the columns, and the loss of DNA must be occurring elsewhere. Accordingly, four more gels, each containing β-lactamase and EGFP PCR products were run to see if increasing the amount of DNA containing agarose by this amount would provide a large enough quantity of DNA to make it through the digestion, ligation, and transformation procedures. Two of the gels showed only bands only for β-lactamase, seen in Figure 23, and two other gels showed bands for both β-lactamase and EGFP.

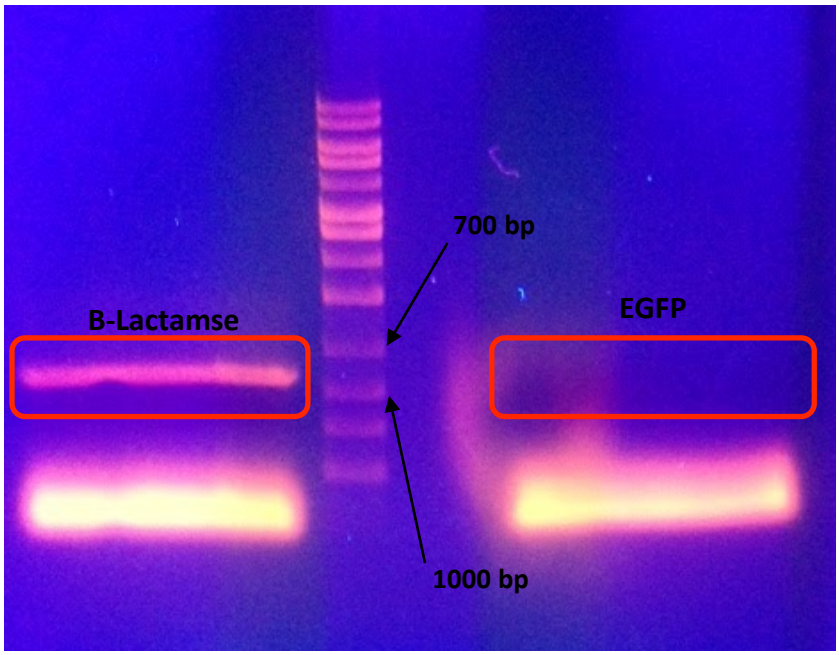


Figure 23: Gel Displaying Only the Band for β-lactamase

In order to determine if the OMEGA Gel Extraction Kit was the cause of the DNA loss, two of the β-lactamase gel slices were extracted through a single column using the OMEGA protocol and the other two β-lactamase gel slices were extracted through a single column using the QIAGEN protocol. Since fewer bands containing EGFP had been acquired from the gels, it was only extracted using the QIAGEN protocol in hopes that it would produce better yields. The

concentrations seen in Table 11 were recorded. In light of doubled concentrations of DNA using the QIAGEN protocol compared to the OMEGA protocol, the QIAGEN kit was used for all subsequent gel extractions.

Table 11: Concentrations of β-Lactamase and EGFP after gel extraction with OMEGA and QIAGEN kits

	Concentration (ng/μL)
β-Lactamase (OMEGA)	14.9
β-Lactamase (QIAGEN)	28.7
EGFP (QIAGEN)	11.1

Optimization of PCR Parameters

Since only β-lactamase gel extracts had been formed at high enough concentrations to proceed with digestion, another PCR of only EGFP was set up with new miniprep made from new overnight cultures. The EGFP PCR products were run through a gel, seen in Figure 24. Though the bands were faint, they were put together and extracted using the QIAGEN protocol, but this extract was found to only have a concentration of 6.1 ng/μL.

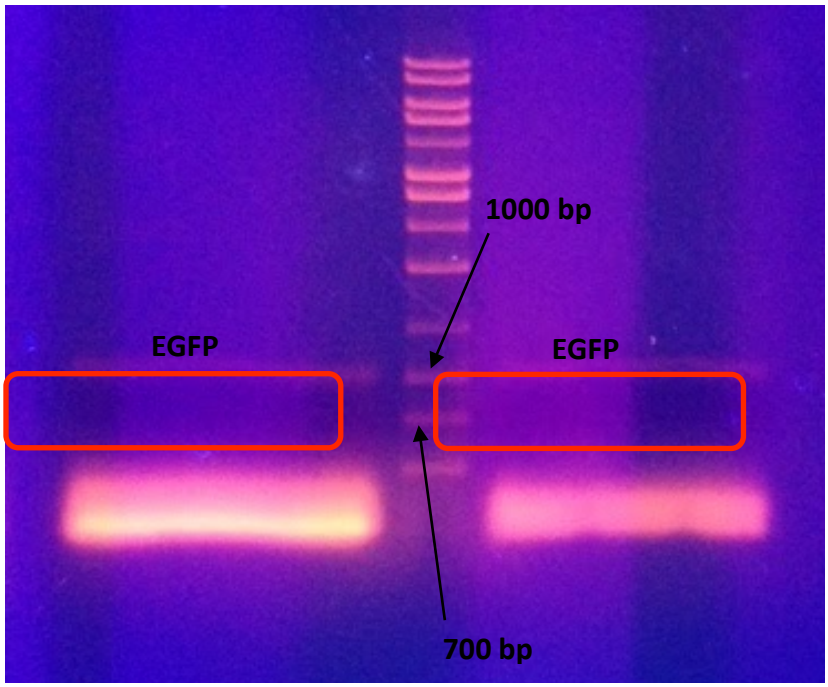


Figure 24: Gel of only EGFP PCR products

As it had now become clear that there must be some other source of the reduction of DNA concentration other than the gel extraction procedure, a plan was developed for the optimization of the PCR protocol. Furthermore, since DNA concentrations had become far too low to proceed with subsequent steps, a new adjusted PCR protocol was developed that would use a much greater concentration of template DNA, 10 µL instead of 4 µL. After a PCR of only EGFP was run under these parameters, another gel was run, seen Figure 25. No DNA was present in this gel, and even the brightness of the primer dimers was decreased. It is hypothesized that the increased salt concentration coming from the increase in elution buffer containing the template DNA prevented the *Taq* polymerase from functioning. Accordingly, this procedure was not used again and the volumes of PCR components were adjusted back.

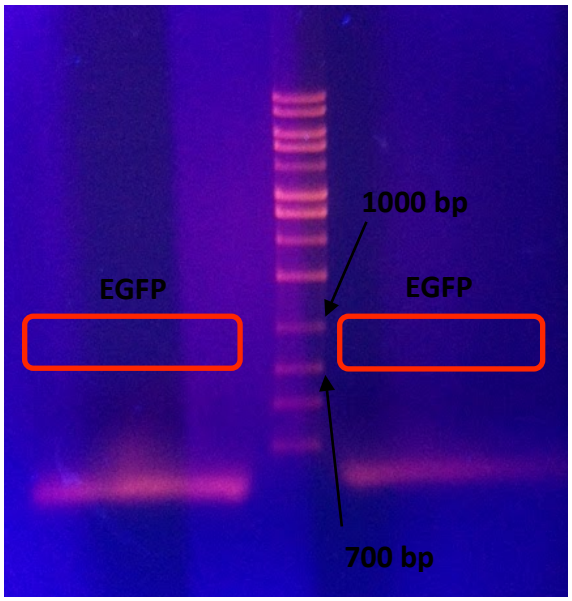


Figure 25: Gel of only EGFP with no PCR products present after increasing template DNA concentration

In a new attempt to increase concentrations of DNA during PCR, the PCR protocol was completely changed. The enzyme *Pfu* Turbo was used in place of *Taq* polymerase and $MgCl_2$, and template DNA concentrations changed back to previous concentrations to further decrease salt concentrations and hopefully increase enzyme activity. The annealing temperature was also further decreased to a temperature optimum for this new enzyme, and the order of the addition of the reactants was adjusted. A PCR reaction was set up for using the new *Pfu* Turbo protocol, but it was not successful and no bands were visible except for primer dimers, as seen in Figure 26. However, at this point, the failed reactions were probably due to a loss of DNA in the miniprep rather than a failure of the *Pfu* Turbo polymerase.

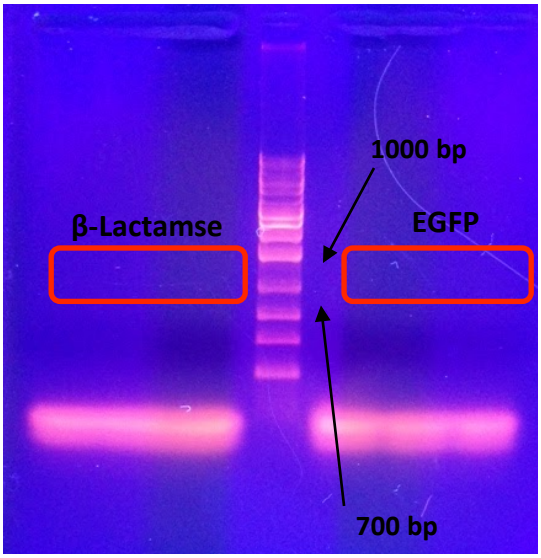


Figure 26: Gel run with β-lactamase and EGFP with no PCR products present after using the *Pfu* Turbo protocol

In an attempt to re-evaluate the failed PCR reaction visualized in the previous figure using the *Pfu* Turbo PCR protocol, the annealing temperature was increased back to 67 °C for 50 cycles to come closer to the melting temperatures of the primers. However, the gel run of these products showed that the PCR reaction was again unsuccessful, as seen in Figure 27.

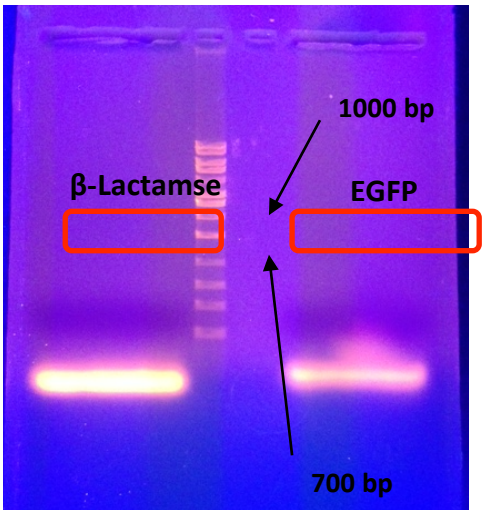


Figure 27: Gel run with β-lactamase and EGFP with no PCR products present after altering the *Pfu* Turbo protocol to a higher annealing temperature

Accordingly, another gel was run using the *Taq* polymerase protocol to see if the *Pfu* Turbo was the source of the errors, and the gel seen in Figure 28 shows the results. This reaction was also not successful. Accordingly, it could not be determined what was causing the loss of DNA at this point.

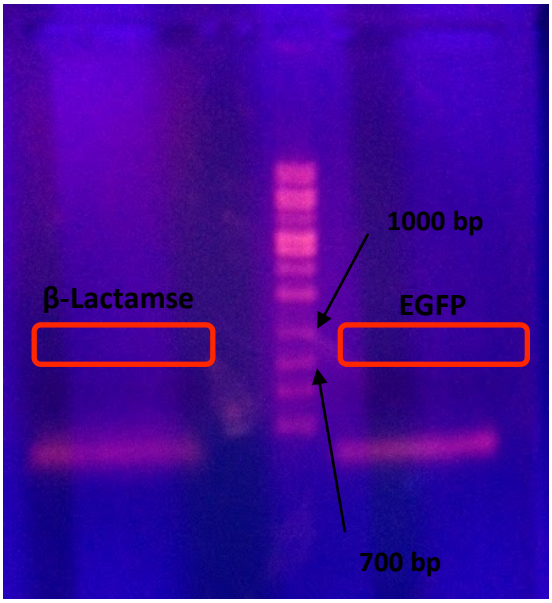


Figure 28: Gel run with β-lactamase and EGFP with no PCR products present after checking Taq polymerase protocol against the Pfu Turbo protocol

It was hypothesized that PCR was unsuccessful because the template DNA being used was too low in concentration to begin with. So, in an attempt to create higher concentration minipreps from the new stocks, overnight cultures were started of pEGFP and pFLAG-MAC and were allowed to grow in the incubator for only 15 hours to prevent overgrowth as a possible source of low miniprep concentration. These cultures were miniprepped according to the OMEGA protocol, and were found using the NanoDrop to have the concentrations seen in Table 12. The concentrations were all lower than expected and the unusual wavelength ratios of 260/280 and 260/230 seemed to indicate an imprecision with blanking of the NanoDrop. Afterwards, the NanoDrop was found to measure the blank anywhere from -0.6 ng/µL to 0.8

ng/μL, but never 0.0 ng/μL. A desirable range of the 260/280 wavelength ratios to demonstrate sample purity would be between 1.80-2.00, and a desirable range for the 260/230 ratios would be between 2.00-2.20. Accordingly, the wavelength ratios could not properly be analyzed to assess the purity of the samples.

Table 12: Concentration of pEGFP minipreps prior to PCR

	Concentration (ng/μL)	260/280	260/230
pEGFP1	26.3	2.00	2.42
pEGFP2	25.4	2.11	14.03
pEGFP3	11.0	2.33	-3.03
pEGFP4	33.9	2.15	5.39
pEGFP5	17.7	2.22	285.34
pEGFP6	15.9	2.33	-14.31

As none of these miniprep concentrations were as high as was desirable, it was hypothesized that the freezing of the cells in the -80 °C freezer was reducing viability, particularly if the power to the freezer had ever gone out and the cells had undergone a thawing and refreezing process. Accordingly, a sample of previously isolated template DNA was used for a transformation procedure into DH5α cells so that cultures could be grown from never frozen cells. To see if the freezing of the cells was reducing viability, a transformation was set up of a sample of the template DNA that had been used for previous reactions, rather than from stocks stored in the -80 °C freezer. However, the transformation of pEGFP (on the left) was unsuccessful, but there was growth on pUC 19 control plasmid (on the right) was successful, seen in Figure 29. The lack of growth of pEGFP but the growth of the positive control showed that the transformation procedure was good, but the pEGFP plasmid was not present in the sample.

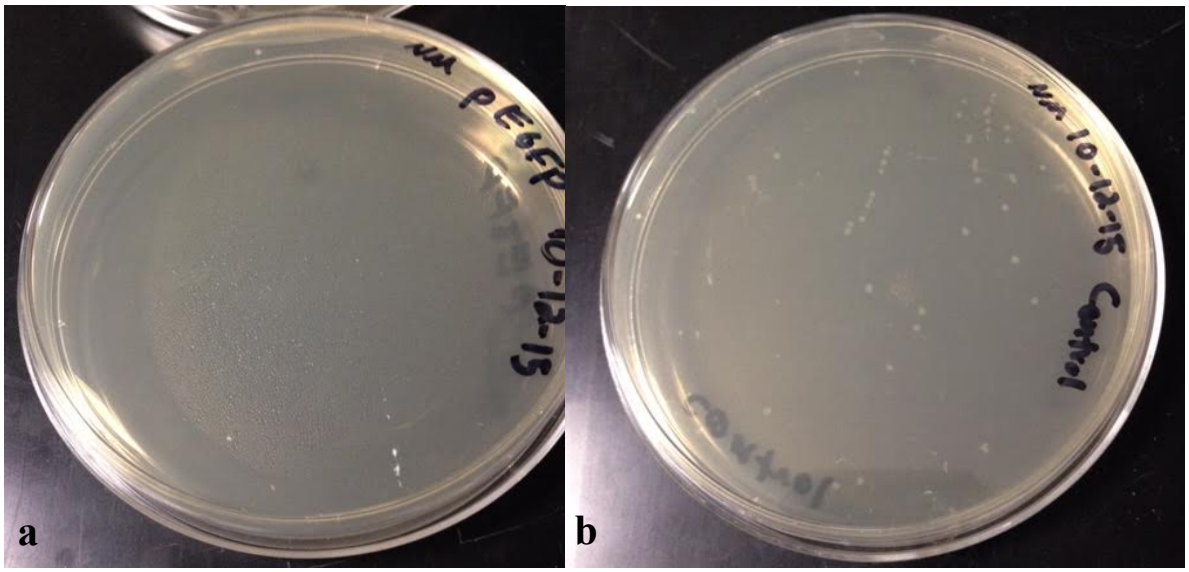


Figure 29: a) Transformation of the pEGFP b) Transformation of pUC 19 control

Since the miniprep tested appeared devoid of the desired DNA, overnight cultures were grown from colonies expressing pEGFP that were picked off a plate from a previous transformation procedure. Then, all 6 cultures were miniprepped using the QIAGEN kit and their concentrations were checked using the NanoDrop to produce the concentrations seen in Table 13. The sample pEGFP1 was set aside in case a need arose for another transformation procedure.

Table 13: Concentration of pEGFP after miniprep from previously transformed DH5α cells

	Concentration (ng/μL)	260/280	260/230
pEGFP1	36.3	1.92	2.22
pEGFP2	7.6	2.08	3.60
pEGFP3	13.8	2.02	3.04
pEGFP4	4.5	2.58	-5.23
pEGFP5	41.3	1.94	2.35
pEGFP6	23.0	2.04	2.65

A new PCR reaction was set up using the standard protocol with the new miniprep of highest concentration along with a new stock of *Taq* polymerase. The PCR products from this reaction were run through a gel, seen in Figure 30.

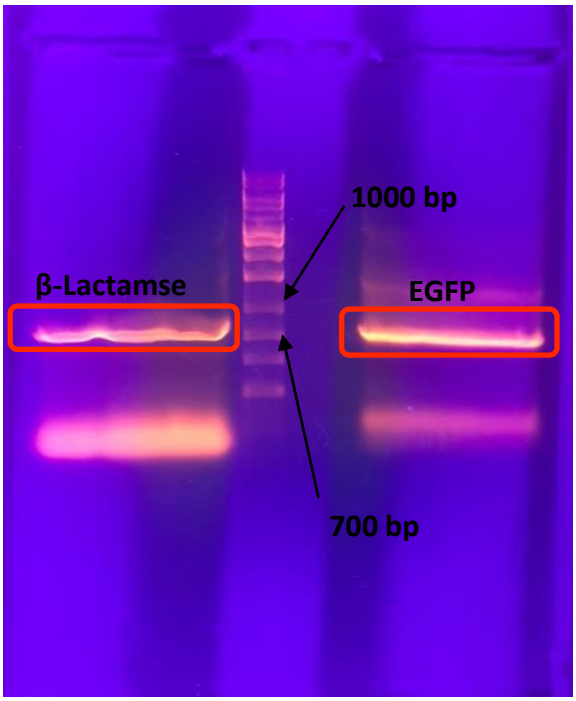


Figure 30: Gel of β-lactamse and EGFP using new miniprep and new *Taq* polymerase

Given the success of this gel, that same miniprep was used as template DNA for a set of PCR reactions at six different annealing temperatures in the gradient thermocycler to optimize the amplification of both genes. The reactant volume had to be adjusted to 40 µL per PCR tube and one sample each for β-lactamse and EGFP were at annealing temperatures of 50.0 °C, 54.0 °C, 58.0 °C, 62.0 °C, 66.0 °C, and 70.0 °C. The products of this reaction were run on two separate gels in order of increasing annealing temperature, seen in Figure 31.

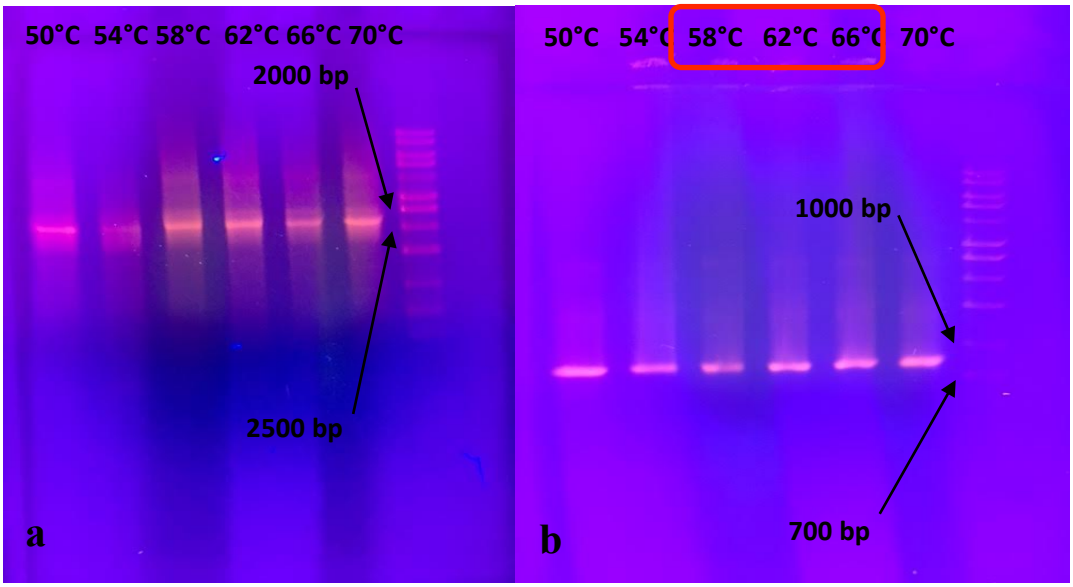


Figure 31: a) Gradient PCR Products of EGFP b) Gradient PCR Products of β-lactamase

The bands on the EGFP gel appear to be between 2000-2500 bp in size, so this is not EGFP. Potentially, this could have been due to the addition of the wrong set of primers. The bands on the gel of β-lactamase appear to be about 800 bp in size, the appropriate size for β-lactamase. Accordingly, a new gradient PCR was set up under the same parameters using only EGFP and were run through the gel in order of increasing annealing temperature, seen in Figure 32. Between, Figure 31 and Figure 32, the band at 58.0 °C appears to be the brightest for EGFP DNA and the temperature range of 62-70 °C appears to be the brightest for β-lactamase DNA, so subsequent PCR reactions were run at these temperatures.

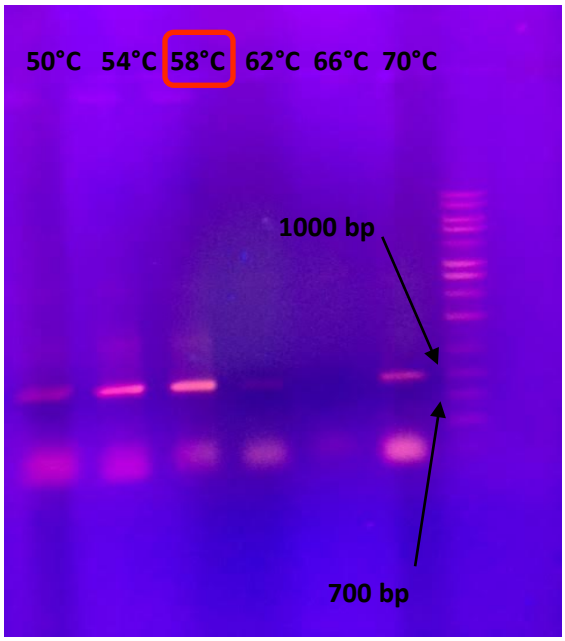


Figure 32: Gradient PCR Products of EGFP

Optimization of Miniprep Parameters

Due to the success of changing from an OMEGA gel extraction kit to a QIAGEN gel extraction kit, a QIAGEN miniprep kit was used to create a new set of pEGFP miniprep. The concentration of these minipreps, which can be seen in Table 14, are roughly five times the concentrations of miniprep DNA produced by the OMEGA kits. Furthermore, both the 260/280 and the 260/230 wavelength ratios are at reasonable values of around 2.00, indicating that the NanoDrop was functioning properly when these concentrations were determined and the samples were uncontaminated. It was concluded after these findings that the QIAGEN kit would be used for any subsequent miniprep procedures.

Table 14: Miniprep concentrations after using QIAGEN miniprep kit

	Concentration (ng/μL)	260/280	260/230
pEGFP1	141.9	1.97	1.96
pEGFP2	154.7	1.98	2.11
pEGFP3	148.6	1.98	2.16
pEGFP4	111.5	2.02	2.25
pEGFP5	128.7	1.99	2.16
pEGFP6	150.7	1.96	2.00

Validation of the Local pH Theory

Since the optimization of PCR parameters, gel extraction protocols, and other procedures were completed, work on this project was focused on completing the validation of the local pH theory by utilizing the ability to generate higher concentrations of DNA. A gel extraction procedure was completed using the QIAGEN kit on the gels slices from the gradient PCR, though the larger gel volume for β-lactamase necessitated that it be split into two separate extraction columns. The concentrations of these extracts were determined using the NanoDrop, seen in Table 15.

Table 15: Concentration of EGFP and B-Lactamase after protocol optimization

	Concentration (ng/μL)
EGFP	23.4
β-Lactamase1	53.4
β-Lactamase2	33.2

These extracts, along with previously amplified DNA and pFLAG-MAC miniprep were sequentially digested with *Eco*RI and *Hin*dIII. The gel that stopped the second digestion can be seen in Figure 33. Bright bands of digested DNA for β-Lactamse, EGFP, and the expression vector pFLAG-MAC were all present, so these bands were cut out to be extracted and ligated to complete the recombinant vectors that will be transformed to finish the molecular cloning stages of the validation of the local pH theory.

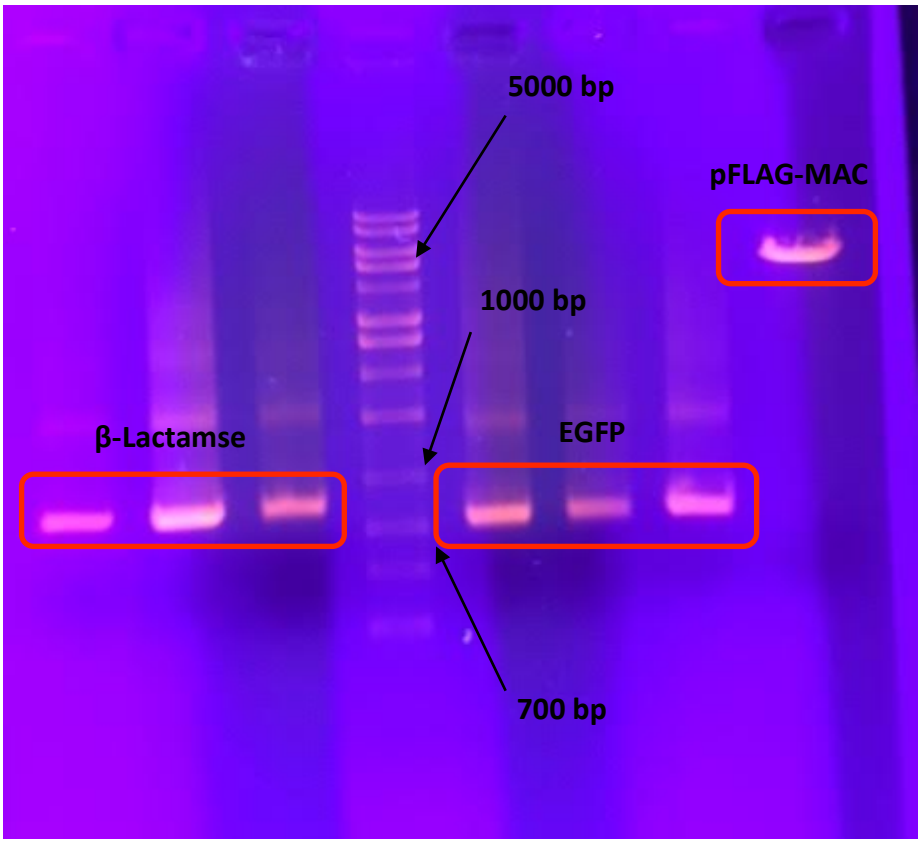


Figure 33: Gel of digestion PCR products after annealing temperature optimization

Conclusions and Future Work

The major goal of this work was to optimize the parameters for the completion of the validation of the local pH theory so that a sensing system can be created to measure the hydrolysis of β-lactam antibiotics. Accordingly, new pEGFP stocks were generated and it was determined that pEGFP was lost in them. The plasmid pEGFP was isolated from other cultures and the gel extraction procedure was optimized, and the QIAGEN kit was found to be more effective. The QIAGEN kit was also found to be more effective for miniprep procedures. A comparison of annealing temperatures through gradient PCR determined that the optimum annealing temperature for this reaction was 58 °C for EGFP and 62-70 °C for β-lactamase.

Future work on this project will be centered using the optimized PCR, gel extraction, and miniprep parameters completed through this work to generate DNA at higher concentrations. This higher concentration DNA will be digested, ligated, and transformed in DH5α cells so that EGFP and β-lactamse can be expressed and purified to move towards the completion of the validation of the local pH theory. Once this is completed, the fusion protein for the *in vivo* system can be both generated and expressed, so that kinetic studies on it can be completed.

Once the development of the sensing system has been finished, it will be able to be used to measure the hydrolysis of β-lactam antibiotics. Accordingly, it will also be able to be used to pursue and find new β-lactamase inhibitors to be used in conjunction with existing β-lactam antibiotics. Furthermore, the completion of this system will allow for the monitoring of bioavailability of β-lactam antibiotics.

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