The Development of Sensing Systems to Measure Hydrolysis of β-lactam Antibiotics

by

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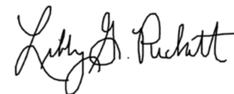

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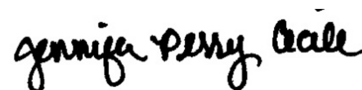

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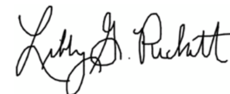

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Abstract

Antibiotic resistance is a prevalent problem in modern society, with almost three million cases of antibiotic resistant infections occurring yearly in the U.S.[1] When antibiotics were developed as a treatment for disease and infection, most bacteria were susceptible to their antibiotic properties. However, over time, as a result of over-prescription and misuse of the drugs, bacteria have developed resistance to the medications. β-lactam antibiotics are a common and effective class of antibiotics, but they too are losing potency as more bacteria develop resistance to them in the form of β-lactam hydrolysis caused by β-lactamase. β-lactamase is an enzyme that catalyzes the cleavage of the β-lactam ring of penicillin antibiotics, rendering them ineffective.

The goal of this project is to create sensing systems that can monitor the hydrolysis of β-lactam antibiotics. The systems will utilize a fusion protein of β-lactamase and enhanced green fluorescent protein (EGFP). β-lactamase, an enzyme responsible for conferring antibiotic resistance, is encoded in the ampicillin resistance (*amp*^{r}) gene of many plasmids. The β-lactam ring of penicillins gives the antibiotics their effectiveness by inhibiting cell wall synthesis during bacterial replication. When β-lactamase catalyzes the cleavage the β-lactam ring, a proton is released, decreasing the local pH. EGFP, a variant of green fluorescent protein (GFP), is a pH-sensitive fluorescent protein that will be used as the reporter protein to monitor the hydrolysis of the β-lactam ring. EGFP has two key mutations in the chromophore region that result in its fluorescence being more intense than that of GFP when excited at 488 nm and allows it to respond to changes in pH. In the assays being developed, the EGFP domain of the fusion protein will respond to the drop in local pH resulting from the β-lactam hydrolysis, leading to a decrease in fluorescence over time.

This project has three directions. The first is the recreation of the *in vitro* fusion protein from the original study to be used for comparison.[50] The second is the validation of the pH theory with the β-lactamase and EGFP proteins being expressed individually and added separately for the assay to demonstrate that the proteins must be in immediate proximity for a decrease in fluorescence to occur. The third is the development of an *in vivo* whole-cell based system using a fusion protein of β-lactamase and EGFP to measure the bioavailability of new β-lactam antibiotics. These aims will be met by isolating the β-lactamase and EGFP genes from a source plasmid, amplifying the genes via polymerase chain reaction (PCR), digesting the genes using restriction enzymes, ligating them into vectors, and transforming them into DH5α competent *E. coli* bacterial cells. After successful transformation of the individual and fusion genes into the *E. coli* cells, the DNA will be sequenced. If the results of sequencing confirm the presence of the target genes, large scale cultures of bacteria expressing the *in vivo* fusion will be created, and the proteins will be purified. Fluorescence assays will be performed on the purified proteins to study their fluorescence characteristics.

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I would like to thank Kimberly Nelson for pushing me to strive to be my best in my academic life, my professional life, and my personal life. She has helped me in more ways than I can count.

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I would like to thank Dr. Jennifer Cecile and Dr. Andrew Bellemer for being second readers. I have always thought highly of them, and I am truly appreciative for helping me grow intellectually, and as a person.

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Introduction

Biosensors

A sensor is "a device that responds to physical stimulus (such as heat, light, sound, pressure, magnetism, or a particular motion) and transmits a resulting impulse (as for measurement or operating a control)."[2] Sensors accomplish this using a recognition element and a transducer. The recognition element responds to changes, and the transducer converts the change into an electronic signal to allow changes in the system to be observed.[3]

Biosensors are a class of sensor that are of interest in this project. A biosensor is an analytical device that "monitors and transmits information about a life process; *especially*: a device consisting of a biological component (such as an enzyme or bacterium) that reacts with a target substance… and emits a signal."[4] The biological component of biosensors acts as the recognition element, and can be an enzyme, antibody, nucleic acid, or receptor, making biosensors more selective and sensitive than other sensors. Biosensors are so sensitive that detections have been made in the femtomolar range.[6] The transducer element is generally based on a reporter protein.[5] Using fluorescent reporter proteins, quantitative measurements can be made through fluorescence analysis in whole cell-based sensing systems, such as by flow cytometry.[7]

Biosensors were first used in 1962 for the enzymatic measurement of blood glucose levels using oxygen electrodes to observe the products of glucose oxidase.[8] Since then, they have experienced many diverse applications. Potentiometric enzyme electrodes with urease are used to measure urea levels. Fiberoptic sensors are used for the *in vivo* measurement of blood gas levels.[9] Potentiometric alternating biosensors are used in the detection of pathogens on foods, and enzymatic biosensors are used in the detection of organophosphate pesticides in milk. Medically, glucose biosensors are used for at-home monitoring of blood glucose levels in individuals with

diabetes mellitus. Fluorescence biosensors are used as imaging agents in cancers and drug discovery.[8] More relevant to this research, GFP fluorescent protein biosensors have been applied to microphysiological systems to monitor enzymatic activity and metabolite production.[10]

In this research, the aim is the creation of sensing systems to measure enzymatic activity of β-lactamase. Sensing systems are a class of sensors that are not "true" sensors, but function similarly. Sensing systems do not utilize an internal transducer, but rather an outside element to measure signals.[11] There are three primary classes of biosensing systems: molecular, cellular, and tissue based. The system used in this research is a whole cell-based biosensing system. Cellular-based systems are advantageous in that they are stable in environments with varying temperature and pH, making them physiologically relevant and allow for bioavailability studies.[12,13] Whole cell-based biosensing systems use a variety of fluorescent reporter proteins, commonly including GFP, aequorin, firefly luciferase, bacterial luciferase, and red fluorescent protein.[10,13]

β-lactam Antibiotics

Penicillin, a commonly used β-lactam antibiotic, was first discovered in 1928 by Alexander Fleming. Fleming had been culturing colonies of *Staphylococcus*, and he noticed that on one dish, a mold was growing. The dish was covered with *Staphylococcus* colonies, except for the area directly surrounding the mold – the zone of inhibition. The mold was later identified as *Penicillium notatum*. It had secreted a liquid that killed and prevented the growth of bacteria. Further experimentation by Fleming showed that the liquid secreted was an effective treatment against other common pathogenic bacteria, such as *Streptococcus* and *meningococcus* (the bacterium responsible for meningococcal meningitis), displaying the toxicity of penicillin towards both Gram-positive and Gram-negative bacteria.[14]

β-lactam antibiotics, also known as penicillins, have a key feature that gives them their effectiveness, a β-lactam ring. The ring feature was discovered in 1945.[14] Figure 1 below depicts the four membered ring containing nitrogen and a carbonyl group.

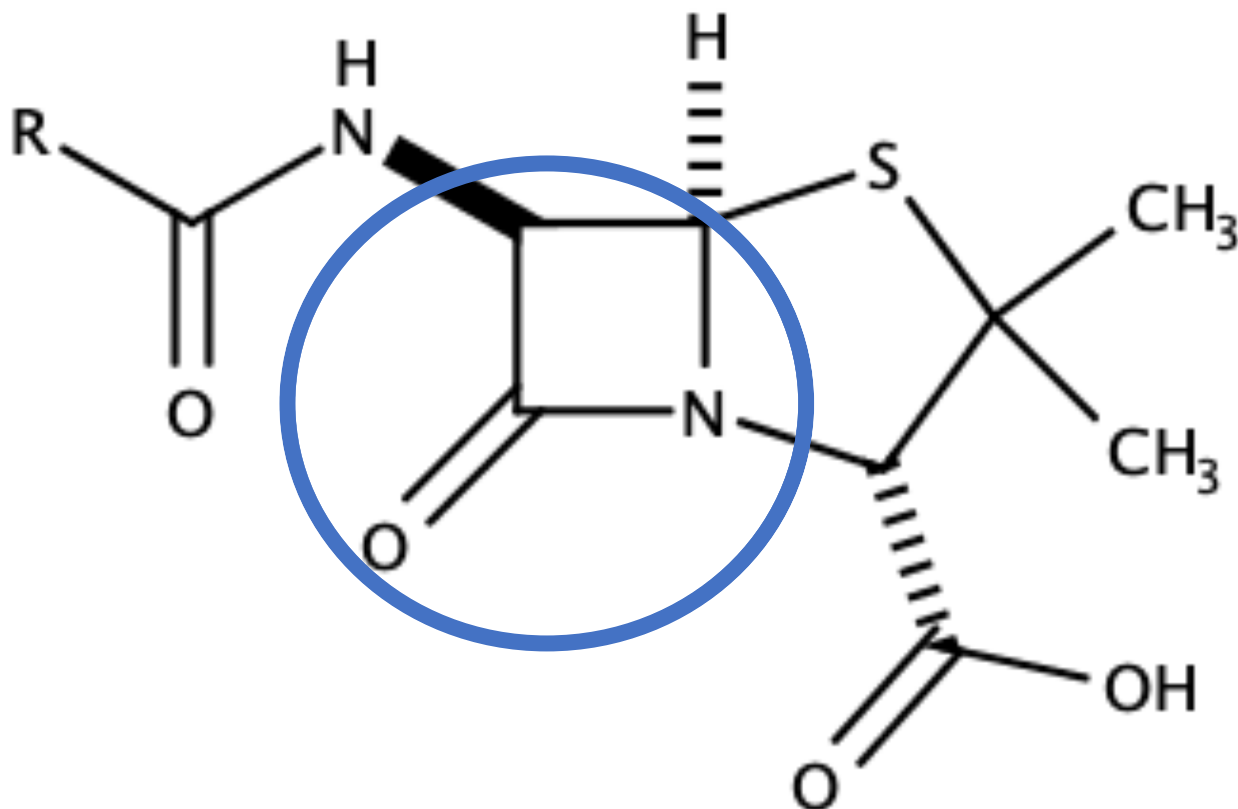


Figure 1. Structure of penicillin with four-membered β-lactam ring encircled

There are a variety of antibiotics that contain the β-lactam ring; they are grouped into four classes based on the varying substituents surrounding the β-lactam ring structure: penicillins, cephalosporins, monobactams, and carbapenems.[15] Figure 2 below depicts representative structures of each class of the antibiotics.

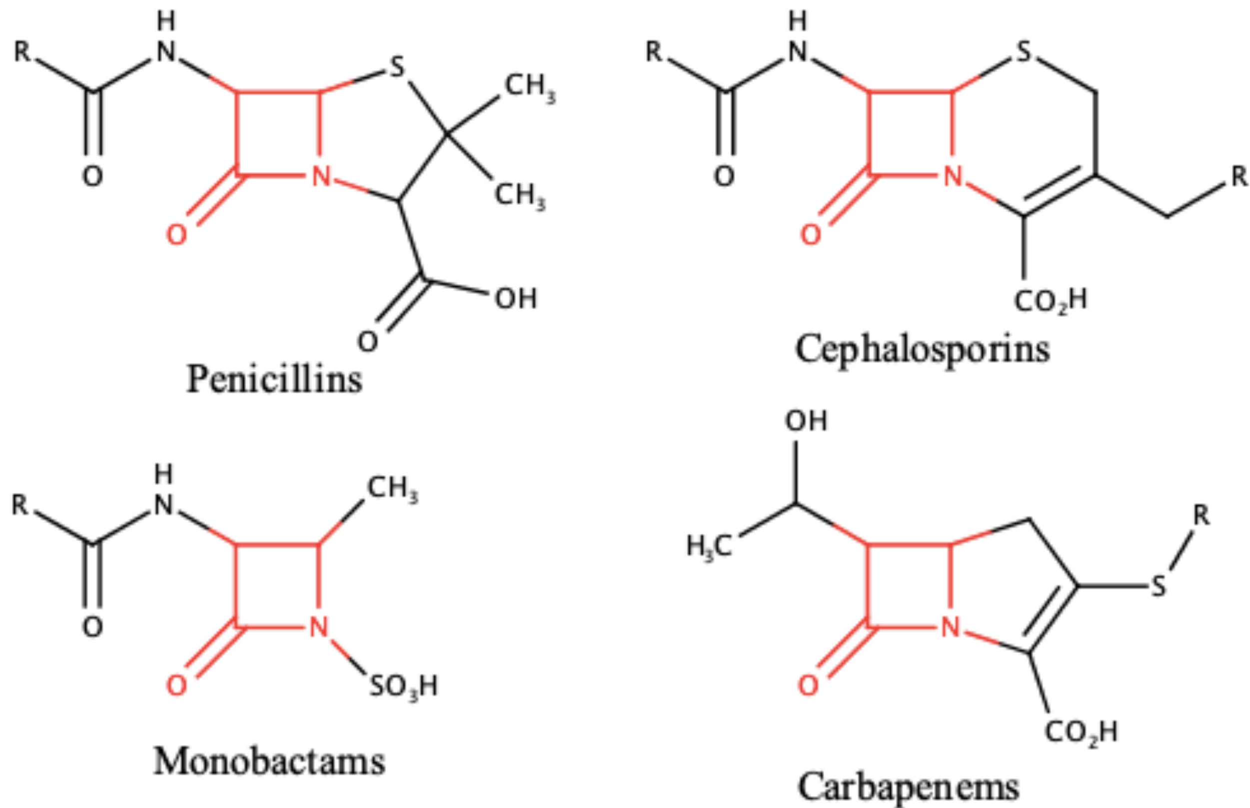


Figure 2. Structure of penicillins, cephalosporins, monobactams, and carbapenems; the β-lactam ring is highlighted in red in each structure

The mechanism of action of β-lactam antibiotics is for the β-lactam ring to covalently bind to and inhibit transpeptidases, carboxypeptidases, and endopeptidases, all essential enzymes in the terminal phases of peptidoglycan cell wall biosynthesis. Due to the affinity of the enzymes for β-lactam antibiotics, they are referred to as penicillin-binding protein (PBPs). PBPs catalyze the reaction that allows the cross-linking of peptidoglycan chains during cell wall synthesis, but this reaction will not occur in the presence of β-lactam antibiotics. With no cell wall present, the bacteria cannot grow or replicate, and are signaled for autolysis.[16]

β-lactamase

Resistance to β-lactam antibiotics is growing for several reasons - two main ones being over-prescribing by doctors and patients failing to follow proper prescription protocols, allowing bacteria to adapt to the drugs by propagation of the *amp*^{r} gene to survive and reproduce, spreading

the resistance.[17] The enzyme that gives resistance to β-lactam antibiotics is penicillinase, also known as β-lactamase. β-lactamase is a hydrolase enzyme that catalyzes the cleavage of the β-lactam ring in penicillins. Once the ring is cleaved, the antibiotic will no longer be able to bind to the peptidoglycan cell wall synthesis enzymes, rendering it ineffective.[16] Figure 3 below depicts the crystal structure of β-lactamase. The enzyme contains 263 amino acids and has a mass of 29 kDa. It contains 13 helices – four 3_{10} helices and nine α-helices – and nine β-strands, with two 3-strand β-sheets.[18]

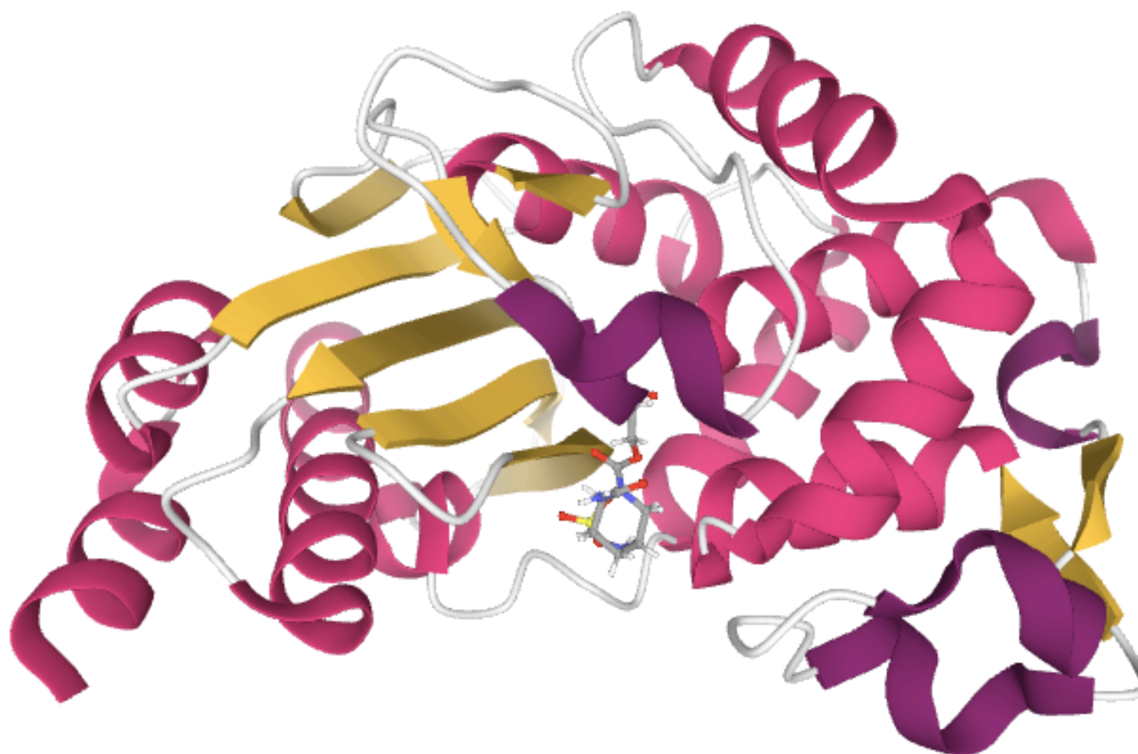


Figure 3. Class A β-lactamase structure from *Escherichia coli* complexed with a penicillin. PDB ID 5NE3 "L2 class A serine-beta-lactamase complexed with avibactam"[18]

There are four classes of β-lactamase enzymes based on their hydrolytic functionality – A, B, C, and D. Classes A, C, and D are serine-based enzymes that cleave peptide bonds in proteins using serine as the nucleophilic amino acid in the active site, whereas class B β-lactamases are metalloenzymes that use one or two zinc ions as catalysts. With greater than 550 known enzymes, class A β-lactamases are the most abundant of the four classes. Despite the differences in the

enzymes, all four classes are thought to be evolutionarily related, and they all catalyze the same reaction.[19,20]

Shortly after the introduction of β-lactam antibiotics, bacterial expression of β-lactamase became prominent. In 1941, almost all strains of *Staphylococcus aureus* were susceptible to penicillin, but many had become resistant by 1944. Currently, more than 95% of *Staphylococcus a*. strains are resistant to β-lactam antibiotics due to β-lactamase expression. β-lactamase is produced more abundantly in Gram-positive bacteria, but Gram-negative bacteria exhibit the enzyme expression as well. [21.] A major reason for the rapid, widespread expression of β-lactamase is that the gene encoding the enzyme can be found in the primary chromosomal DNA, or the secondary plasmid DNA.[22] Plasmid DNA is commonly transferred between bacteria, both intraspecifically and interspecifically, by bacterial conjugation, allowing the *amp^r* gene to be spread with little deterrence.[23]

β-lactamase is commonly used as a selectable marker in molecular biology, DNA recombination, and genetic engineering. Genes are amplified, digested, and ligated into vector plasmids to construct a recombinant plasmid to be transformed into bacterial cells. Because the enzyme allows for bacterial growth on selective media containing ampicillin antibiotic, it can be used to identify successful transformations and propagation of target genes for efficient DNA cloning.[24]

β-lactam antibiotics have numerous benefits. Due to this, much research has been done to find ways to deactivate β-lactamase for the continued use of antibiotics, in spite of increasing resistance. A popular method is the administration of cocktails containing β-lactamase inhibitors alongside β-lactam antibiotics. For example, Augmentin is a cocktail that contains amoxicillin and clavulanic acid. β-lactamase inhibitors are drugs that block the activity of β-lactamase, preventing

the degradation of β-lactam antibiotics. These β-lactamase inhibitors have very little antibiotic activity on their own but serve a supplemental purpose for antibiotics, allowing it to interact with target bacteria even if they express β-lactamase. The inhibitors contain a β-lactam ring which allows them to bind in the active site of the β-lactamase enzyme. Once bound, the inhibitors undergo a conformational change to create a more reactive species that attacks other amino acids in the active site, inactivating the β-lactamase enzyme. Common examples of β-lactamase inhibitors are clavulanic acid, sulbactam, and tazobactam. These molecules contain a β-lactam ring like those found in β-lactam antibiotics. β-lactamase binds to the inhibitor, leaving less enzyme available to catalyze the cleavage of the β-lactam ring in the antibiotics.[25] Figure 4 below depicts the structures of the inhibitors.

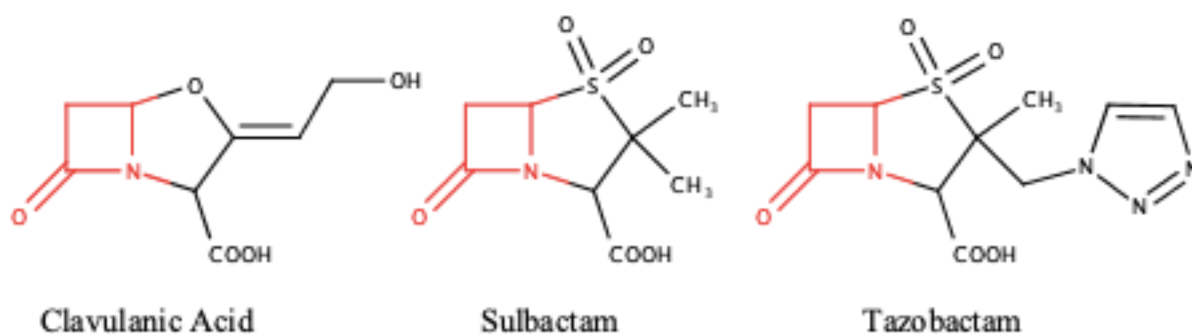


Figure 4. Structure of β-lactamase inhibitors clavulanic acid, sulbactam, and tazobactam; the β-lactam ring structure is highlighted in red

Green Fluorescent Protein (GFP) and Enhanced Green Fluorescent Protein (EGFP)

In the first century A.D., a Roman natural philosopher, Pliny the Elder, described the green light emitted by glowing jellyfish in the Mediterranean Sea. He noted that the slime could be removed from the jellyfish and the luminescence transferred to other objects.[26] It was not until 1962 that GFP was first discovered and isolated from the jellyfish, *Aequorea victoria*, by Shimomura, Johnson, and Saiga.[27] In the jellyfish *Aequorea v.*, GFP fluoresces when luminescent energy from the bioluminescence of aequorin, a blue luminescent protein, is transferred to it as a

result of Förster resonance energy transfer (FRET).[28] FRET is the transfer of energy between two chromophores – a donor in its excited electronic state and an acceptor – as a result of radiationless dipole-dipole coupling.[29] Figure 5 below depicts the jellyfish *Aequorea v.*

Figure 5. *Aequorea victoria* jellyfish with GFP green fluorescence[30]

GFP is used as a quantitative reporter protein, as its pH-dependent fluorescence can be measured using a spectrofluorometer. GFP has stable fluorescence from pH 6 to 10, but fluorescence decreases at pH < 5 and increases at pH > 10.[31] It is often used as the reporter protein of choice over other luminescent reporter proteins (such as aequorin and luciferase), because it is stable at biological pHs and is autofluorescent. The autofluorescence is the result of three amino acid residues – Ser[65], Tyr[66], and Gly[67] – in the central α-helix that form the chromophore region of GFP.[32] GFP has two excitation peaks – a λ_{max} at 395 nm and a second at 475 nm – and an emission peak at 509 nm, giving the protein its green fluorescence.[33] The utilization of GFP is also advantageous in that it retains its fluorescence properties when expressed in a species from which it did not originate.[34] Due to their stabilities at normally

denaturing conditions, such as basic pH, high salinity, and in the presence of organic solvents and detergents, GFP and its variants have been expressed in many species, including bacteria, plants, and animals. Figures 6 and 7 below depicts examples of GFP expression in various organisms.

Figure 6. Transgenic rhesus monkeys expressing EGFP under ambient light and under UV light[35]

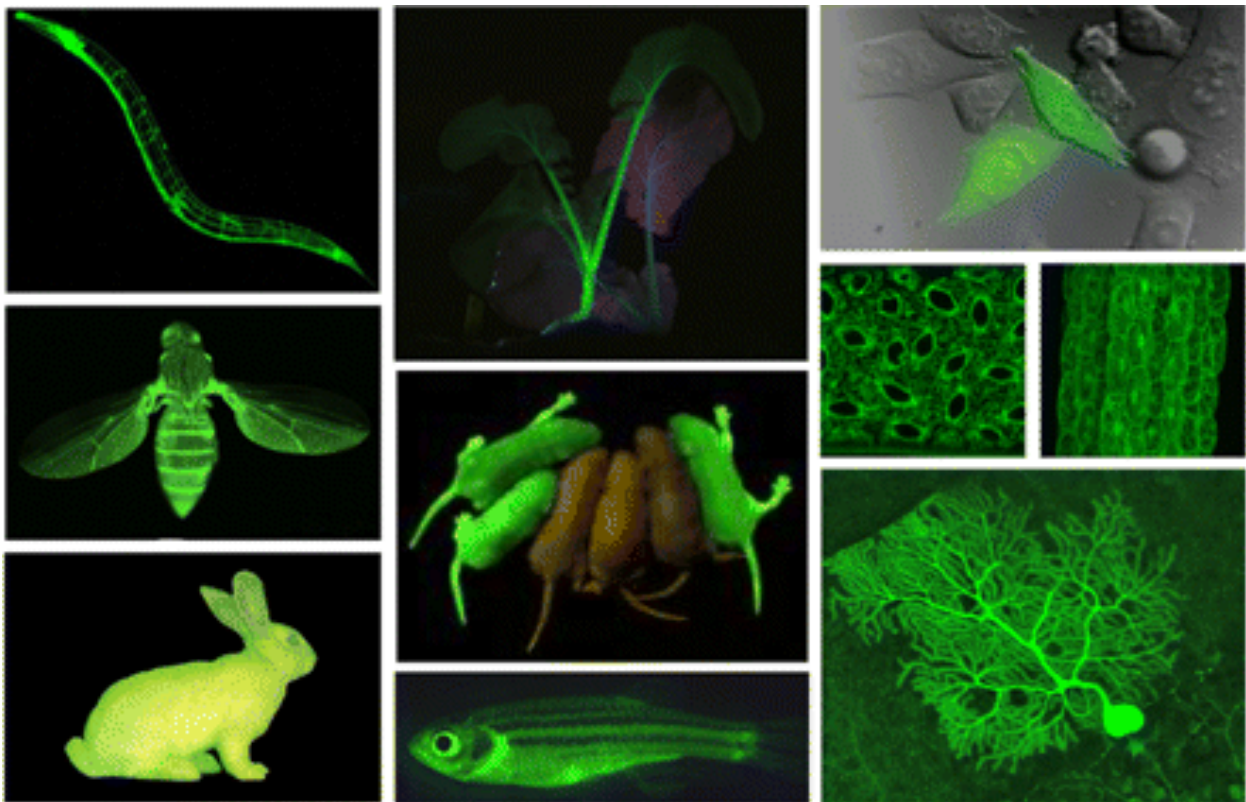


Figure 7. Transgenic animals, plants, and cells expressing GFP under UV light[36]

GFP has many applications as a reporter protein. It does not lose its fluorescence when fused to another protein, making it very useful to monitor localization of proteins, and it can be

used for non-invasive measurements of gene expression, protein localization, intracellular protein targeting, and pH by observing its fluorescence.[37,38] This has allowed for GFP to be used in the study of gene transfer in human stem cells and bacterial transformations.[39] Sensing systems using GFP and GFP derivatives have been used to detect lysine content, calcium binding proteins, L-arabinose, copper, lead, and cadmium, highlighting the diverse applications of GFP as a reporter.[34,40,41]

GFP has a size of 26.9 kDa and is comprised of 238 amino acid residues.[42] The protein contains 11 β-sheets arranged in a β-barrel formation with a diameter of 24 to 30 Å.[32,43,44] The β-barrel is almost perfectly symmetrical, with a flaw in the seventh and eighth strands that is believed to be necessary for proper protein folding since the β-barrel forms before the chromophore region folds into its active form.[45] The chromophore region consists of Ser^{65}, Tyr^{66}, and Gly^{67} residues in the central α-helix of the protein.[32] The β-barrel structure protects the chromophore region, which gives it stability in the denaturing conditions previously described.[45] Figure 8 below depicts the structure of GFP.

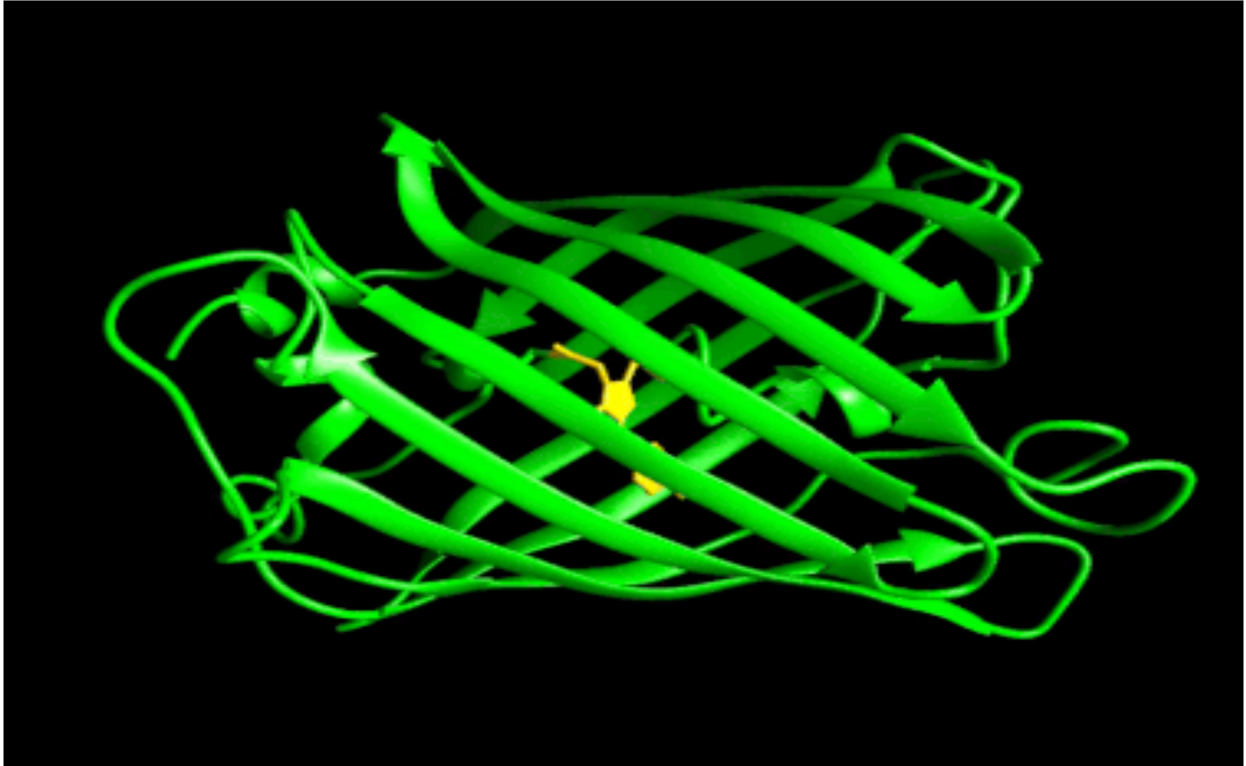


Figure 8. Ribbon structure of GFP with the fluorescent chromophore highlighted in yellow. PDB ID 1EMA "GREEN FLUORESCENT PROTEIN FROM AEQUOREA VICTORIA"[45]

Wild type GFP (wtGFP), while useful, could be improved upon. It has a low brightness, a delay between protein synthesis and fluorescence emergence, and complex photoisomerization.[46] Due to this, variations of GFP were developed for use in research. Examples are enhanced green fluorescent protein (EGFP), enhanced blue fluorescent protein (EBFP), blue fluorescent protein 2 (BFP2), enhanced yellow fluorescent protein (EYFP), enhanced cyan fluorescent protein (ECFP)), and GFPuv (18 times brighter than wtGFP).[47] The various spectral properties of the variants mean they have their own unique signatures, their emission wavelengths. This is useful to tag different proteins with the variants and allows for simultaneous detection of multiple proteins. Like GFP, each of the variant's fluorescence is also pH dependent.

EGFP, a red-shifted variant of GFP mentioned above, is used in this research. EGFP has two key mutations that shift its excitation maximum from 395 nm to 488 nm and increases its

fluorescence intensity. EGFP fluoresces 35 times brighter than GFP when excited at 488 nm, making it much more desirable than GFP for laboratory studies.[39] The increase in fluorescence intensity is the result of an increase in extinction coefficient caused by the two mutations. The mutations are F64L just outside the chromophore region and S65T just within the chromophore region. The mutations make it more useful in lab work as the argon laser, which emits light at 488 nm, is one of the most common instrumental lasers. The 488 nm wavelength is also very common in filter sets for many analytical instruments.[47]

The fluorescence of EGFP can also be used to quantitatively measure gene expression in specifically designed systems due to its pH-dependent fluorescence. This is the result of the protonation state of a hydroxyl group in the chromophore region of EGFP. When deprotonated at a high pH, there is a high level of fluorescence due to a greater abundance of electrons in the system. When it is protonated at a low pH, there is a low level of fluorescence due to a lower abundance of electrons in the system.[48] EGFP is also beneficial in that the mutations optimize the protein for expression in mammalian cells.[47] Over the years, EGFP and other GFP variants have been expressed in normal lab animals, such as mice and monkeys, and even in household animals, such as cats and dogs. Figure 9 below depicts the pH-dependent fluorescence of EGFP.

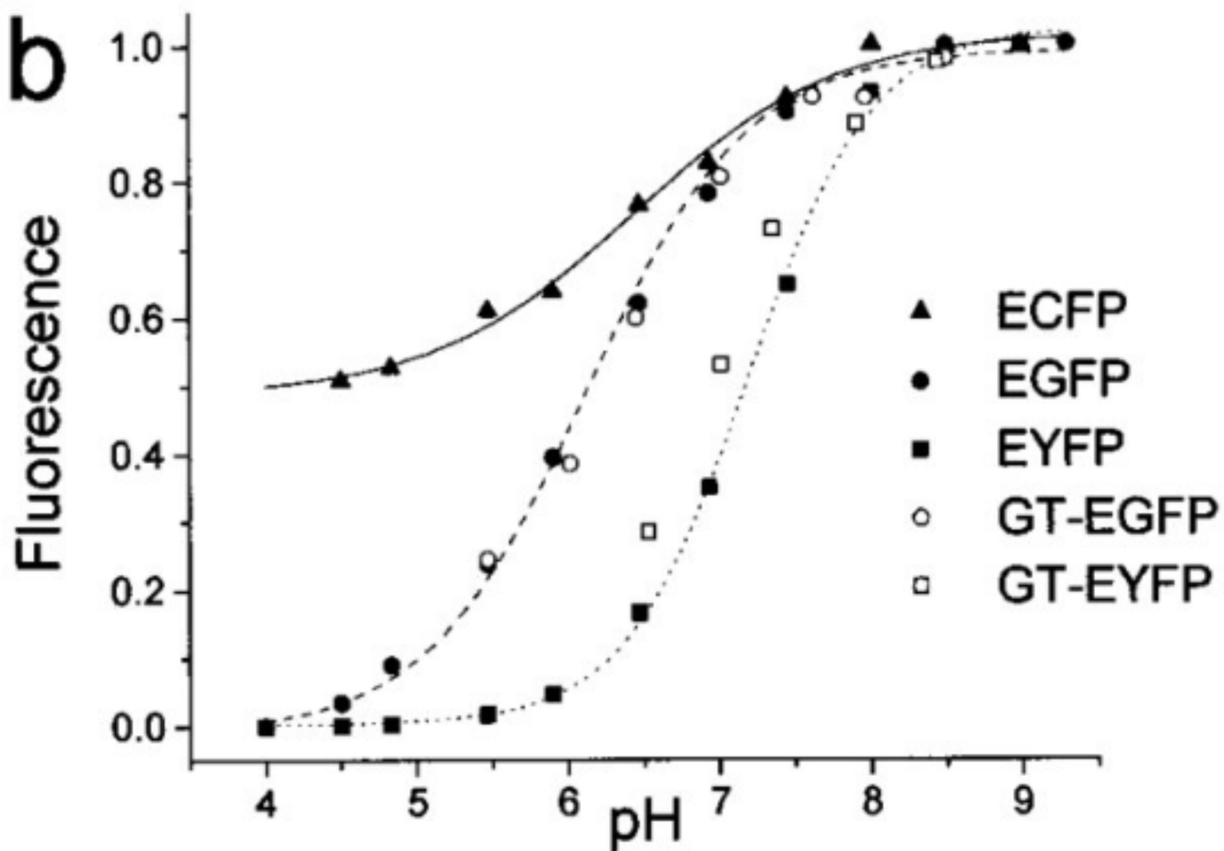


Figure 9. The pH-dependent fluorescence of GFP variants[49]

EGFP fluorescence is dependent on the concentration of EGFP as well. The concentration and fluorescence have a linear relationship up to an absorbance of 0.05, but the linearity is then lost. However, the relationship still follows a predictable, logarithmic relationship after. Figure 10 below depicts the linear and logarithmic relationship.

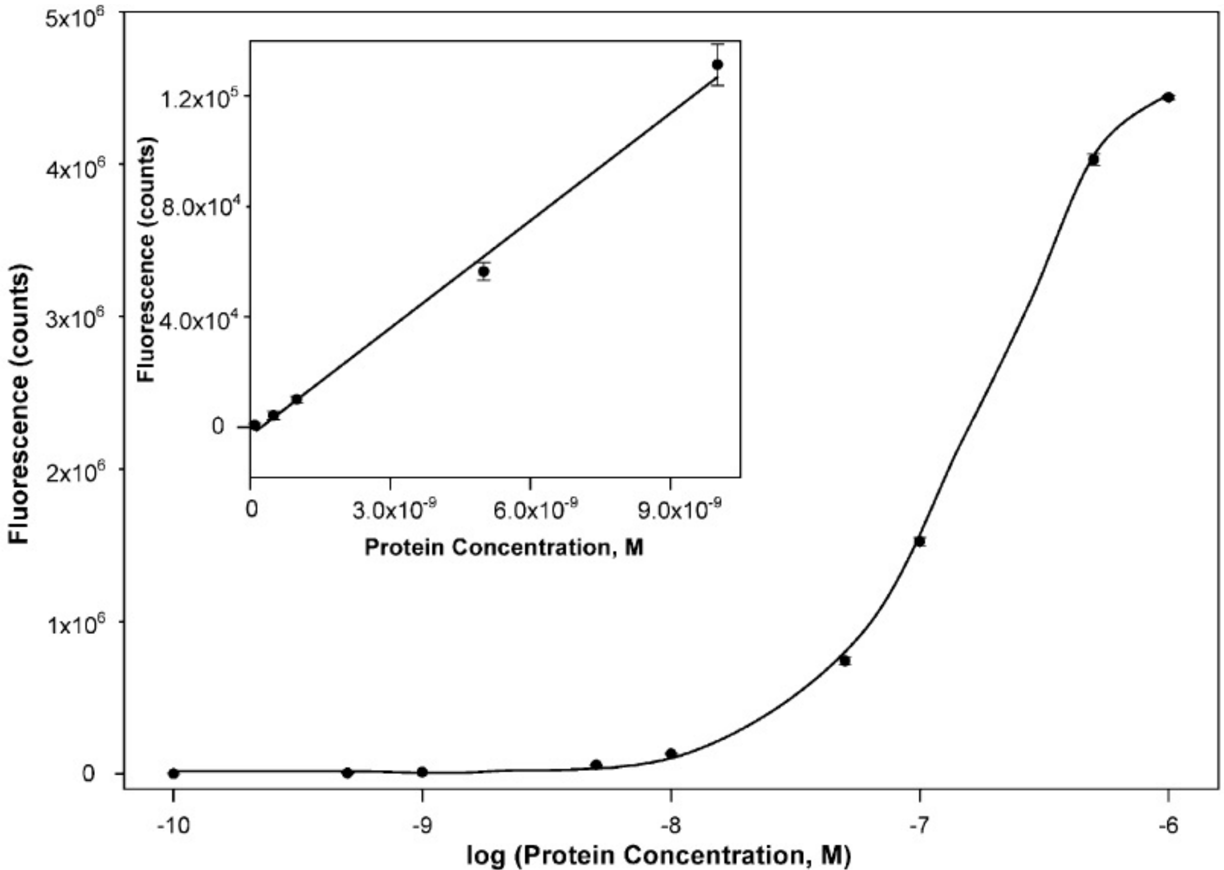


Figure 10. Relationship of fluorescence vs. concentration of EGFP-β-lactamase fusion protein[50]

Principle of the Assays

The pH theory states that for a sensing system to be effective, the genes for β-lactamase and EGFP must be directly fused together so that they are in close enough proximity for the decrease in local pH to impact the fluorescence of the EGFP domain. An *in vitro* construct of β-lactamase will be recreated. There is an expected decrease in fluorescence over time as β-lactamase cleaves the β-lactam rings of ampicillin, leading to a local pH around the fusion protein. The genes will be isolated and expressed separately. The lack of close-proximity will result in β-lactamase activity having no effect on the fluorescence of EGFP, because the local pH around β-lactamase is decreasing, but the bulk pH remains unchanged and EGFP is not close enough to β-lactamase for the local pH change to affect it. An *in vivo* construct of β-lactamase and EGFP will be constructed to be expressed in a whole-cell sensing system. Like the *in vitro*

fusion protein, there is an expected decrease in fluorescence over time as a result of the direct proximity of the β-lactamase and EGFP domains of the fusion protein. Figure 11 below depicts a summary of the principle of the assays.

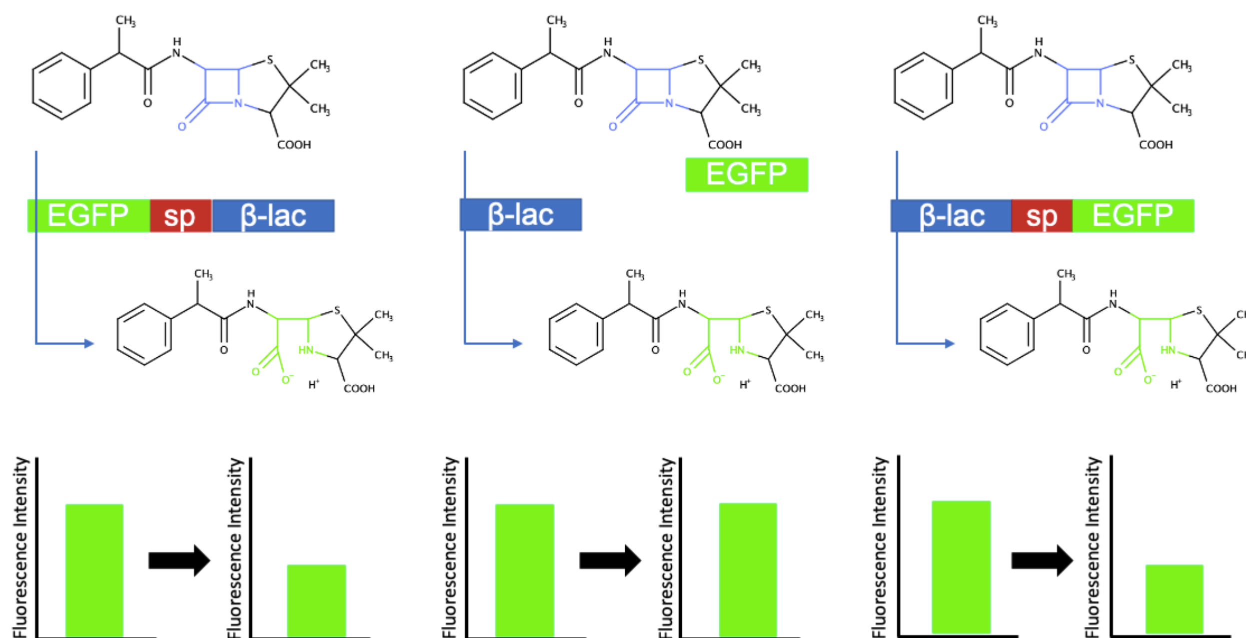


Figure 11. Summary of the principle of the *in vitro*, individual, and *in vivo* assays.

In Vitro Fusion Protein

In this portion of the project, the original *in vitro* fusion protein, created by Puckett et. al., will be recreated. pEGFP, the source plasmid for β-lactamase and EGFP genes, is isolated from stock cultures, and the genes for β-lactamase and EGFP are selectively amplified using polymerase chain reaction (PCR). Rather than just amplifying the genes alone, a tail containing a restriction site will be placed on one end of the sequences for β-lactamase and EGFP, and a spacer region will be placed at the other ends of the genes, depicted in Figure 12 below. Following the first round of PCR, a second, overlap extension PCR will be run to fuse the two genes into one, using the spacer region to facilitate the fusion. For the *in vitro* fusion protein, the EGFP gene will be placed before the β-lactamase gene. After being fused together, the fusion gene will be digested using *Hin*dIII and *Eco*RI restriction enzymes and ligated into a pFLAG-MAC expression vector for

transformation into *E. coli* cells. Figure 12 below depicts a summary of the *in vitro* fusion portion of the project.

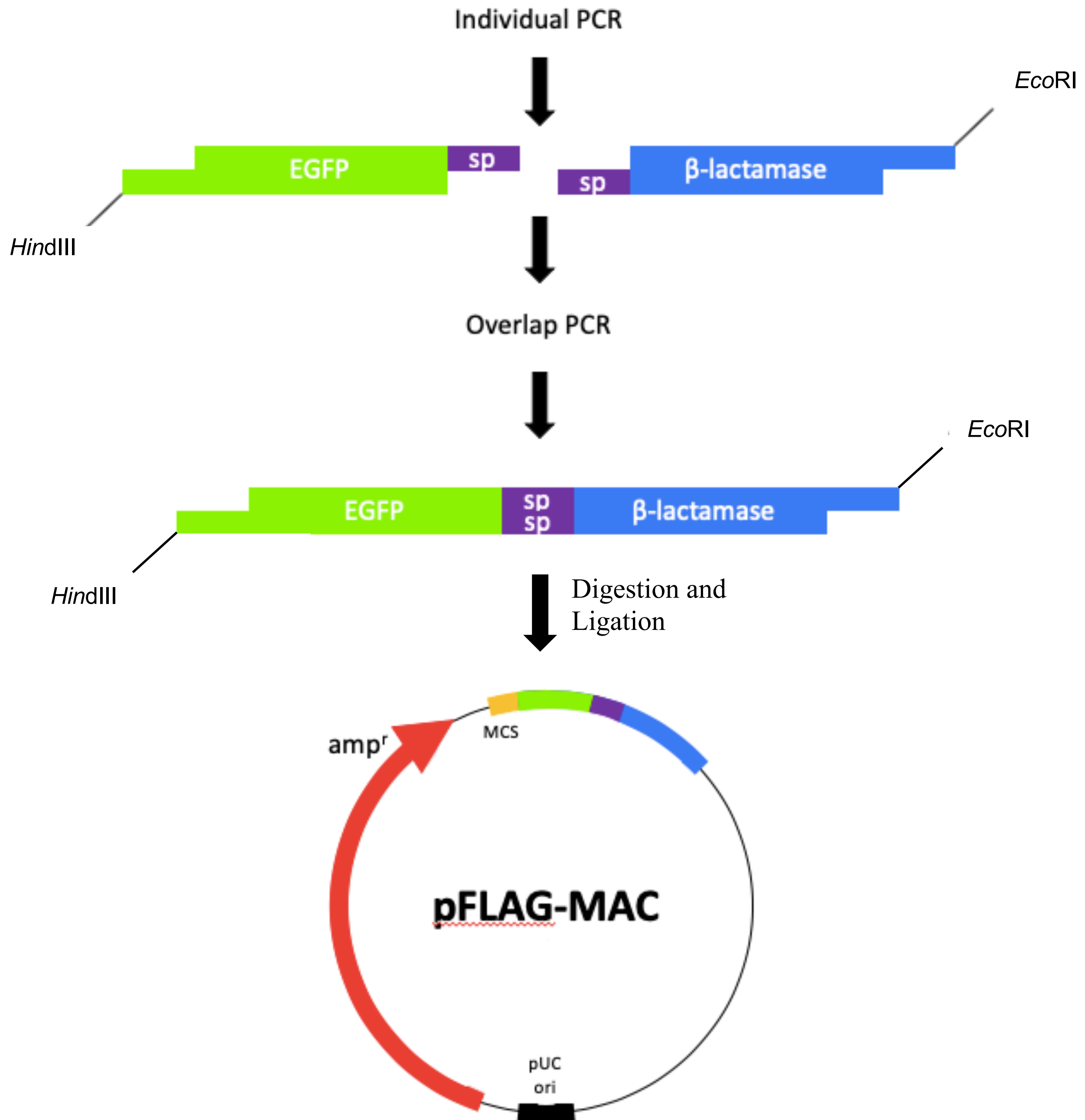


Figure 12. Amplification, digestion, and ligation of *in vitro* fusion protein into pFLAG-MAC vector plasmid

Validation of the pH Theory

In this portion of the project, the genes for β-lactamase and EGFP are isolated and amplified individually. Rather than fusing the two together, they are digested using *Hin*dIII and *Eco*RI restriction enzymes and ligated into separate pFLAG-MAC expression vectors to create two new plasmids -- pLP003 and pLP004 -- for transformation into *Escherichia coli* to validate the local pH theory. Figure 13 below depicts a summary of the digestion and ligation of the individual genes into the pFLAG-MAC expression vectors.

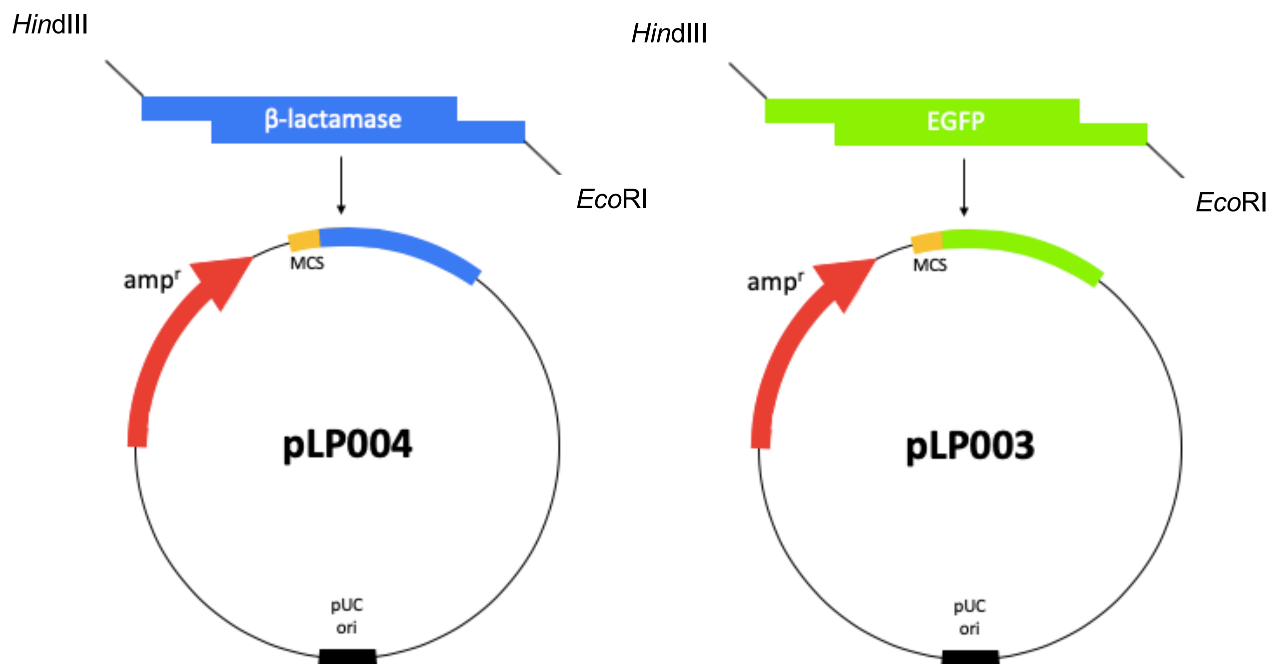


Figure 13. Digestion and ligation of β-lactamase and EGFP, individually, into pFLAG-MAC for validation assay

For the first two portions of the project -- the individual portion and the *in vitro* portion -- the vector plasmid pFLAG-MAC was chosen. The presence of a FLAG affinity octapeptide tag allows the use of anti-FLAG affinity gels to aid in purification of the proteins from cellular material.

In Vivo Fusion Protein

The molecular biology techniques used in the creation of the *in vivo* fusion protein portion of the project are very similar to those used in the *in vitro* portion. There are three primary differences; the order of the genes, the expression vector used, and the restriction enzymes used during the digestion.

During the *in vitro* portion of the project, the EGFP gene was inserted upstream of the β-lactamase gene. Because the fluorescence of EGFP domain of the fusion protein was studied *in vitro*, the order of the genes did not matter. However, in the *in vivo* portion of the project, the fusion protein must be expressed in the periplasm of the cell so that interactions with antibiotics crossing the cell membrane will occur. The gene for β-lactamase contains a signal peptide that directs the protein to the periplasm, so the β-lactamase gene must be upstream of the EGFP gene. For that reason, the order of the genes becomes extremely important. Figure 14 below depicts the order of the genes in the *in vitro* and *in vivo* portions of the project.

Figure 14. *In vitro* and *in vivo* fusion genes with signal peptide present on β-lactamase gene for *in vivo* fusion gene

For the *in vitro* portion of the project, pFLAG-MAC was being used as the expression vector because pFLAG-MAC contains the *amp^r* gene. For the *in vivo* portion, an expression vector had to be used that did not encode for ampicillin resistance, because the fusion gene should contain the only hydrolytic activity for β-lactamase. pET28b was chosen as the new expression vector, because it encodes for kanamycin resistance, allowing the confirmation of a successful ligation and transformation if the transformed *E. coli* cells were able to grow in the presence of kanamycin. The ampicillin resistance can be identified as a property of the insert, and not the expression vector.

Figure 15 below depicts the recombinant pET28b vector plasmid with the *in vivo* fusion protein ligated in.

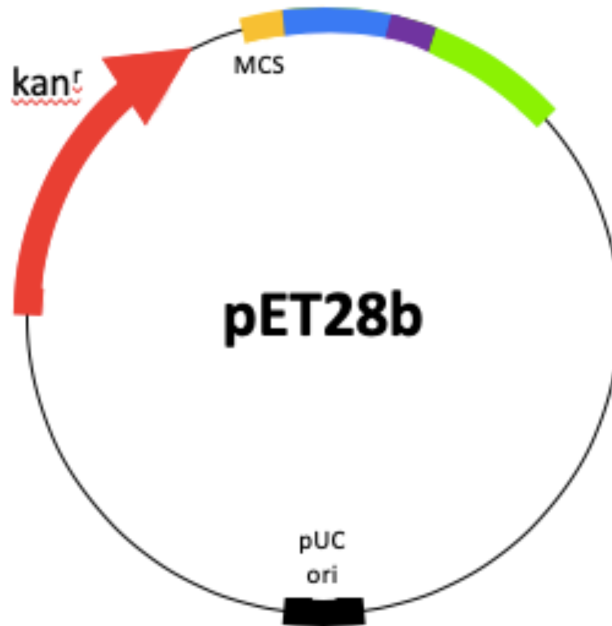


Figure 15. pET28b plasmid with *in vivo* fusion gene inserted

During the *in vitro* portion of the project, the restriction enzymes used were *Eco*RI and *Hin*dIII. For the *in vivo* portion of the project, the restriction enzymes used were *Hin*dIII and *Xho*I. The purpose of using *Xho*I, as opposed to *Eco*RI, during the *in vivo* portion is that in the pET28b vector plasmid, the *Hin*dIII restriction site falls before the *Xho*I restriction site. *Eco*RI was not used because it falls before the *Hin*dIII restriction site. It made it easier to keep things organized between the *in vitro* and *in vivo* portions to use a different enzyme set. Figure 16 below depicts the *in vivo* fusion gene with the *Hin*dIII and *Xho*I restriction sites attached to tails of the gene.

Figure 16. *In vivo* fusion gene with *Hin*dIII and *Xho*I restriction sites attached

The *in vivo* fusion gene above will be ligated into the pET28b vector plasmid. Restriction enzymes in will be used to digest the plasmid and the *in vivo* fusion gene to generate "sticky-ends" that will allow the fusion gene to be inserted into the vector plasmid. In pET28b, the restriction site for *Hin*dIII falls upstream of *Xho*I; β-lactamase will be transcribed prior to EGFP. Once translated, the signal peptide will direct the protein to the periplasm of the cell, where the expressed fusion protein can react with ampicillin that crosses the cell membrane. The local pH will decrease as a proton is released with each cleavage event. EGFP is a pH-dependent fluorescent protein, so as β-lactamase activity increases, the drop in local pH will lead to decreased fluorescence. Figure 17 below depicts a representation of the of the protein being directed to the periplasm and the fluorescence over time expected as the fusion protein is active.

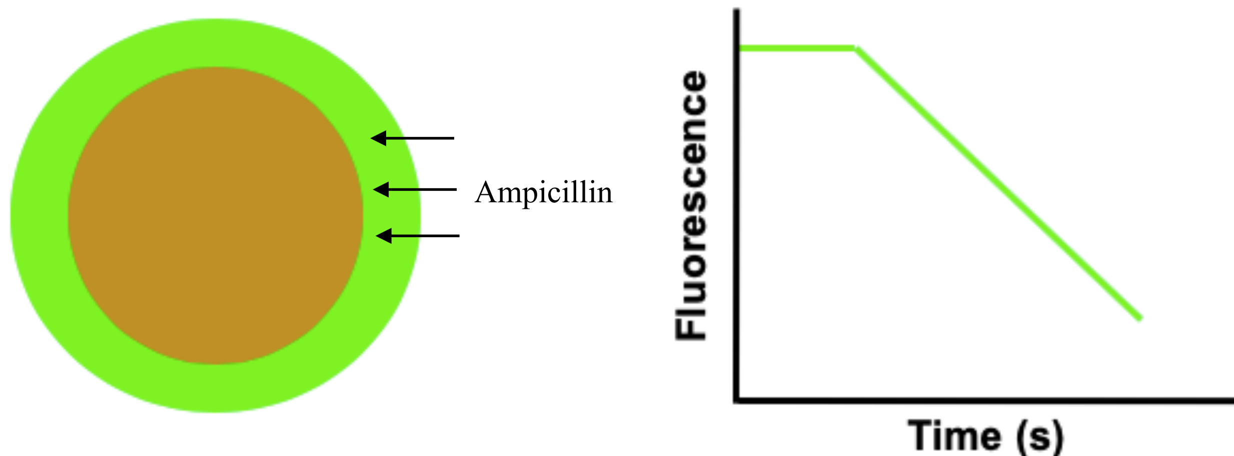


Figure 17. Representation of fusion protein in the periplasm of cell and expected change in fluorescence over time of fusion protein activity

Previous Work on this Project

Prior to the start of this research, work had already been completed on the project. A major accomplishment was the creation of the EGFP-β-lactamase fusion protein to be used for *in vitro* fluorescence studies. Figure 18 below depicts the gene, cloned in the vector plasmid pFLAG-MAC. Using this fusion protein, it was verified that the hydrolysis of β-lactam antibiotics by β-lactamase can be monitored over time using the pH-dependent fluorescence of EGFP.[50]

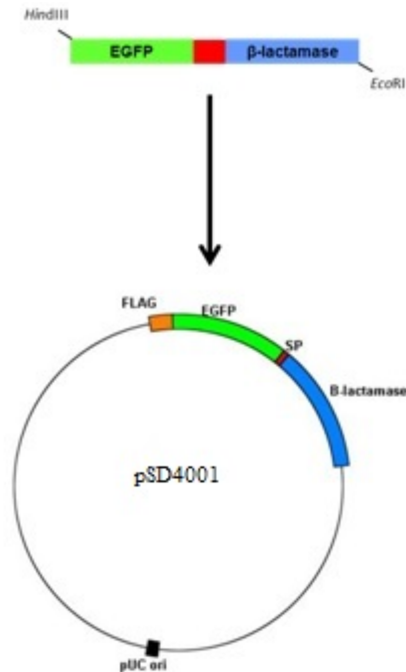


Figure 18. Plasmid pSD4001 with expression of EGFP-β-lactamase fusion gene[50]

This plasmid has been transformed into DH5α competent *E. coli* cells, protein expression induced with isopropyl β-D-1-thiogalactopyranoside (IPTG), and the protein purified with anti-FLAG affinity gel. *In vitro* studies were completed using this protein. The EGFP domain of the fusion protein was demonstrated to retain the pH-dependent fluorescence when fused to β-lactamase. Figure 19 below depicts the pH-dependent fluorescence of the *in vitro* fusion protein.

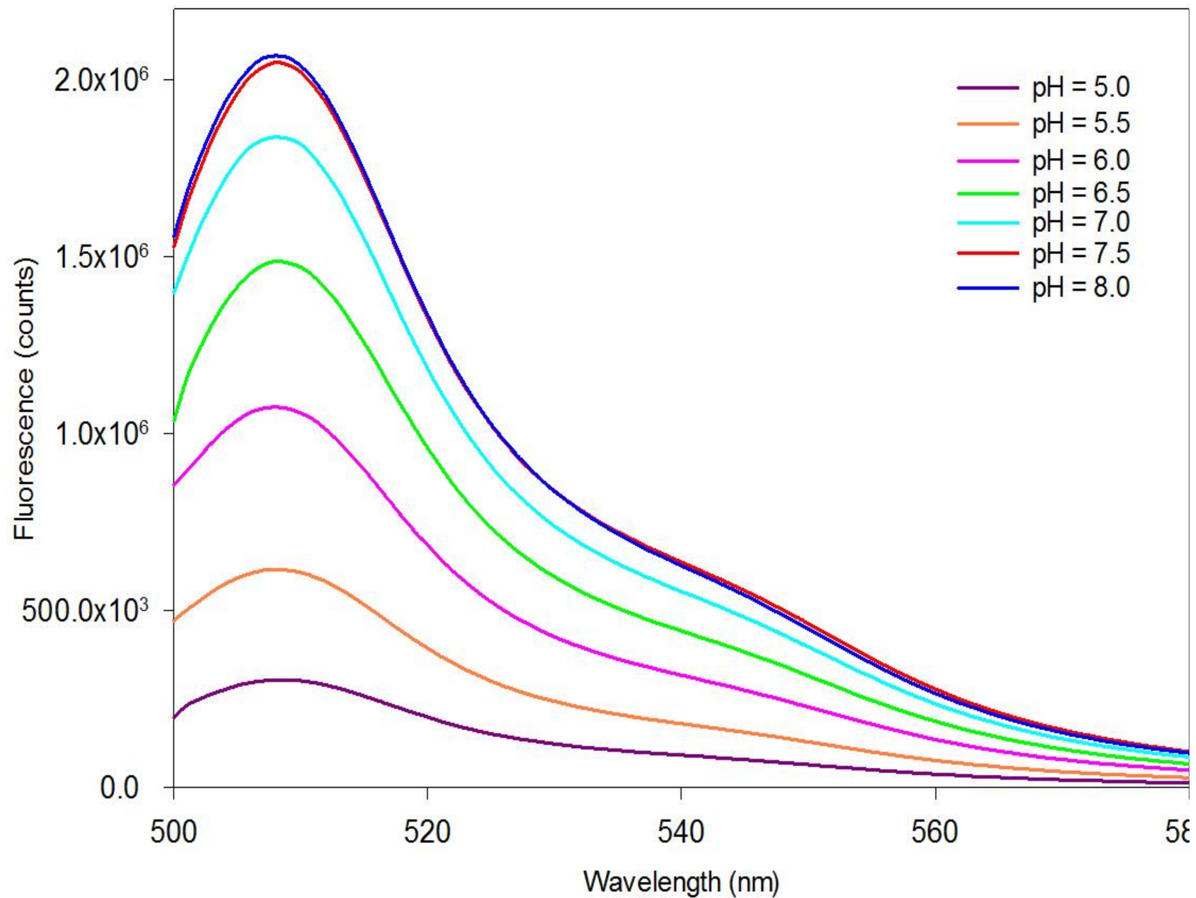


Figure 19. pH-dependent fluorescence of *in vitro* fusion protein; decreased pH leads to decreased fluorescence

An important study that was performed looked at the protein's response to ampicillin (a β-lactam antibiotic); as the ampicillin interacted with the β-lactamase domain of the fusion protein, the β-lactam ring was cleaved, releasing a proton, lowering the local pH of the system. The EGFP domain of the fusion protein responded to the lowered pH with a decrease in the intensity of the fluorescence, this interaction is illustrated in the data collected by Puckett et. al.[50] Figure 20 below depicts the decrease in fluorescence in response to the decrease in pH.

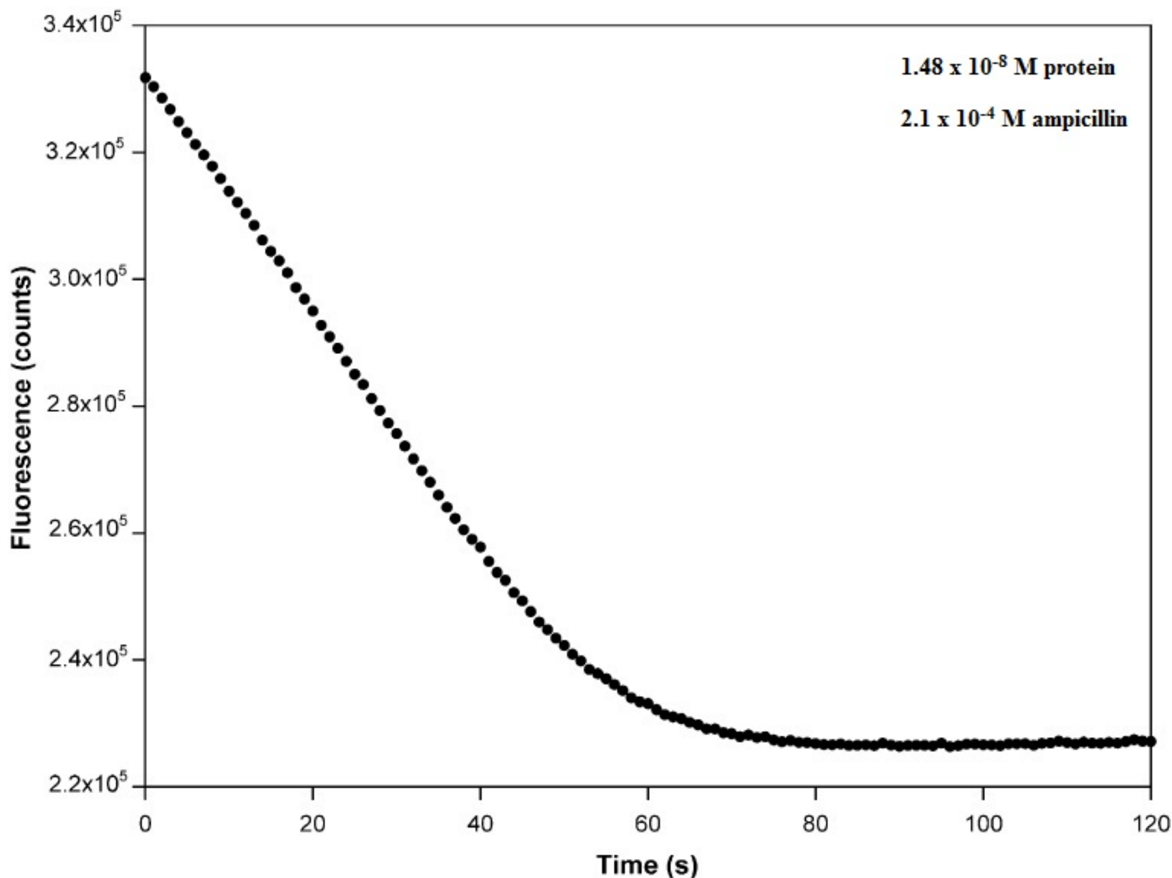


Figure 20. Change in fluorescence over time as a result of fusion protein-ampicillin interaction[50]

A portion of this research is seeking to replicate this data in order to compare results from utilizing the individual proteins. The separate proteins will be added together at the same concentration of the fusion protein. It is hypothesized that the two proteins must be in close proximity (fused together) in order to monitor the local pH change that occurs with β-lactam hydrolysis.

Aim of Project

The *in vitro* EGFP-β-lactamase fusion protein will be recreated to confirm previous findings and to validate the pH theory. An *in vivo* β-lactamase-EGFP fusion protein will be constructed to be used in a whole-cell sensing system.

After the β-lactamase-EGFP *in vivo* fusion gene has been successfully cloned and transformed into DH5α competent *E. coli* cells, protein expression will be induced using IPTG, in

the same way that previous work did with the *in vitro* fusion protein. Whole-cell, *in vivo* fluorescence studies will be performed in the presence of ampicillin. The data gathered should be similar to the data gathered in the *in vitro* studies; a decrease in fluorescence over time. The expected difference in the *in vitro* and *in vivo* data is that the *in vivo* data will contain a lag before the decrease in fluorescence is observed since the antibiotic must cross the cell membrane – thus allowing for bioavailability studies.

For the *in vivo* portion of the project, it was not necessary to have a FLAG affinity tag to aid in purifying the protein from cellular material, because the fluorescence studies will be performed *in vivo*. The protein will remain in the cell and its antibiotic-resistant/fluorescence activity will be directly observed. The *in vivo* fusion protein will have the potential to be used in whole-cell bioavailability assays, indirectly measuring the activity of β-lactamase in response to treatment with β-lactam antibiotics, as well as studying inhibitors, by observing the change in fluorescence of the EGFP domain of the protein.

Methods and Materials

Apparati

Bacterial overnight cultures were prepared on a VWR shaker table and incubator (Cornelius, OR). Polymerase chain reaction (PCR) was performed in an Eppendorf Mastercycler Personal Thermocycler (Hamburg, Germany). PCR optimization was performed in an Applied Biosystems Veriti 96 Well Plate Thermal Cycler from ThermoFisher Scientific (Asheville, NC). Gel electrophoresis was run in an IBI Quickscreen QS-710 (New Haven, CT) connected to a Thermo EC105 power supply (Asheville, NC). Gels were visualized using a VWR UVP UV transilluminator (Cornelius, OR). Enzyme digestions were performed in a VWR 1208 water bath (Cornelius, OR). Bacterial plates were grown in a VWR Shel Lab 1500E incubator (Cornelius, OR). Concentrations were determined using a ThermoScientific NanoDrop 2000C Spectrophotometer (Asheville, NC). Also used throughout the experiment were a Tuttnauer 3870ELV autoclave (Shemesh, Israel), an Eppendorf 5424C centrifuge (Westbury, NY), a Mettler Toledo AL 54 analytical balance (Columbus, OH), and a ThermoScientific -80°C freezer (Asheville, NC). In addition to the instruments, Eppendorf 1.5 mL microcentrifuge tubes (Westbury, NY), Fisher Scientific Fisherbrand petri dishes (Pittsburgh, PA) and 14 mL Becton Dickinson Falcon tubes (Franklin Lakes, NJ) were also used.

Reagents:

Luria Bertani (LB) broth and LB agar were purchased from Difco (Lawrence, KS). PCR MasterMix was purchased from ThermoScientific (Asheville, NC). *Hind*III, *Eco*RI, digestion buffer H, digestion buffer M, loading dye, DH5α cells, pUC19 DNA, SOC media, DNA ligase, ligase buffer, agarose, and DNA ladder were purchased from Invitrogen (Carlsbad, LA). RedSafe^{TM} nucleic acid staining solution was purchased from VWR (Cornelius, OR). Ethidium

bromide, ampicillin sodium salt, and kanamycin sulfate were purchased from Sigma Aldrich (St. Louis, MO). Primers were ordered from Eurofins MWG Operon (Huntsville, AL). The pEGFP vector was purchased from Clontech (Mountain View, CA), the pFLAG-MAC vector was purchased from IBI Kodak (New Haven, CT), and the pET28b vector was donated by Ece Karatan (Appalachian State University). Tris-acetate-ethylenediaminetetraacetic acid buffer (TAE buffer) (made one liter at 25x concentration, then diluted to 1x with DI water) was mixed in house with tris base (121.0 g) and glacial acetic acid (28.6 mL) from EM Science (Gibbstown, NJ) as well as EDTA (18.6 g Na_2EDTA) from EMD Chemicals (Gibbstown, NJ). Miniprep, gel extraction, and PCR purification kits were purchased from Qiagen (Valencia, CA). Sterile techniques were used.

LB Broth and Agar Plates:

LB broth was prepared by adding 8 g of LB broth powder to 400 mL of DI water in a glass storage bottle. The broth solution was autoclaved. After autoclaving, 20 mg of kanamycin sulfate was added for a concentration of 50 μg/mL for LB-kan. For LB-amp, 40 mg of ampicillin sodium salt was added for a concentration of 100 μg/mL.

LB plates were prepared by adding 14 g of LB agar powder to 400 mL of DI water in a glass storage bottle. The agar solution was autoclaved and left to cool to less than 50°C. After cooling, 20 mg of kanamycin sulfate was added for a concentration of 50 μg/mL for the plates for pET28b colonies. For plates for pEGFP colonies, 40 mg of ampicillin sodium salt was added for a concentration of 100 μg/mL. The agar solution was poured into petri dishes to about two-thirds full, allowed to cool and solidify, and stored upside down in the refrigerator.

Bacterial Cultures and MiniPrep:

Bacterial cultures of pEGFP were prepared and grown by adding a toothpick scraped across *E. coli* transformed with the source DNA from stock culture from the -80°C freezer. Bacterial

cultures of pET28b were prepared and grown by adding a toothpick scraped across the source DNA from plates of pET28b and pEGFP to a 15-mL Falcon tube with 3 mL of LB-kanamycin broth for pET28b or LB-ampicillin broth for pEGFP. The cultures were incubated for 16 hours overnight on the shaker table at 37°C and 200 rpm.

The DNA was isolated from the bacterial cells using the QIAprep Spin Miniprep Kit according to the Qiagen protocol. First, the bacterial cultures were transferred into a 1.5-mL Eppendorf microcentrifuge tube, centrifuged at 13,000 rpm for 1 minute, and decanted until only the pellet remained. This was performed for each bacterial culture until none remained in the Falcon tube. The remaining pellet was resuspended in 250 μL of Buffer P1 by vortexing. Once the pellet was resuspended 250 μL of Buffer P2 were added and the mixture was inverted 6 times. Then, 350 μL of Buffer N3 were added and the mixture was inverted 6 times again. The tube was centrifuged at 13,000 rpm for 10 minutes. After centrifugation the supernatant was transferred into a QIAprep spin column and centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded. The spin column was washed with 750 μL of Buffer PE and centrifuged at 13,000 rpm for 1 minute. The spin column was removed from the tube, the bottom of the column was wiped with a Kimwipe. To elute the DNA from the column 30 μL of Buffer EB were added and left to sit for 1 minute. The column was centrifuged at 13,000 rpm for 1 minute. The spin column was discarded and the flow-through containing the isolated DNA was stored in the 4°C refrigerator for later use.

Polymerase Chain Reaction and Gel Electrophoresis

After isolation of plasmids using MiniPrep, the genes for EGFP and β-lactamase each had to be isolated from the pEGFP plasmid and amplified using polymerase chain reactions (PCR). The PCR reaction mixtures contained 21 μL of sterile water, 2 μL of DNA, 1 μL of reverse primer,

1 μL of forward primer, and 25 μL of PCR MasterMix. Table 1 below depicts the primers used for the individual gene isolation in the validation of the pH theory portion of the project.

Table 1. Primers used in PCR mixture for individual gene isolation in the validation of the pH theory portion of the project

β-lactamase	Forward Primer	5’ - ACCATCGCAGTAAAGCTTCAC CCAGAAACGCTGGTGAAAGTA - 3’
	Reverse Primer	5’ - ACCGCCGCAGTGAATTCATTA CCAATGCTTAATCAGTGAGGC - 3’
EGFP	Forward Primer	5’ - ACCTACGCAGTTAAGCTTGT GAGCAAGGGCGAGGAGCTG - 3’
	Reverse Primer	5’ - TACACCGCAGTGAATTCAT TACTTGTACAGCTCGTCCAT - 3’

The underlined sequence of each primer is the recognition sequence for the restriction enzymes. Both forward primers contain the sequence for *Hin*dIII and both reverse primers contained the sequence for *Eco*RI. These restriction sites were chosen, because they are unique to the MCS of the pFLAG-MAC vector. Table 2 below depicts the PCR program parameters.

Table 2. PCR parameters for gene isolation from pEGFP

Stage	Temperature	Time
Denaturing	94°C	1 Minute
Annealing	50°C	1 Minute
Extending	72°C	2 Minutes
Number of Cycles	50	

After PCR, the DNA was separated using gel electrophoresis. The gel was made by dissolving 0.5 g agarose in 50 mL 1x TAE buffer. The 1x TAE buffer was made by diluting 40 mL 25x TAE buffer (121.0 g Tris base, 28.6 mL glacial acetic acid, and 18.6 g Na_2EDTA, diluted to 1 L with deionized water (DI)) to 1 L. To the solution, 2 μL of RedSafe^{TM} nucleic acid staining

solution, or 3 µL of ethidium bromide, were added. The solution was poured into a gel casting tray and allowed to cool. After the gel solidified, the products from the PCR were mixed with 5 µL loading buffer and placed in the wells of the gel in 25 µL aliquots. Five microliters of 1 kb ladder was added to a single well to use as a reference. The gel was run in 1x TAE buffer at 100 V for 40 minutes. When finished running, the gel was transferred to a UV transilluminator to view the bands. Both EGFP and β-lactamase bands appear around 800 base pairs. The desired bands were excised, placed in 1.5 mL Eppendorf tubes, and stored in the refrigerator for DNA extraction from the gel.

In order to extract the DNA from the gel, the bands were processed using a gel extraction kit from Qiagen. First, Buffer QG was added at a ratio of 3:1 buffer to gel volume. The tube was held in hand until the gel melted, with intermittent vortexing. The solution was transferred to a spin column and centrifuged at 13,000 rpm for 1 minute, with the flow-through being discarded. The spin column was washed with 750 µL Buffer PE and centrifuged at 13,000 rpm for 1 minute twice, discarding the flow-through each time. The outside of the column was wiped with a Kimwipe. The column was placed in a 1.5 mL Eppendorf tube and 30 µL Buffer EB was added to the center of the column. It was left to sit for 1 minute, then centrifuged at 13,000 rpm for 1 minute. The spin column was discarded and the flow-through was stored in the refrigerator for later use.

When PCR was performed for the fusion proteins, the procedure was slightly different. Two PCR programs were run separately. In each run, the contents remained much the same as the PCR for the individual genes, but different primers were used. The first PCR program used the same parameters as the PCR for individual genes, with the only difference being the primers. Table 3 depicts the primers used for the *in vitro* fusion protein PCR. Table 4 depicts the primers used for

the *in vivo* fusion protein PCR. The different primers included an overlap section later used to fuse the genes together. The restriction sites are underlined, and the spacer region is italicized.

Table 3. Primers used in PCR mixture for recreation of *in vitro* fusion protein

EGFP	Forward Primer	5’-ACCACCGCAGTA<u>AAGCTT</u>ATG GTGAGCAAGGGCGAGGAG-3’
	Reverse Overlap Primer	5’-*TCCTCCTCCTCC*CTTGTA CAGCTCGTCCATGCC-3’
β-lactamase	Forward Overlap Primer	5’-ATGGACGAGCTGTACAAG*GGAGGA* *GGAGGA*CACCCAGAAACGCTGGTGAAA-3’
	Reverse Primer	5’-ACCGCCGCAGTA<u>GAATTC</u>TTA CCAATGCTTAATCAGTGAGGC-3’

Table 4. Primers used in PCR mixture for creation of *in vivo* fusion protein

β-lactamase	Forward Primer Fusion Step 1	5’ - TTATTATTATTAATGAGTA TTCAACATTTCCGTGTCGCC - 3’
	Forward Primer Fusion Step 2	5’ - ACCACCGCAGTA<u>AAGCTT</u> ATGAGTATTCAACATTTCCGT-3’
	Reverse Overlap Primer	5’-*TCCTCCTCCTCC*CCAATG CTTAATCAGTGAGGC-3’
EGFP	Forward Overlap Primer	5’-TCACTGATTAAGCATTGG*GGAGGA* *GGAGGA*ATGGTGAGCAAGGGCGAGGAG-3’
	Reverse Primer Fusion Step 1	5’ - AGGAGGAGGAGGTTACTTG TACAGCTCGTCCATGCCGAG - 3’
	Reverse Primer Fusion Step 2	5’-ACCGCCGCAGTA<u>CTCGAG</u>TTA CTTGTACAGCTCGTCCATGCC-3’

After the first round of PCR was completed, the bands for β-lactamase and EGFP were extracted together in the same tube in order to create a mixture of the DNA for both genes. The mixture was used in the second round of PCR. The primers used in the second round of PCR did not include the overlap primers, only the β-lactamase forward fusion Step 2 primer and EGFP

reverse fusion Step 2 primer were used. Figure 21 below depicts the fusion Step 1 and Step 2 overlap amplifications.

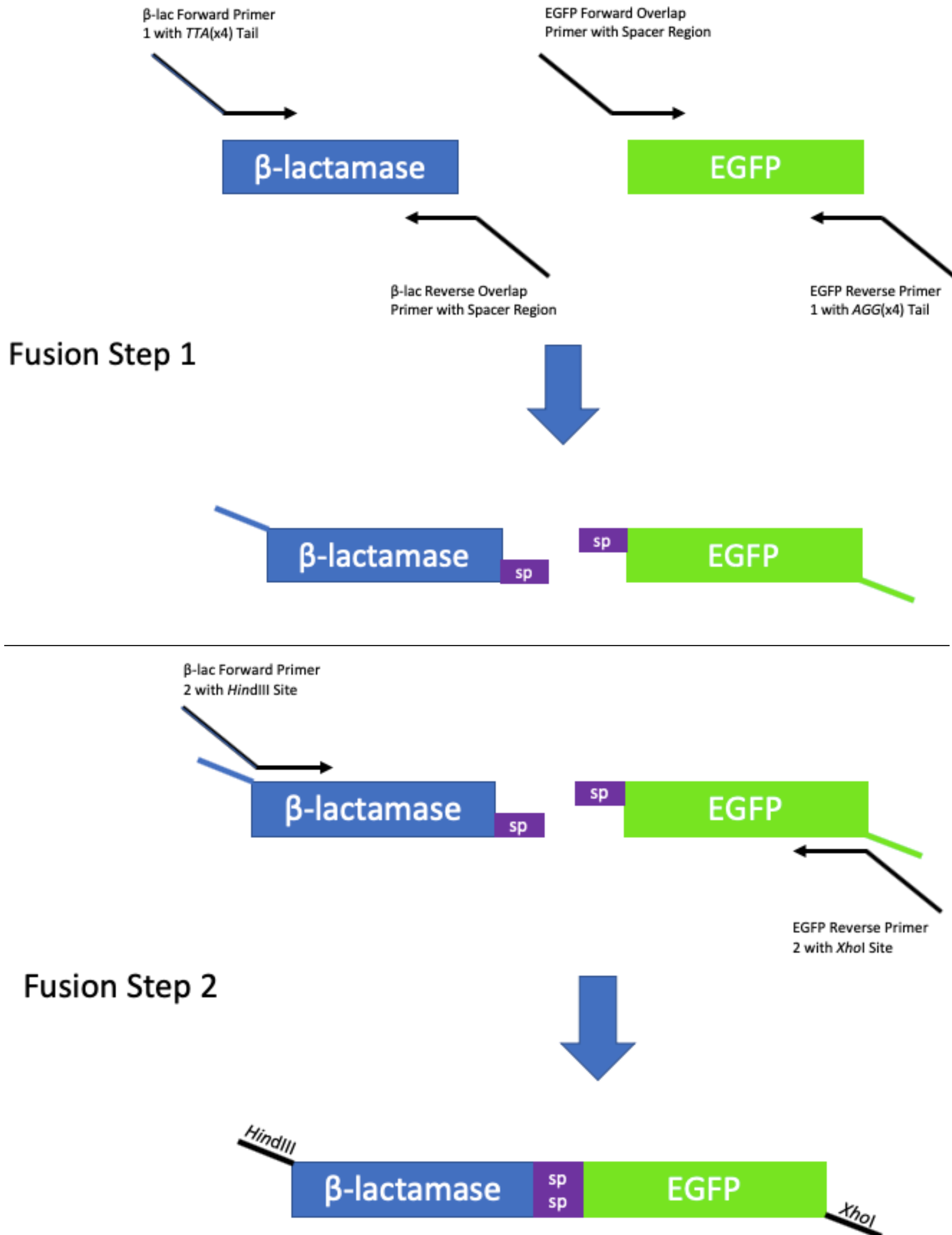


Figure 21. Illustration of steps one and two of fusion overlap PCR

The parameters for the PCR program were different, with two sets of conditions being used. Table 5 below depicts the PCR program parameters. Due to the larger size of just over 1500 bp for the fusion gene the extension time was increased. After the second round of PCR, another gel was run, and the bands were extracted using the above protocol for gel extraction.

Table 5. PCR parameters used for overlap extension PCR for *in vitro* and *in vivo* fusion genes

Stage	Temperature	Time
Denaturing	94°C	1 Minute
Annealing	45°C	1 Minute
Extending	72°C	3.5 Minutes
Number of Cycles	20	
Denaturing	94°C	1 Minute
Annealing	50°C	1 Minute
Extending	72°C	3.5 Minutes
Number of Cycles	30	

Enzyme Digestion and Purification

After the DNA was removed from the gel, the next step was to use a specific restriction endonuclease to create sticky ends. Both the EGFP and β-lactamase genes were digested, as well as the pFLAG-MAC vector. Digestion of the vector was necessary so that the genes could be inserted into it. Digestions were performed sequentially, using *Hin*dIII then *Eco*RI, or concurrently using both *Hin*dIII and *Eco*RI. *Hin*dIII and *Xho*I was used for the *in vivo* fusion protein. The reaction mixture for each of the enzymes contained 25.5 μL DNA, 3 μL enzyme buffer, and 1.5 μL restriction enzyme. Each digestion was left in the water bath at 37°C for 3 hours. Once

FastDigest^{TM} enzymes were obtained, the digestion was left in the water bath at 37°C for 15 minutes.

After the 3 hours or 15 minutes, the first digestion was stopped by PCR purification. The purification was completed using a Qiagen QIAquick PCR Purification kit as follows. Five volumes of Buffer PB were added to 1 volume of digested product and mixed. The DNA was bound by centrifuging at 13,000 rpm for 1 minute with the flow-through being discarded. The solution was washed with 750 µL Buffer PE and centrifuged twice at 13,000 rpm for 1 minute, with flow-through being discarded. The column was placed in a 1.5 mL Eppendorf tube, and the DNA was eluted by adding 30 µL Buffer EB to the center of the column and letting it sit for 1 minute. It was then centrifuged at 13,000 rpm for 1 minute. After the *Eco*RI digestion was completed, the contents of the tube were run on a gel, the desired bands were excised, and the DNA was extracted using the above protocol. After completion of both digestions, the products were stored in the refrigerator for later use.

Nanodrop

The concentration of both the gene inserts and vector were determined using the NanoDrop. The instrument was first cleaned by adding 3 µL of sterile water to the pedestal. After 2 minutes, the pedestal was dried by dabbing with a Kimwipe. Two microliters of elution buffer were placed on the pedestal to blank the instrument. Samples were then added to the pedestal in 2 µL aliquots with the elution buffer being ran in between each sample to reblank the instrument. After samples were run, the pedestal was again cleaned with 3 µL of sterile water and dabbed dry with a Kimwipe.

Ligation and Transformation

The concentrations determined from the NanoDrop were used to determine the appropriate volumes to be used in the ligation. Equation 1 depicts the formula used for the calculation. The solution of the equation was then divided by the concentration of the insert. The equation had 3 constants, the kilobasepairs of the insert (0.8), the kilobasepairs o the vector (5), and the ratio of the insert to the vector (3). The destination vector used for the validation of the pH theory and *in vitro* assay portions of the project was pFLAG-MAC. The destination vector used for the *in vivo* assay portion of the project was pET28b. For the ligation, 2 μL of T4 DNA ligase, 4 μL ligase buffer, and the determined amounts of insert and vector, forming a 3:1 insert:vector ratio, were used. Water was added to the mixture to bring it to a volume of 20 μL. The ligation was completed by holding the mixture at 16°C for at least 12 hours overnight.

Equation 1. Ligation calculation using Nanodrop concentration results

$$\frac{\text{nanograms of vector} \times \text{kilobasepairs of insert (0.8)} \times \text{ratio of insert to vector (3)}}{\text{kilobasepairs of vector (5)}} = \mu\text{L of insert added}$$

Transformations were performed to acquire plated colonies expressing the pET28b vector and pEGFP source plasmid. Transformations were also performed for the individual gene expression with pLP003 and pLP004 plasmids. First, 2.5 μL of plasmid were transferred into 25 μL of DH5α competent cells in Falcon tubes. The contents were incubated on ice for 15 minutes, heat shocked in a 42°C water bath for 45 seconds and incubated on ice again for 2 minutes. After the second incubation on ice, 350 μL of SOC media was added to the plasmid and cell mixture. It was then placed on the shaker table at 37°C and 200 rpm for 45 minutes. After the incubation in

the shaker table, 10 μL and 20 μL aliquots of the mixture were spread on agar plates with the appropriate antibiotic and incubated for 24-48 hours at 37°C.

Results and Discussion

Recreation of the In Vitro Fusion Protein

For the recreation of the *in vitro* fusion protein, the genes for β-lactamase and EGFP were successfully isolated with the gene spacer attached. Figure 22 below depicts the gel image following amplification of the individual genes with the primers for the *in vitro* fusion protein. The high background observed is likely indicative of the staining dye binding to the gel matrix, the gel being contaminated with DNA, or the electrophoresis chambers needing cleaning to remove contaminants.

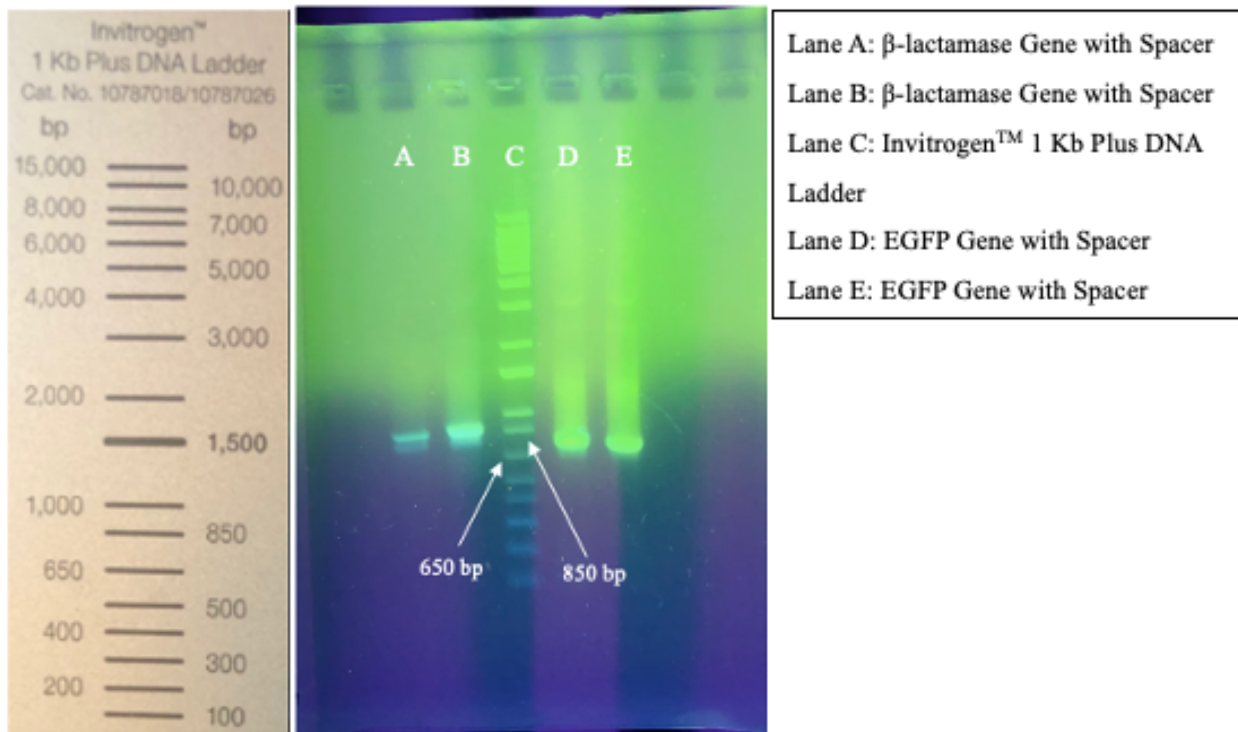


Figure 22. Invitrogen™ DNA size ladder and gel image of amplified β-lactamase and EGFP with spacer attached using *in vitro* fusion protein primers

After isolation and amplification of the individual genes, fusion PCR was performed but the two individual genes could not be fused together into one as no bands of the appropriate size were observed in the gel image following overlap PCR. More work needs to be done to recreate

the fusion gene. Although it has been accomplished in the past, the results have not been replicated. Adjustments involving new polymerases and PCR conditions will be looked at in order to create the *in vitro* fusion protein, as *Pfu* polymerase was used in the previous creation of the *in vitro* fusion protein.

Validation of the pH Theory

PCR optimization was performed to determine the ideal annealing temperatures of the primers used for the isolation of the β-lactamase and EGFP genes. Gradient PCR was performed with an annealing temperature range of $50^{\circ}C$ to $70^{\circ}C$, in increments of $4^{\circ}C$. Figures 23 and 24 below depict the gel images obtained from gel electrophoresis following gradient PCR. The gel images show that the optimum annealing temperature for both the β-lactamase and EGFP primers is $50^{\circ}C$, as it produced the most intense bands. This temperature was used as the annealing temperature in subsequent PCR amplifications of the individual genes. At the bottom end of each lane are primer dimers, by-products of PCR in which the primer molecules hybridized.

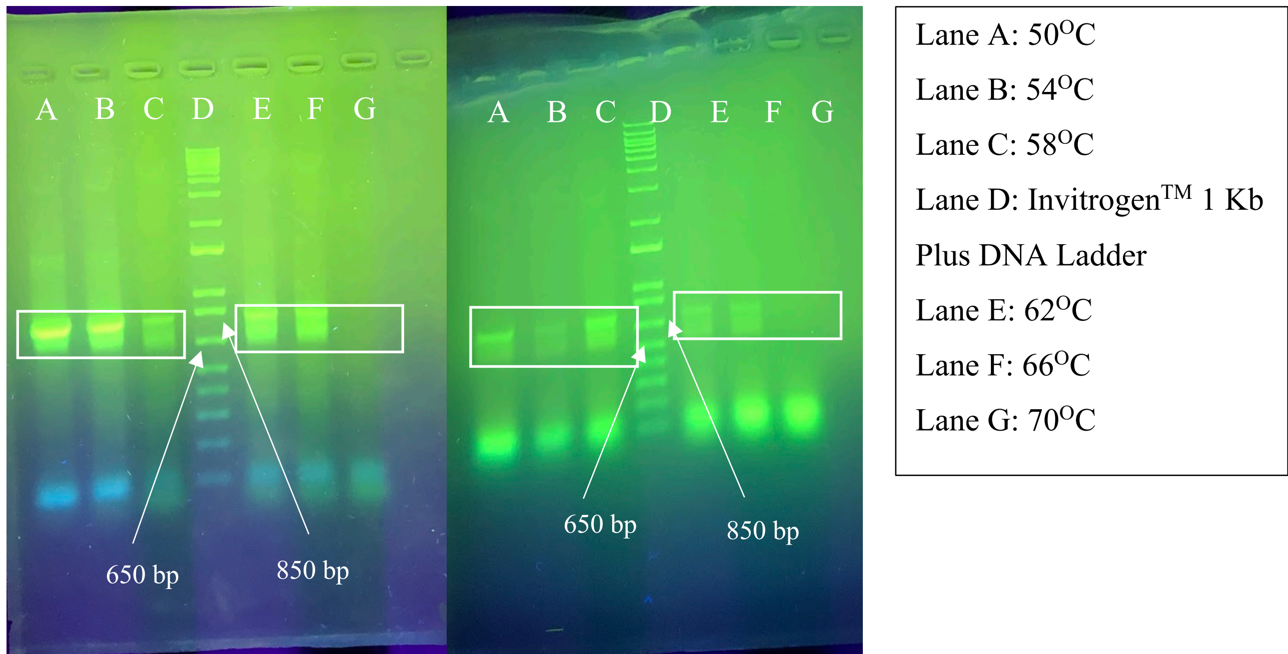


Figure 23. Gel image of β-lactamase after PCR annealing optimization

Figure 24. Gel image of EGFP after PCR annealing optimization

In the validation of the pH theory assay, the genes for both β-lactamase and EGFP were successfully isolated from the pEGFP plasmid and amplified via PCR. Figure 25 below depicts the gel image obtained from gel electrophoresis following PCR. The bands for β-lactamase and EGFP fall between the 650 and 850 bp ladder bands, the appropriate locations for the two genes; β-lactamase and EGFP have gene sizes of 789 bp and 714 bp, respectively. The DNA was isolated by gel extractions performed on excised gel samples.

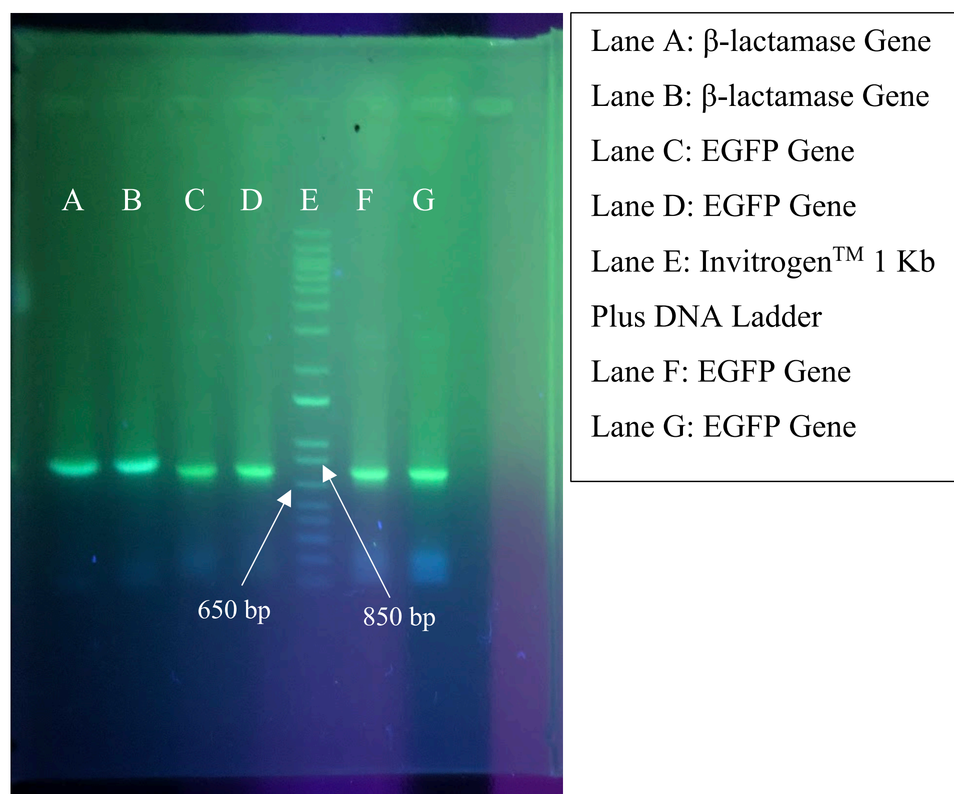


Figure 25. Gel image of amplified β-lactamase and EGFP obtained following PCR

After the bands were excised and the DNA was extracted, the concentrations of the amplified DNA were determined using the NanoDrop instrument prior to digesting with restriction enzymes. After digestions, the concentrations were again determined. This was performed to get concentrations for the calculations required for the ligation of the genes into the pFLAG-MAC vector. However, after performing ligation and transformation of the genes, no colony growth occurred. A positive control of pUC19 DNA had been transformed as well, and growth occurred

for the pUC19 colonies. This indicated that the error had occurred during the ligation and not the transformation. Initially, the gel extraction procedure was very inefficient; large amounts of DNA were lost through the process, resulting in percent yields of DNA at less than 20% from gel extractions following digestion. The process went through significant testing to optimize it and reduce the DNA loss. The EGFP gene was used as the DNA sample for each scenario. Several variables were altered in the protocol to determine the ideal process for the greatest DNA recovery. To optimize the process, four variables were altered: 1) the number of times the dissolved gel sample was run through the column, 2) the binding speed at which the column was centrifuged to bind the DNA, 3) the number of times the bound sample was washed with PE buffer, and 4) the amount of time the sample was let stand with EB buffer for elution. Table 6 below depicts the results of the optimization process, with the concentrations of the samples before and digestion with *Eco*RI and *Hin*dIII restriction enzymes. Based on the percent recoveries of each scenario, the best combination was determined to be running the dissolved gel through the column two times, binding the DNA at 7,000 rpm, washing the bound sample one time with PE buffer, and letting the sample stand with elution buffer for three minutes before centrifugation.

Table 6. EGFP DNA concentrations before and after digestion for the optimization of the gel extraction protocol

Variable	Conc. Before Dig.	Conc. After Dig.	% Recovery
1) 1 Time	81.4 ng/µL	14.0 ng/µL	17.2%
1) 2 Times	72.4 ng/µL	43.0 ng/µL	59.4%
1) 3 Times	77.8 ng/µL	47.2 ng/µL	60.7%
2) 2,000 rpm	93.1 ng/µL	69.7 ng/µL	74.9%
2) 7,000 rpm	87.9 ng/µL	68.8 ng/µL	78.3%
2) 13,000 rpm	69.8 ng/µL	10.7 ng/µL	15.3%
3) 1 Wash	83.4 ng/µL	17.8 ng/µL	21.4%
3) 2 Washes	73.5 ng/µL	13.3 ng/µL	18.1%
3) 3 Washes	76.1 ng/µL	14.9 ng/µL	19.6%
4) 1 Minute	71.7 ng/µL	14.2 ng/µL	19.8%
4) 3 Minutes	78.2 ng/µL	52.6 ng/µL	67.3%
4) 6 Minutes	84.8 ng/µL	56.4 ng/µL	66.5%

The optimum combination of variables described above was used in gel extractions, yielding consistent percent recoveries of over 80%. Table 7 below depicts the concentrations of β-lactamase and EGFP extracted from the gel in Figure 25 above, in addition to the pFLAG-MAC vector, before and after digestion using the optimized gel extraction protocol.

Table 7. DNA concentrations before and after digestion of DNA with *Eco*RI and *Hin*dIII restriction enzymes, and percent recovery

Gene	Conc. Before Dig.	Conc. After Dig.	% Recovery
β-lactamase (1)	78.7 ng/μL	67.2 ng/μL	85.4%
β-lactamase (2)	83.1 ng/μL	71.4 ng/μL	85.9%
EGFP (1)	63.8 ng/μL	52.7 ng/μL	82.6%
EGFP (2)	71.1 ng/μL	62.1 ng/μL	87.3%
EGFP (3)	78.4 ng/μL	64.9 ng/μL	82.8%
EGFP (4)	80.3 ng/μL	71.2 ng/μL	88.7%
pFLAG-MAC	124.8 ng/μL	107.5 ng/μL	86.1%

After digestions, the determined concentrations of extracted DNA were used to calculate the volume of DNA solution to add to the ligation solution. This is as far as the 'validation of pH theory' portion of the project went. After ligations were performed, the resulting recombinant plasmids were transformed into DH5α cells. Either the ligation or transformation proved to be unsuccessful each time as no growth occurred on agar plates following transformation. It is likely that it was the ligations that proved to be unsuccessful as transformations using the pEGFP vector plasmid (which contains both β-lactamase and EGFP genes) were successful, as were transformations performed using pUC19 DNA as a positive control.

Development of the In Vivo Fusion Protein

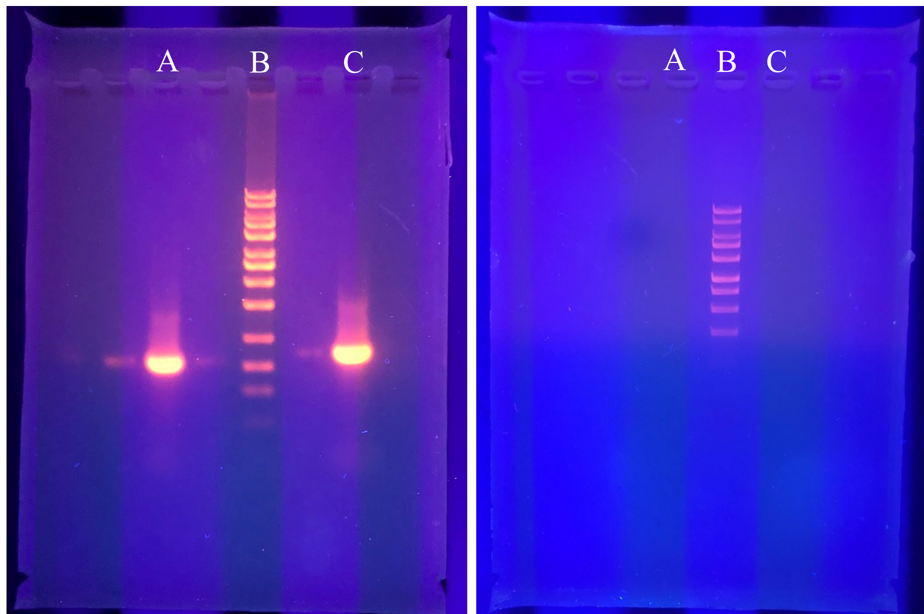
Unlike the *in vitro* portion of the project, the *in vivo* portion relies an alternative antibiotic resistance other than ampicillin resistance, as ampicillin resistance is being conferred by transforming *E. coli* cells with a plasmid containing the fusion gene of β-lactamase and EGFP. Due to this, pFLAG-MAC could not be used as the expression vector. Instead, pET28b, a plasmid vector containing the gene for kanamycin resistance is being used. Frozen stocks of pET28b were

used to create overnight cultures to be miniprepped. Using the miniprepped pET28b DNA, transformations were performed to maintain a supply of vector plasmid DNA. Using the resulting cultures, minipreps were created to be used in ligations with the *in vivo* fusion protein.

Initially, the primer set of *in vivo* primers had been incorrectly designed and were not appropriate for this experiment. The primers contained restriction sites for *Hin*dIII and *Eco*RI and were placed in the wrong orientation for ligation of the desired recombinant gene into the pET28b vector plasmid; the EGFP gene would be ligated in upstream of β-lactamase and would have been transcribed first, negating the goal of the *in vivo* portion of the project. In addition, the position of the restriction sites would have caused a frame-shift mutation when the construct was translated. Therefore, even if the fusion gene had been constructed, it would not have been ligated into the vector or transcribed by the bacteria. Table 8 below depicts the incorrectly designed primers. The restriction sites are underlined. Figures 26 and 27 below depicts the gel image resulting from the first and second fusion PCR procedure using the incorrectly designed primers.

Table 8. Incorrectly designed primers for *in vivo* fusion gene

β-lactamase Forward with *Hin*dIII	5’ – ACCACCTACTTAAGCTTAAT GAGTATTCAACATTTCCGT – 3’
EGFP Reverse with *Eco*RI	5’ – ACCACCTACTGAATTCATT TACTTGTACAGCTCGTCCAT – 3’



Left Gel Image
Lane A: EGFP
Lane B: Invitrogen™ 1 Kb Plus DNA Ladder
Lane C: β-lactamase
Right Gel Image
Lane A: EGFP
Lane B: Invitrogen™ 1 Kb Plus DNA Ladder
Lane C: β-lactamase

Figure 26. Gel image of individually amplified genes using incorrectly designed primers

Figure 27. Gel image of unsuccessful overlap PCR extension using incorrectly designed primers

New primers were used containing the correct restriction sites – *Hin*dIII and *Xho*I – and additional tails off the 5' ends of the β-lactamase forward primer and EGFP reverse primer. Adding the tail should increase the likelihood of the primers in the second PCR procedure binding and allow a more successful fusion. Table 9 below gives the sequence of the new primers with the tail added italicized. After the primers were ordered and arrived, they were resuspended in TE buffer, pH 7.4.

Table 9. New primers used in the first *in vivo* PCR procedure

β-lactamase Forward	5' - *TTATTATTATTA*ATGAGTATTCAACATTTCCGTGTCGCC - 3'
EGFP Reverse	5' - *AGGAGGAGGAGG*TTACTTGTACAGCTCGTCCATGCCGAG - 3'

Using the above primers, and two overlap primers for β-lactamase and EGFP, the genes were individually isolated and amplified with the 5' tails and 3' gene spacer attached. Figure 28 below depicts the gel image following amplification of the individual genes with the primers for the *in vivo* fusion protein.

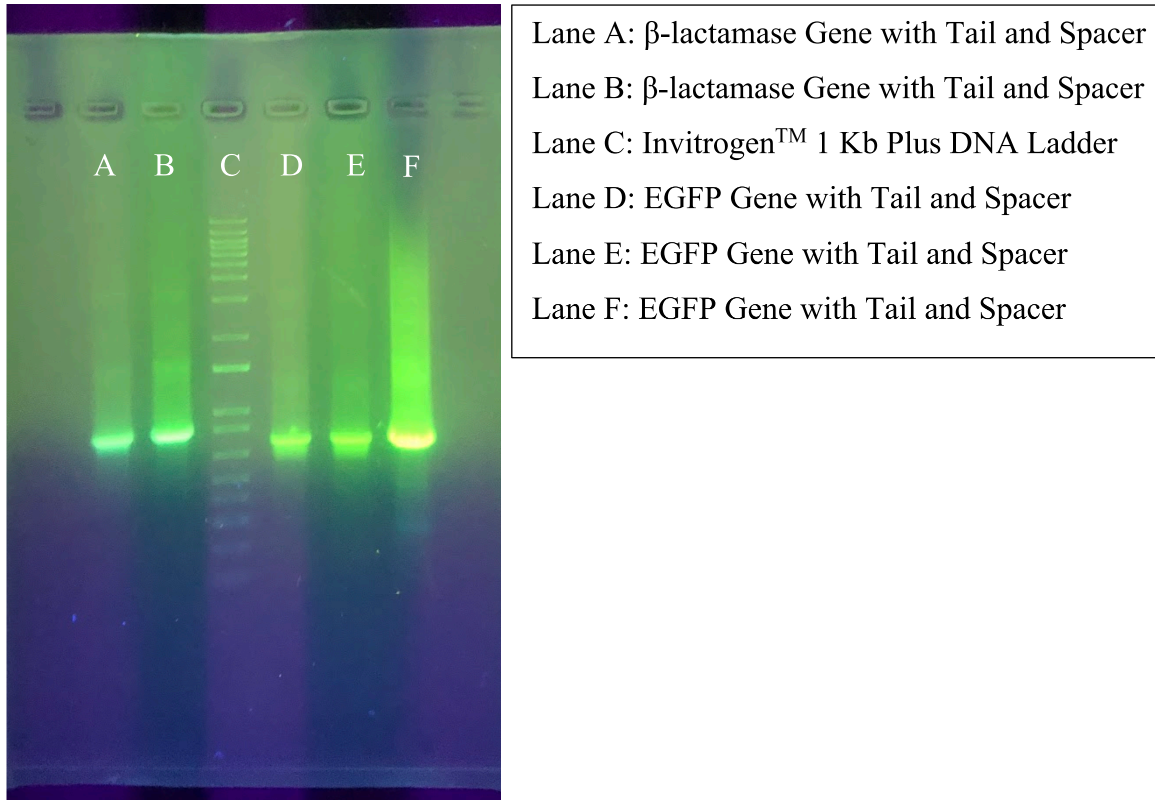


Figure 28. Gel image of amplified β-lactamase and EGFP with tail and spacer attached using *in vivo* fusion protein primers

After isolation and amplification of the individual genes, fusion PCR was performed but the two individual genes were not be fused together into one. A PCR protocol that had not been optimized was utilized for the *in vivo* construct. As such, alterations to the conditions in which PCR is performed will be looked into, including the polymerase used, the reaction temperature/time conditions, and the solution in which the reaction takes place so that the process can be optimized. Figure 29 below depicts the gel image obtained in the gradient PCR for the fusion PCR procedure. The reaction was unsuccessful, indicating that some parameters, likely other than annealing temperature of the primers, must be altered and observed. The gradient reaction run below was run with an annealing temperature range of 45°C to 65°C, in increments of 4°C. At the bottom end of the gel, primer dimers can be observed.

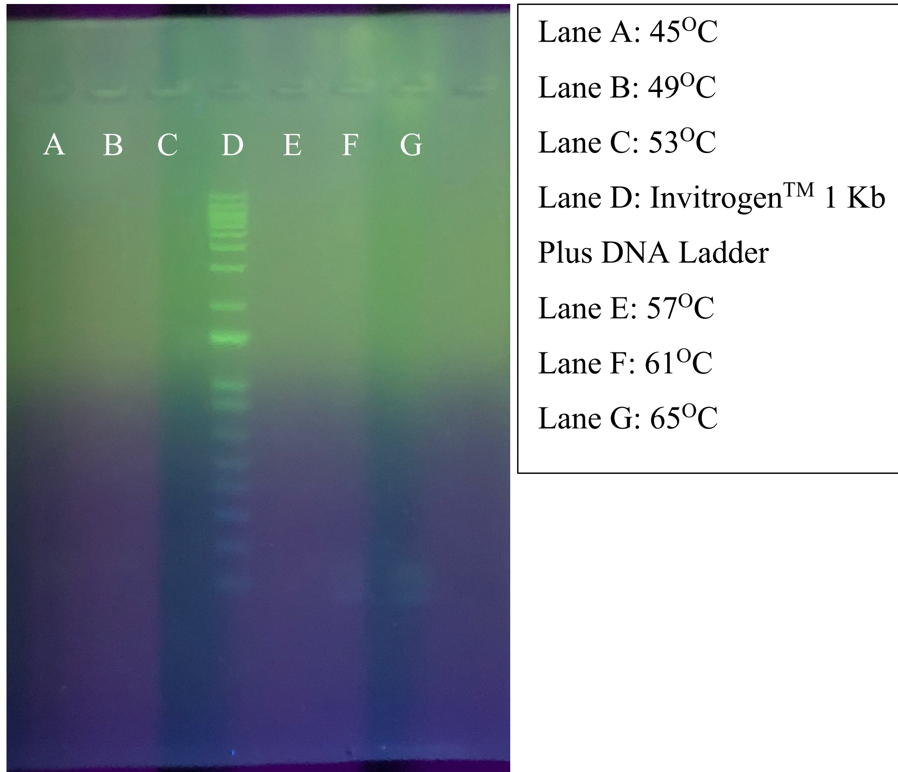


Figure 29. Gel image of gradient fusion PCR products for optimal annealing temperature of primers

Conclusions and Future Work

The individual genes for β-lactamase and EGFP were successfully isolated from the plasmid pEGFP and amplified via PCR with primers containing the restriction site sequences for *Hin*dIII and *Eco*RI restriction enzymes. The genes were digested using the restriction enzymes and yielded suitable DNA concentrations for ligation into a vector plasmid. However, after ligation and transformation, no colony growth was observed for the β-lactamase and EGFP transformed bacteria. Error occurred during ligation of the digested genes into the pFLAG-MAC vector as a positive control of pUC19 DNA during transformation resulted in bacterial growth on selective media. Initially, low concentrations following gel extraction were the cause of unsuccessful ligations. After gel extraction optimization, the likely error is impurities, such as agarose, in the extracted DNA. β-lactamase and EGFP must still be successfully ligated into the pFLAG-MAC expression vector and transformed into DH5α cells for the validation of the pH theory.

The genes for β-lactamase and EGFP, after being isolated, were successfully amplified via PCR in the first step of fusion PCR for the recreation of the *in vitro* fusion protein using the *in vitro* primers, having the spacer attached. However, the overlap step of fusion PCR was not successful, so the *in vitro* fusion gene must still be recreated. The *in vitro* fusion gene will be digested with *Hin*dIII and *Eco*RI restriction enzymes and ligated into pFLAG-MAC for expression, and the protein will be used for comparison in fluorescence measurements. The isolated β-lactamase and EGFP were successfully amplified via PCR in the first fusion PCR for the creation of the *in vivo* fusion protein using the newly designed *in vivo* primers, having the spacer and tail attached. However, the overlap second step of fusion PCR was not successful, so the *in vivo* fusion gene must still be created. The *in vivo* fusion gene will be digested with *Xho*I and *Hin*dIII restriction enzymes and ligated into pET28b for expression.

In order to improve the experimental design and increase the success of the project in the future, alternative protocols should be considered. The primary issue in this research is the fusion of the β-lactamase and EGFP genes into a single fusion construct. Currently, the genes are being fused via overlap extension PCR using *Taq* polymerase. Literature searches reveal that *Taq* polymerase is a cheaper, useful polymerase for the amplification of individual products, but is an inefficient enzyme for the construction of fusion products. *Taq* polymerase lacks 3' to 5' exonuclease proofreading activity. Not only does this inhibit the fidelity of the enzyme, it also limits the size of the amplicon as well. *Taq* has been demonstrated to sometimes be unable to amplify DNA sequences in the size range of 1500 to 2000 bp.[51] The fusion product of β-lactamase and EGFP is just over 1500 bp. In addition, *Taq* leaves single A-overhangs at the 3' ends of DNA sequences, disrupting overlapping regions during the overlap extension fusion PCR.[52] *Taq*'s inability to efficiently fuse together gene products highlights a flaw in the experimental design. As such, future research should be performed using high-fidelity enzymes capable of efficiently generating larger constructs. Experimentation performed by Puckett et. al. using *Pfu* polymerase yielded a fusion protein to be used for *in vitro* fluorescence studies.[50] *Pfu* and *Pfu* Turbo are high-fidelity DNA polymerases with 3' to 5' exonuclease activity used during PCR for the generation of larger amplicons with fewer base-substitution errors. *Pfu* has been used in fusion PCR to generate products up to 20 kb in size, and up to 35 kb fragments in non-fusion amplification.[51,53] A limitation of *Pfu* is that it is slower than *Taq*, requiring longer elongation cycles, up to twice as long, to amplify the DNA.[54] To overcome the reduced accuracy and fidelity of *Taq* and the increased elongation times of *Pfu*, mixtures of *Taq* and *Pfu* acting synergistically have been used, taking advantage of *Taq*'s speed and *Pfu*'s proofreading.[51,55,56] *Taq* PLUS DNA Polymerase is a polymerase mixture that contains both *Taq* and *Pfu*. *Taq* PLUS has an error rate of 7.5×10^{-5} per

nucleotide per cycle. *Taq* has an error rate of $1.5x10^{-4}$ per nucleotide per cycle, while *Pfu* has an error rate of $5.1x10^{-6}$ per nucleotide per cycle.[57,58] The error rates indicate that by mixing the *Taq* and *Pfu* polymerase, the error decreases from that of *Taq* alone, but is still greater than that of *Pfu* alone. *Taq* was being used in a MasterMix to reduce error in the PCR steps of the experiment. It is recommended to use *Pfu* over *Taq* or a mixture, as *Pfu* has been demonstrated to be effective for fusing β-lactamase and EGFP, and the increased time of PCR is a small price to pay for increased success. Another polymerase for consideration is Phusion, a high-fidelity, high-processivity polymerase. Phusion polymerase has a novel *Pyrococcus*-like 3' to 5' exonuclease proofreading domain fused to a protein DNA-binding domain, allowing it to 'clamp' on to DNA and stay attached over long strands of DNA to be amplified for increased processivity. Phusion, like *Pfu*, has 3' to 5' exonuclease activity to improve fidelity. Phusion has an error rate of $3.9x10^{-6}$ per nucleotide per cycle, comparable to that of *Pfu*.[57] Phusion has been demonstrated to be effective in overlap extension PCR repeatedly, making it a prime candidate as the PCR polymerase to be used for future work on this experiment.[59, 60,61] Both *Pfu* and Phusion have been demonstrated as high-fidelity enzymes and have a high efficacy for overlap extension PCR. However, Phusion has a greater processivity than *Pfu*, making Phusion the better candidate as it will have comparable fidelity with increased processivity, reducing times required for PCR. Similar to Phusion, Q5 polymerase from New England Biolabs could be considered. Like Phusion, Q5 polymerase has a higher fidelity than *Taq* and is faster than *Pfu*. In addition, it can come in a mastermix, like *Taq*, keeping with the goal of reducing human error during preparation of PCR reaction mixtures.[62]

Another issue encountered, though less major, was the presence of primer dimers following PCR. Primer dimers form as a PCR byproduct when the primer molecules hybridize to each other

due to complementary bases in the primer sequences. When primer dimers form, they reduce PCR efficiency by competing for resources needed for amplification of target DNA and increasing non-target products of PCR.[63] The simplest method to reduce their formation is the optimization of the annealing temperature via gradient PCR. Though this has been performed for the individual genes and primers, it should be performed again with new polymerases, and for the validation of the optimal annealing temperatures of the current primers, and for the determination of the optimum annealing temperature for the overlap primers. The addition of dimethyl sulfoxide (DMSO) is also a possible solution. DMSO optimizes the specificity of product formation in PCR, inhibiting the formation of secondary DNA structures and binding to DNA to prevent the reannealing of single-stranded DNA. DMSO is also known to facilitate the annealing of primers with template DNA, increasing target product formation in PCR. A 5% DMSO solution has been determined to be optimal.[64]

In addition to running traditional PCR, real time, or quantitative PCR (qPCR) needs to be run to observe the DNA amplification process as it is happening. qPCR uses dye-based fluorescent labeling to accomplish this, taking fluorescence measurements during each cycle as the DNA is amplified. This allows the observance of when the DNA begins to amplify logarithmically and allows the quantification of the amount of DNA present. By running qPCR, it will be possible to observe at what stage the DNA begins to amplify logarithmically.[65] When experimenting with the various polymerases, qPCR and PCR optimization procedures will have to be performed extensively to determine the optimal conditions for the various polymerases to get ideal amplicon yield and results. Figure 30 below depicts a diagram of qPCR, where C_q is the cycle at which logarithmic amplification begins.

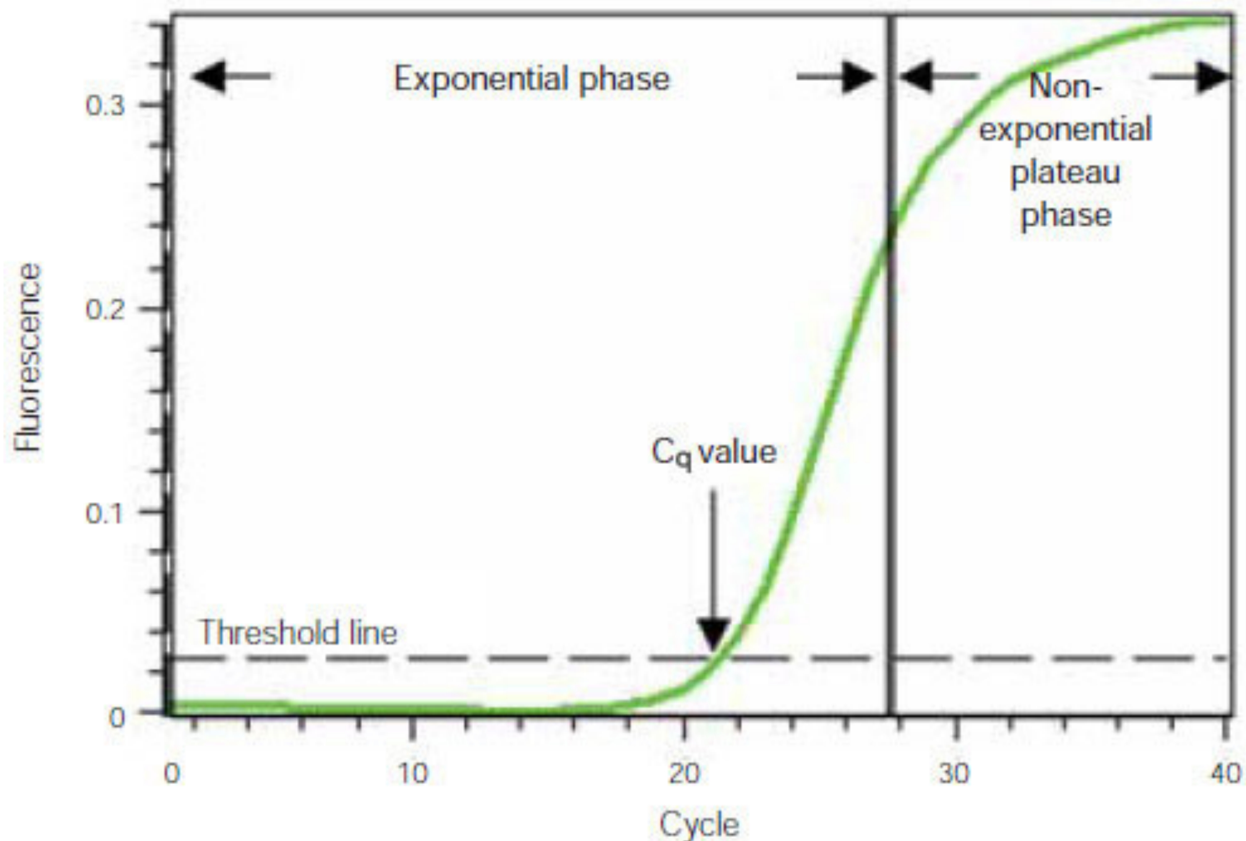


Figure 30. Diagram of qPCR.[66]

An alternative method for construction of the fusion protein is to use expressed protein ligation. EPL is a post-translational modification method in which the C-terminus of a protein is modified to contain a thioester, while the N-terminus of another is modified to contain a cysteine residue, allowing the conjugation of the proteins by the formation of a native peptide bond between the two modifications.[67] EPL has been used for the preparation of fusion proteins. Yu et. al. used EPL to synthesize a fusion construct of EGFP and synthetic cell penetrating peptide. The EGFP thioester was prepared by intein-mediated purification with an affinity chitin-binding tag (IMPACT) system, used to generate an EGFP-intein-chitin binding domain (EGFP-I-CBD) protein, which then allowed for on-column cleavage of purified EGFP-I-CBD to generate the EGFP thioester. The EGFP thioester was incubated in ligation buffer with the synthesized cell penetrating peptide, resulting in a fusion protein of the two.[68] A vital limitation of this technique

is that it is a post-translation modification. Currently, performing the *in vivo* portion of this research would be very difficult using EPL, but research into such protocols is ongoing. Hauser and Ryan performed EPL using an N-terminal cysteine containing fragment that was generated *in vivo* using a pelB fusion protein. They subjected a recombinant fusion protein comprised of the N-terminal residues 1-111 of apolipoprotein E, intein, and CBD to 2-mercaptoethanesulfonic acid to cleave it. A second fusion protein of a pelB leader peptide fused to apolipophorin III residues 1-91 was directed to the periplasm of *E. coli* cells when cultured. Here, peptidase cleaving activity generated the N-terminal cysteine. The two fusion proteins, apolipoprotein E-intein-CBD and pelB-apolipophorin III, were successfully ligated to yield a fusion hybrid apolipoprotein.[69]

Another prospective method to overlap fusion PCR is the PCR cut-and-ligate method to ligate them together as a single gene. Webb et. al. used this method to fuse GFP with various proteins involved in the sporulation in *Bacillus subtilis*. The gene for GFP was fused with sspE-2g, cotE, csfB, and gerE. GFP was ligated into pBluescriptSKII, generating pCW8. Using this plasmid, the other genes were amplified via PCR, digested with restriction enzymes, and ligated upstream of GFP into pCW8 to generate other plasmids to be used in the studies. By removing the stop codons of the various other genes, they would be transcribed as GFP fusion constructs, allowing the observation and localization of the proteins involved in sporulation.[70] More recently, a similar fusion method was used in which a plasmid containing GFP had biotin protein ligase ligated into it to fuse GFP and biotin protein ligase, allowing for the detection of Cu^{2+} and Zn^{2+} cations in aqueous solution.[71] A method similar to this was also used by the Puckett research group to fuse the genes for organophosphorus hydrolase (OPH) and EGFP. OPH and EGFP were individually amplified via PCR with an *Xho*I restriction site added to each one, *Hin*dIII added to one gene, and *Eco*RI added to the other. The two pieces were then digested with *Xho*I and ligated

together at the restriction site. A second digestion and ligation are performed using *Eco*RI and *Hin*dIII with the pFLAG-MAC vector, resulting in the ligation of the fusion construct into the vector for expression. The genes for β-lactamase and EGFP could be individually amplified via PCR with the restriction sites added to the ends using primers, and digested. The two could then be ligated together to create the fusion construct, without the need for overlap PCR being performed. Once the fusion gene was constructed, using a different set of restriction enzymes so as not to cleave the construct, the fusion gene and vector plasmid could be digested and then ligated together to create the desired recombinant plasmid. Alternatively, EGFP could be ligated into a plasmid, and then β-lactamase could be ligated in upstream with the stop codon removed, allowing for the continuous transcription and translation of the fusion protein. After successfully ligating the fusion gene, a single fusion protein would be transcribed and translated by bacterial cells, allowing fluorescence studies to be performed. Figure 31 below depicts a summary the PCR cut-and-ligate method that could be used for the creation of the *in vivo* fusion construct to be ligated into the pET28b vector.

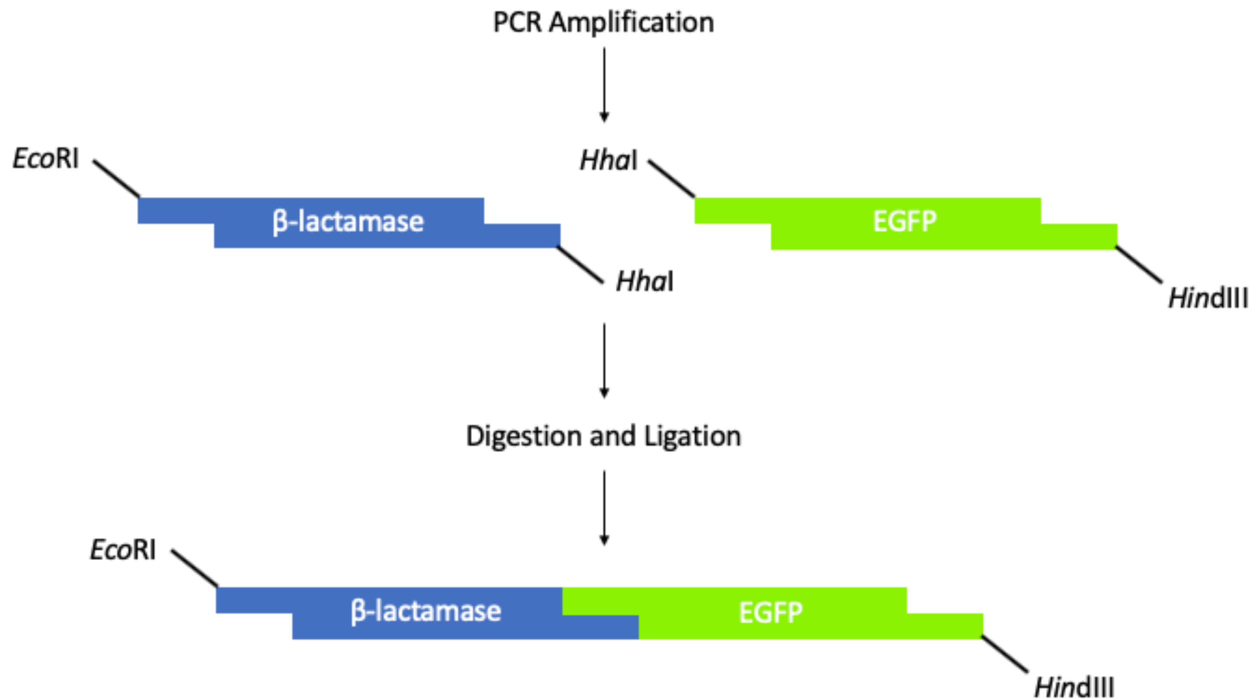


Figure 31. β-lactamase and EGFP PCR cut-and-ligate method

A final consideration for alternative methodology is the use of USER (uracil-specific excision reagent) fusion. Geu-Flores et. al. demonstrated the use of USER fusion to combine the fusion of three PCR products into one with the cloning of the fusion product into a vector in one step, as opposed to two PCR setups, a digestion, and a ligation. When performing overlap extension PCR, multiple PCR setups must be run, introducing the opportunity for human and instrument error. In their experimentation, Geu-Flores et. al. amplified three DNA sequences individually using *Pfu* Turbo. The primers of the three individual sequences contained complementary overhangs for the fusion of the genes. The purified the products with Qiagen QIAquick PCR purification kit. The PCR products, along with a stock of PacI/Nt.BbvCI pre-digested plasmid, were run on an agarose gel to estimate concentration, and they mixed them in a 10:10:10:1 molar ratio (with 10 being amplicons and 1 being the digested vector plasmid). The mixtures were treated with USER™ enzyme mix. USER™ enzyme mix contains uracil DNA glycosylase and DNA glycosylase-lyase endonuclease VIII. The result was a successful fusion and

ligation of the three sequences into a pre-digested vector in one step requiring only one hour.[72] While this method seems promising, it is recommended first investing time in the use of *Pfu* or Phusion polymerase an optimizing the PCR protocols for those enzymes, as it would likely be the simplest solution for the issues faced in the research with the least amount of change in methodology. As *Taq* is an inefficient enzyme for larger constructs, this should improve the future success of the project.

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References

1. CDC. Antibiotic Resistance Threats in the United States, **2019**. Atlanta, GA: U.S. Department of Health and Human Services, CDC; **2019**.
2. Sensor. *Merriam-Webster.com*: **2020**.
3. Thevenot, D. R.; Toth, K.; Durst, R. A.; Wilson, G. S., ELECTROCHEMICAL BIOSENSORS: RECOMMENDED DEFINITIONS AND CLASSIFICATION. *Analytical Letters* **2001**, *34* (5), 635-59.
4. Biosensor. *Merriam-Webster.com*: **2020**.
5. Bhalla, N.; Jolly, P.; Formisano, N.; Estrela, P., Introduction to biosensors. *Essays in Biochemistry* **2016**, *60* (1), 1-8.
6. Sansone, L.; Macchia, E.; Taddei, C.; Torsi, L.; Giordano, M., Label-free optical biosensing at femtomolar detection limit. *Sensors nad Actuators B: Chemical* **2018**, *255* (1), 1097-104.
7. Feliciano, J.; Liu, Y.; Daunert, S., Novel reporter gene in a fluorescent-based whole cell sensing system. *Biotechnology and Bioengineering* **2006**, *93* (5), 989-97.
8. Mehrotra, P., Biosensors and their applications – A review. *Journal of Oral Biology and Craniofacial Research* **2016**, *6* (2), 153-9.
9. Murugaiyan, S. B.; Ramasamy, R.; Gopal, N.; Kuzhandaivelu, V., Biosensors in clinical chemistry: An overview. *Advanced Biomedical Research* **2014**, *3*, 67.
10. Senutovitch, N.; Vernetti, L.; Boltz, R.; DeBiasio, R.; Gough, A.; Taylor, D. L., Fluorescent protein biosensors applied to microphysiological systems. *Experimental Biology and Medicine* **2015**, *240* (6), 795-808.
11. Puckett, L. G., An Integrated Approach to the Design and Development of Sensing Systems for Clinical and Pharmaceutical Application. University of Kentucky, **2003**.
12. Daunert, S.; Barrett, G.; Feliciano, J. S.; Shetty, R. S.; Shrestha, S.; Smith-Spencer, W., Genetically Engineered Whole-Cell Sensing Systems: Coupling Biological Recognition with Reporter Genes. *Chemical Reviews* **2000**, *100* (7), 2705-38.
13. Close, D. M.; Ripp, S.; Sayler, G. S., Reporter Proteins in Whole-Cell Optical Bioreporter Detection Systems, Biosensor Integrations, and Biosensing Applications. *Sensors* **2009**, *9* (11), 9147-74.

14. American Chemical Society International Historic Chemical Landmarks. Discovery and Development of Penicillin. http://www.acs.org/content/acs/en/education/whatischemistry/landmarks/flemingpenicillin.html (accessed April 13, 2020).
15. Kong, K. F.; Schneper, L.; Mathee, K., Beta-lactam Antibiotics: From Antibiosis to Resistance and Bacteriology. *APMIS* **2011**, *118* (1), 1-36.
16. Fernandes, R.; Amador, P.; Prudêncio, C., β-Lactams: chemical structure, mode of action and mechanisms of resistance. *Reviews in Medical Microbiology* **2013**, *24* (1), 7-17.
17. Laxminarayan, R.; Duse, A.; Wattal, C.; et al., Antibiotic resistance-the need for global solutions. *The Lancet Infectious Diseases* **2013**, *13* (12), 1057-98.
18. Calvopina, K.; Hinchliffe, P.; Brem, J.; Heesom, K. J.; Johnson, S.; Cain, R.; Lohans, C. T.; Fishwick, C. W. G.; Schofield, C. J.; Spencer, J.; Avison, M. B., Structural/mechanistic insights into the efficacy of nonclassical beta-lactamase inhibitors against extensively drug resistant Stenotrophomonas maltophilia clinical isolates. *Molecular Microbiology* **2017**, *106*, 492-504.
19. Philippon, A.; Slama, P.; Dény, P.; Labia, R., A Structure-Based Classification of Class A β-Lactamases, a Broadly Diverse Family of Enzymes. *Clinical Microbiology Reviews* **2016**, *29* (1), 29-57.
20. Bush, K.; Fisher, J. F., Epidemiological expansion, structural studies, and clinical challenges of new β-lactamases from gram-negative bacteria. *Annual Review of Microbiology* **2011**, *65*, 455-78.
21. Neu, H. C., The crisis in antibiotic resistance. *Science* **1992**, *257* (5073), 1064-73.
22. Wu, C.; Lin, C.; Zhu, X.; et al., The β-Lactamase Gene Profile and a Plasmid-Carrying Multiple Heavy Metal Resistance Genes of *Enterobacter cloacae*. *International Journal of Genomics* **2018**, *2018*, 4989602.
23. Bacterial conjugation. *Wikipedia*: **2020**. https://en.wikipedia.org/wiki/Bacterial_conjugation
24. Jang, C. W.; Magnuson, T., A Novel Selection Marker for Efficient DNA Cloning and Recombineering in *E. coli*. *PLOS ONE* **2013**, *8* (2), e57075.
25. Drawz, S. M.; Bonomo, R. A., Three Decades of β-Lactamase Inhibitors. *Clinical Microbiology Reviews* **2010**, *23* (1), 160-201.

26. Pliny, J. B.; Riley, H. T., The natural history of Pliny, Book XXXII. Remedies derived from aquatic animals. Chapter 52 - Other aquatic productions. Adarca or Calamochnos: three remedies. Reeds: eight remedies. Think of the sæpia. Gaius Plinius Secundus (Pliny the Elder). AD77. Bohn HG, London (**1855**).
27. Shimomura, O.; Johnson, F. H.; Saiga, Y., Extraction, Purification and Properties of Aequorin, a Bioluminescent Protein From the Luminous Hydromedusan, Aequorea. *Journal of Cellular and Comparative Physiology* **1962**, *59*, 223-39.
28. Morise, H.; Shimomura, O.; Johnson, F. H.; Winant, J, Intermolecular energy transfer in the bioluminescent system of Aequorea. *Biochemistry* **1974**, *13* (12), 2656-62.
29. Sekar, R. B.; Periasamy, A.; Fluorescence resonance energy transfer (FRET) microscopy imaging of live cell protein localizations. *Journal of Cell Biology* **2003**, *160* (5), 629-33.
30. Murdock, A., How basic research on jellyfish led to an unexpected scientific revolution. *University of California* **2017**. https://www.universityofcalifornia.edu/news/how-basic-research-jellyfish-led-unexpected-scientific-revolution
31. Campbell, T. N.; Choy, F. Y. M., The Effect of pH on Green Fluorescent Protein: a Brief Review. *Molecular Biology Today* **2001**, *2* (1), 1-4.
32. Ormö, M.; Cubitt, A. B.; Kallio, K.; Gross, L. A.; Tsien, R. Y.; Remington, S. J., Crystal structure of the Aequorea victoria green fluorescent protein. *Science* **1996**, *273* (5280), 1392-5.
33. Stepanenko, O. V.; Verkhusha, V. V.; Kuznetsova, I. M.; Uversky, V. N.; Turoverov, K. K., Fluorescent Proteins as Biomarkers and Biosensors: Throwing Color Lights on Molecular and Cellular Processes. *Current Protein and Peptide Science* **2008**, *9* (4), 338-69.
34. Shetty, R. S.; Deo, S. K.; Liu, Y.; Daunert, S., Fluorescence-based sensing system for copper using genetically engineered living yeast cells. *Biotechnology and Bioengineering* **2004,** *88* (5), 664-70.
35. Niu, Y.; Yu, Y.; Bernat, A.; et al., Transgenic rhesus monkeys produced by gene transfer into early-cleavage-state embryos using a simian immunodeficiency virus-based vector. *Proceedings of the National Academy of Sciences of the United States of America* **2010**, *107* (41), 17663-7.

36. Chalfie, M., GFP: Lighting up life. *Proceedings of the national Academy of Sciences of the United States of America* **2009**, *106* (25), 10073-80.
37. Kneen, M.; Farinas, J.; Li, Y.; Verkman, A. S., Green Fluorescent Protein as a Noninvasive Intracellular pH Indicator. *Biophysical Journal* **1998**, *74* (3), 1591-9.
38. Snapp, E., Design and Use of Fluorescent Fusion Proteins in Cell Biology. *Current Protocols in Cell Biology* **2005**, *Chapter 21*, 21 4 1-21 4 13.
39. Cheng, L.; Du, C.; Murray, D.; Tong, X.; Zhang, Y. A.; Chen, B. P.; Hawley, R. G., A GFP reporter system to assess gene transfer and expression in human hematopoietic progenitor cells. *Gene Therapy* **1997**, *4* (10), 1013-22.
40. Chalova, V. I.; Zabala-Diaz, I. B.; Woodward, C. L.; Ricke, S. C., Development of a whole cell green fluorescent sensor for lysine quantification. *World Journal of Microbiology and Biotechnology* **2007**, *24* (3), 353-9.
41. Shetty, R. S.; Deo, S. K.; Shah, P.; Sun, Y.' Rosen, B. P.; Daunert, S., Luminescence-based whole-cell-sensing systems for cadmium and lead using genetically engineered bacteria. *Analytical and Bioanalytical Chemistry* **2003**, *376* (1), 11-7. Epub 2003 Mar 28.
42. Prendergast, F. G.; Mann, K. G., Chemical and Physical Properties of Aequorin and the Green Fluorescent Protein Isolated From Aequorea Forskålea. *Biochemistry* **1978**, *17* (17), 3448-53.
43. Helms, V.; Straatsma, T. P.; McCarmmon, J. A., Internal Dynamics of Green Fluorescent Protein. *The Journal of Physical Chemistry B.* **1999**, *103* (16), 3263-9.
44. Brejc, K.; Sixma, T. K.; Kitts, P. A.; Kain, S. R.; Tsien, R. Y.; Ormö, M.; Remington, S. J., Structural basis for dual excitation and photoisomerization of the Aequorea victoria green fluorescent protein. *Proceedings of the National Academy of Sciences of the United States of America* **1997**, *94* (6), 2306-11.
45. Stepanenko, O. V.; Stepanenko, O. V.; Kuznetsova, I. M.; Verkhusha, V. V.; Turoverov, K. K., Beta-Barrel Scaffold of Fluorescent Proteins: Folding, Stability and Role in Chromophore Formation. *International Review of Cell and Molecular Biology* **2013**, *302*, 221-78.
46. Cubitt, A. B.; Heim, R.; Adams, S. R.; Boyd, A. E.; Gross, L. A.; Tsien, R. Y., Understanding, improving and using green fluorescent proteins. *Trends in Biochemical Sciences* **1995**, *20* (11), 448-55.

47. Clontech *Living Colors^{TM} Fluorescent Proteins User Manual*.

48. Niwa, H.; Inouye, S.; Hirano, T.; Matsuno, T.; Kojima, S.; Kubota, M.; Ohashi, M.; Tsuji, F. I., Chemical nature of the light emitter of the Aequorea green fluorescent protein. *Proceedings of the National Academy of Sciences of the United States of America* **1996**, *93* (24), 13617-22.

49. Llopis, J.; McCaffery, J. M.; Miyawaki, A.; Farquhar, M. G.; Tsien, R. Y., Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. *Proceedings of the National Academy of Sciences* **1998**, *95* (12), 6803-8.

50. Puckett, L. G.; Lewis, J. C.; Bachas, L. G.; Daunert, S., Development of an assay for beta-lactam hydrolysis using the pH-dependence of enhanced green fluorescent protein. *Analytical Biochemistry* **2002**, *309* (2), 224-31.

51. Barnes, W. M., PCR amplification of up to 35-kb DNA with high fidelity and high yield from λ bacteriophage templates. *Proceedings of the National Academy of Sciences of the United States of America* **1994**, *91*, 2216-20.

52. Sambrook, J.; Fritsh, E. F.; Maniatis, T., Molecular cloning: a laboratory manual. **2001**. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

53. Shevchuk, N. A.; Bryksin, A. V.; Nusinovich, Y. A.; Cabello, F. C.; Sutherland, M.; Ladisch, S., Construction of long DNA molecules using long PCR-based fusion of several fragments simultaneously. *Nucleic Acids Research* **2004**, *32* (2), e19.

54. Ishino, S.; Ishino, Y., DNA polymerses as useful reagents for biotechnology – the history of developmental research in the field. *Fronteirs in Microbiology* **2014**, *5*, 465.

55. Śpibida, M.; Krawczyk, B.; Olszewski, M.; Kur, J., Modified DNA polymerases for PCR troubleshooting. *Journal of Applied Genetics* **2017**, *58* (1), 133-42.

56. Cline, J.; Braman, J. C.; Hogrefe, H. H., PCR Fidelity of *Pfu* DNA Polymerase and Other Thermostable DNA Polymerases. *Nucleic Acids Research* **1996**, *24* (18), 3546-51.

57. Potapov, V.; Ong, J. L., Examining Sources of Error in PCR by Single-Molecule Sequencing. *Public Library of Science ONE* **2017**, *12* (1), e0169774.

58. Taq PLUS DNA Polymerase Protocol. Cat. # 786-850. Geno Biosciences. URL: https://www.gbiosciences.com/image/pdfs/protocol/786-850_protocol.pdf

59. Wäneskog, M.; Bjerling, P., Multi-fragment site-directed mutagenic overlap extension polymerase chain reaction as a competitive alternative to the enzymatic assembly method. *Analytical Biochemistry* **2014**, *444*, 32-7.
60. Bryksin, A.; Matsumura, I., Overlap Extension PCR Cloning. In: Polizzi K., Kontoravdi C. (eds). *Synthetic Biology* **2013**. Methods in Molecular Biology (Methods and Protocols), vol. 1073. Humana Press, Totowa, NJ.
61. Bryksin, A. V.; Matsumura, I., Overlap extension PCR cloning: a simple and reliable way to create recombinant plasmids. *Biotechniques* **2018**, *48* (6).
62. Ricardo, P. C.; Françoso, E.; Arias, M. C., Fidelity of DNA polymerases in the detection of intraindividual variation of mitochondrial DNA. *Mitochondrial DNA Part B* **2019**, *5* (1), 108-12.
63. Brownie, J.; Shawcross, S.; Theaker, J.; Whitcombe, D.; Ferrie, R.; Newton, C.; Little, S., The elimination of primer-dimer accumulation in PCR. *Nucleic Acids Research* **1997**, *25* (16), 3235-41.
64. Chester, N.; Marshak, D. R., Dimethyl Sulfoxide-Mediated Primer Tm Reduction: A Method for Analyzing the Role of Renaturation Temperature in the Polymerase Chain Reaction. *Analytical Biochemistry* **1993**, *209* (2), 284-90.
65. Bustin, S. A.; Benes, V.; Garson, J. A. et. al., The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry* **2009**, *55* (4), 611-22.
66. Bio-Rad, U.S.A. What is Real-Time PCR (qPCR)? **2020**. URL: https://www.bio-rad.com/en-us/applications-technologies/what-real-time-pcr-qpcr?ID=LUSO4W8UU
67. Berrade, L.; Camarero, J. A., Expression Protein Ligation: A Resourceful Tool to Study Protein Structure and Function. *Cellular and Molecular Life Sciences* **2009**, *66* (24), 3909-22.
68. Yu, H.H.; Nakase, I.; Pujals, S.; Hirose, H.; Tanaka, G.; Katayama, S.; Imanishi, M.; Futaki, S., Expressed protein ligation for the preparation of fusion proteins for endotoxin removal and intracellular delivery. *Biochimica et Biophysica Acta (BBA) – Biomembranes* **2010**, *1798* (12), 2249-57.

69. Hauser, P. S.; Ryan, R. O., Expresssion protein ligation using an N-terminal cysteine containing fragment genereated *in vivo* from a pelB fusion protein. *Protein Expression and Purification* **2007**, *54* (2), 227-33.
70. Webb, C. D.; Decatur, A.; Teleman, A.; Losick, R., Use of Green Fluorescent Protein for Visualization of Cell-Specific Gene Expression and Subcellular Protein Localization during Sporulation in *Bacillus subtilis*. *Journal of Bacteriology* **1995**, *177* (20), 5906-11.
71. Sorenson, A. E.; Schaeffer, P. M., A new bivalent fluorescent fusion protein for differential Cu(II) and Zn(II) ion detection in aqueous solution. *Analytica Chimica Acta* **2020**, *1101*, 120-8.
72. Geu-Flores, F.; Nour-Eldin, H. H.; Nielson, M. T.; Halkier, B. A., USER fusion: a rapid and efficient method for simultaneous fusion and cloning of multiple PCR products. *Nucleic Acids Research* **2007**, *35* (7), e55.

Vita

Samuel Wyatt Guy was born in Pilot Mountain, North Carolina on November 5, 1997 to Charles and Wendy Guy. He graduated *Summa Cum Laude* from Appalachian State University with University and Departmental Honors. He has a Bachelor's of Science in Chemistry with a concentration in Biochemistry and a certificate in Forensic Science.

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