AN ABSTRACT OF THE THESIS OF

Carl Peterson for the Master of Science

in Physical Sciences presented on July 11, 2014

Title: Synthesis of Thermostable DNA Polymerase/Fluorescent Protein Chimeras for PCR Study

Abstract approved:_____

(Dr. Kim Simons, Thesis Advisor)

The Polymerase Chain Reaction (PCR) is an invaluable technique in modern biochemistry that uses DNA polymerase enzymes from extremophile microbe species together with thermocycling to amplify a specific sequence of DNA. Another revolution in biochemistry is the use of fluorescent proteins which are reporter molecules of varying hues and intensities. This research combines PCR enzymes and fluorescent proteins. We have found a few fluorescent proteins that exhibit thermostability, refolding properties, or both. We plan to combine PCR polymerase enzymes with a fluorescent protein resistant to high temperature inactivation, specifically tdTomato or ZsGreen. A successful chimera can be used to elucidate the reaction taking place as well as a possible optimization of the processes involved. Four polymerase/fluorescent protein combinations were created with one chimera showing fluorescent activity.

Keywords: PCR, fluorescent proteins, chimeras, DNA polymerase, thermostable

SYNTHESIS OF THERMOSTABLE DNA POLYMERASE/FLUORESCENT PROTEIN CHIMERAS FOR PCR STUDY

A Thesis

Presented to

The Department of Physical Sciences

EMPORIA STATE UNIVERSITY

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

By

Carl David Peterson

July 2014

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Approved by the Department Chair

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Dean of the Graduate School and Distance Education

ACKNOWLEDGEMENTS

First and foremost, I would like to express my gratitude to Dr. Kim Simons. When I walked into his office at the beginning of my graduate studies he had no obligation to take me in as a student (who he had never met) and provide all that he has over the course of my graduate student career. In no conceivable reality could I have expected a better advisor. I would also like to thank the rest of the faculty at Emporia State University, specifically the chemistry department, and I hope that all goes well in the future. Finally, I would like to thank my wife Robyn who has been such an endless source of support and encouragement throughout.

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CHAPTER 1 INTRODUCTION

1.1 POLYMERASE CHAIN REACTION (PCR)

The Polymerase Chain Reaction (PCR) is an indispensable technique for studies involving RNA or DNA.[1] From one DNA template huge numbers of copies of a specific sequence are produced, yielding large quantities to be visualized and manipulated. Costs are minimal, the reaction takes place in a few hours, and the preparation is relatively simple. In the words of Dr. Kary Mullis, the inventor of PCR, the technique "requires no more than a test tube, a few simple reagents, and a source of heat."[2] This ability to amplify a specific DNA sequence has been such a revolutionary technique that Kary Mullis received the 1993 Nobel Prize in Chemistry for its invention.[3] The technique is used for genetic fingerprinting in paternity testing, studies of genetic diseases, forensic science, detection and diagnosis of infectious diseases, and DNA cloning laboratory manipulations.[4] Figure 1.1 shows an overview of three cycles of PCR.

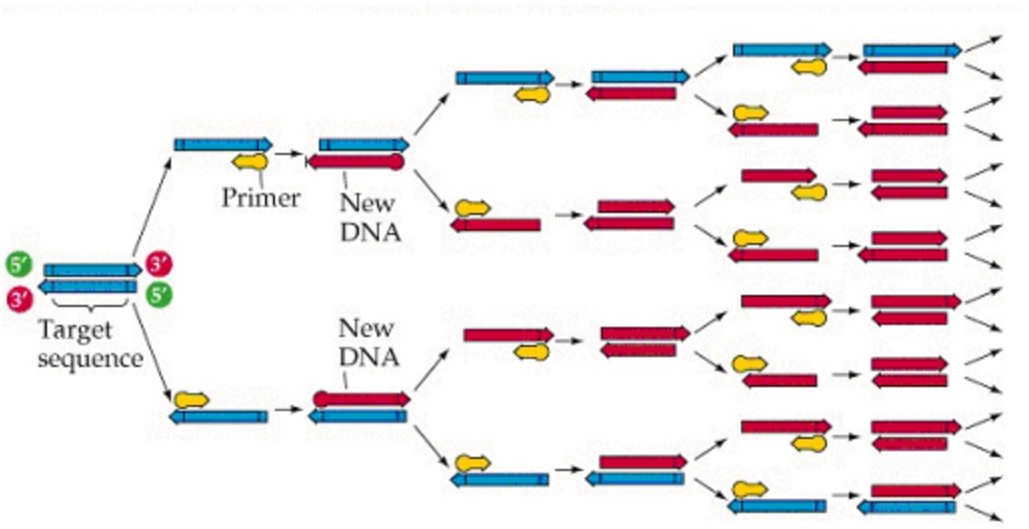

Figure 1.1 Three Cycles of PCR.

The original double stranded DNA segment on the left is undergoing successive copying events (each pair of horizontal arrows) which after many cycles result in exponential amplification of the target sequence. Original template (blue) is separated with heat (diagonal arrows) to allow for the binding of primers (yellow). New copies of DNA sequence are shown in red and the arrow on each DNA sequence shows directionality of antiparallel sequence extension. Copies from the reaction are used as templates in the next rounds of amplification. After 36 cycles there are 2^{36} or ~68 billion copies originating from the initial segment of DNA assuming enough reaction materials are present and 100% efficiency.[5]

For a cycle of PCR, the first step is the dissociation of the double stranded DNA into individual strands by temperature elevation. Then, the temperature is reduced to allow for the binding of primers in a process called annealing. The binding of specifically designed oligonucleotide primer molecules, usually 15-60 nucleotides long, to their complementary sequences on the template at the ends of the target sequence to be amplified serve as anchors for the DNA polymerase molecules. After the primers have bound to the template binding sites at low temperature, the temperature is increased to the ideal range for the DNA polymerase enzyme. When the polymerase molecules bind the primer/template complex, extension of the oligo begins and the template is copied. The DNA polymerase enzyme will bind and begin adding nucleotides to the primer sequence even at lower temperatures, albeit at a slower rate than at optimal temperature for the enzyme. This low temperature extension of the primer sequence increases the avidity of association between the template and its growing complement which keeps them together as the temperature is elevated for optimal polymerase enzyme activity. The polymerase adds to the sequence being extended by polymerizing deoxynucleotide triphosphate (dNTP) molecules to the 3' end of the primer molecule antiparallel with and according to the complementary nucleotides of the sequence being copied. This means it only copies in one direction (Figure 1.2). In PCR, the primers are required for selectivity of the target sequence, and the polymerase is responsible for copying the DNA. Once the polymerase completes a copy of the target sequence, the process is repeated by thermocycling, causing a chain reaction of amplification of the target sequence. This is where the name "Polymerase Chain Reaction" comes from and the more detailed sequence of events can be seen in Figure 1.2.

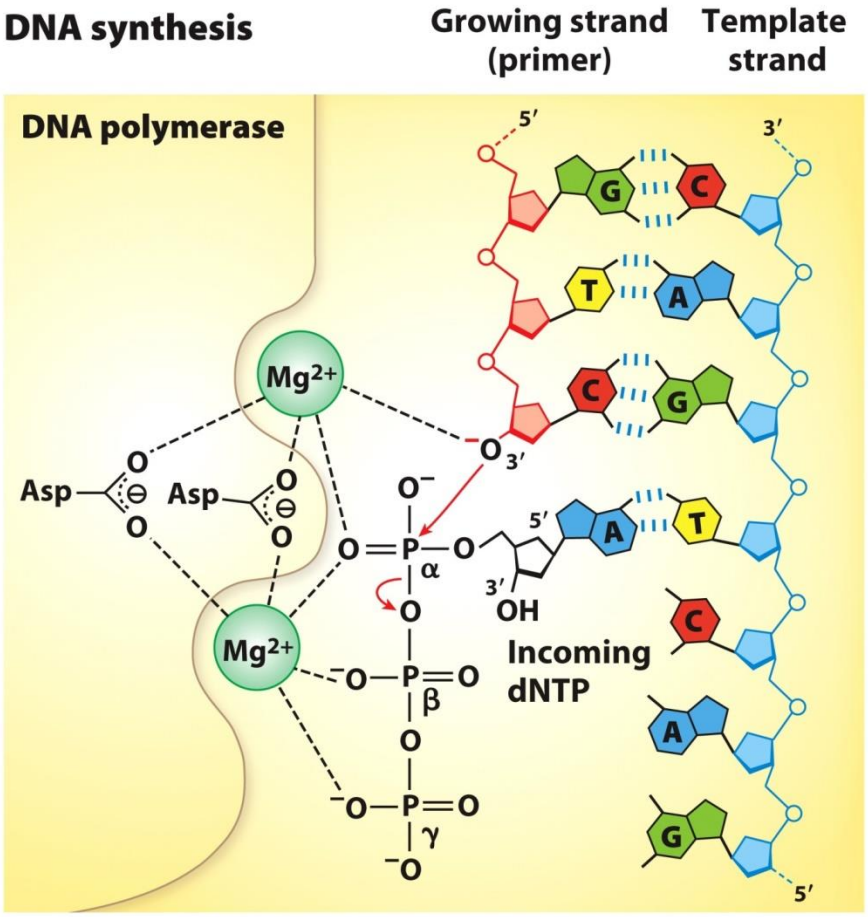

Figure 1.2 Addition of dATP by DNA Polymerase to the Growing Strand.

The DNA template strand (blue deoxy-phosphate polymer) and the geometry of the polymerase active site determine which specific nucleotide molecule can be added to the 3' end of the growing strand (in this case, dATP matching to the thymine). The growing strand associates with the template strand through the additive affinities of multiple hydrogen bonds between complementary base pairs. The DNA polymerase continues down the template strand adding nucleotides to the growing strand by polymerizing the phosphate groups of each incoming nucleotide. Aspartate groups in the active site of the DNA polymerase serve to stabilize the Mg^{2+} ions that catalyze the polymerization reaction.[6]

The original enzyme employed for copying DNA in each cycle of PCR is *Taq* DNA polymerase. This enzyme was first isolated in 1976 from the thermophilic bacterium *Thermus aquaticus* living in the lower geyser basin at Yellowstone National Park. The bacteria lives at sustained temperatures of 50-80°C and was later discovered at other high temperature habitats around the world.[7] *Taq* polymerase operates optimally at 70-72°C, which is prohibitive for most enzymes and its thermostability allows for the double stranded DNA to be first separated by highly elevated temperatures (up to 95°C). Once the single strands are exposed, the temperature is reduced to the working range for primers to bind and the polymerase to begin copying. As a result of *Taq* polymerase being resistant to inactivation from elevated temperatures, the enzyme remains functional through extended thermal cycling. Because of the unique characteristics of *Taq* polymerase and other thermostable polymerases, universities, biotechnology companies, and other organizations spend billions of dollars on them each year.

Another related DNA polymerase protein is *Pfu* polymerase.[8] This protein comes from the archaea species *Pyrococcus furiosus* which was first discovered in 1986 in geothermal ocean vents near Vulcano Island in Italy. This species is classified as a hyperthermophile because it survives at temperatures of 70-103°C and thrives at ~100°C. The use of *Pfu* polymerase for PCR applications has gained favor in recent years because this polymerase has some advantages over *Taq* polymerase including higher fidelity and processivity as well as greater thermostability to resist inactivation after repeated heating and cooling cycles.[9][10] However, *Pfu* polymerase also has a lower extension velocity than *Taq* polymerase so more time must be spent at the copying (elongation) period during thermocycling.[9][11]

1.2 GREEN FLUORESCENT PROTEIN (GFP)

The characterization of Green Fluorescent Protein and the subsequent mutagenic engineering of GFP to produce many fluorescent proteins of varying color, intensity and functional conditions have allowed the fusion of target proteins and fluorescent protein tags with specific known characteristics to track them. Osamu Shimomura was the first to study and characterize GFP beginning in the 1960's, which then allowed Martin Chalfie to clone the GFP gene and use GFP as a protein fusion partner to track intracellular gene expression.[12] Once the relevance of GFP was understood by Roger Tsien he worked to describe the chromophore formation and then create an array of different fluorescent proteins that absorb and emit light at different regions of the spectrum. This group of three researchers were collectively awarded the 2008 Nobel Prize in Chemistry for their work.[13][14] The GFP gene is originally from the jellyfish species *Aequeoria victoria*; however, GFP has been recombinantly produced in many organisms evolutionarily distant from the original species including bacteria, yeasts, and mice. The protein structure of GFP can be seen in Figure 1.3.

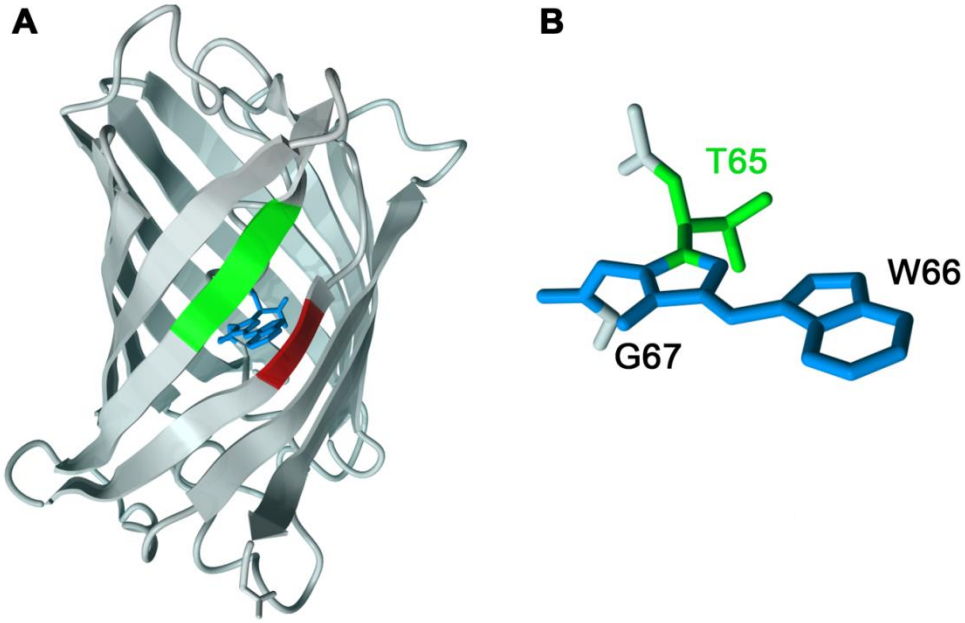

Figure 1.3 Tertiary Structure of GFP and Fluorophore.

(A) Protein structure of GFP (**B**) close-up of the amino acids (threonine, tryptophan, and glycine) constituting the fluorophore.[15]

As seen in Figure 1.3, the tertiary structure of GFP with eleven β-sheets surrounds the covalently bonded 4-(p-hydroxybenzylidine)imidazolidin-5-one chromophore and an α-helix (not shown) running up the axis of the cylinder.[16] The three internal amino acids of Thr65-Trp66-Gly67 autocyclize and conjugate to form the chromophore.[17] The structure of the original fluorescent protein from *Aequeoria victoria*, exhibits certain characteristics that are common to most fluorescent proteins in use today.[18] These characteristics include a barrel shape structure of β-sheets surrounding the internal fluorophore generated from 2 or more amino acids.[18] For a fluorescent protein to have these characteristics there must be a minimum ~700 base pairs for the gene of a monomeric fluorescent protein. Various mutations to the chromophore amino acid composition or protein side chain structure allow for changes in the hydrogen bonding network or electron stacking interactions that affect the emission intensity, color, and photostability of the protein.[19][20]

Other fluorescent proteins used as fluorescent protein tags were discovered in different species including *Zoanthus* and *Discosoma* genera of coral, although the fluorescent protein genes used today for labeling purposes are mainly derived from *Aequeoria victoria*.[21][22] The fluorescent proteins have also often been engineered through either random or site-directed mutagenesis to enhance various desirable characteristics; for example, fluorescence intensity, emission color, stability at pH levels, non-aggregation, or anoxic maturation to list a few.[23][24] The engineered S65T + F64L mutant Enhanced Green Fluorescent Protein (EGFP) is generally held as the gold standard against which all other fluorescent proteins are judged because EGFP has N- and C-terminal attachment permittivity, brightness, resistance to photobleaching, rapid maturation, functionality at

37°C and monomeric properties.[25][26] The common theme among these fluorescent proteins is that they absorb light at a higher energy (often ultraviolet and thus, not visible to the human eye) and emit it at a lower energy within the visible range.[27]

The tagging of cellular proteins with a fluorescent protein to create a "chimera" has been such a revolutionary technique allowing for the equal coexpression of both allowing for the cellular protein to be visualized by the visible light emitted by its tag. This can be used to create fluorescence microscopy images illustrating the localization, concentrations, and even orientations (using different techniques) of these intracellular proteins as seen in Figure 1.4.

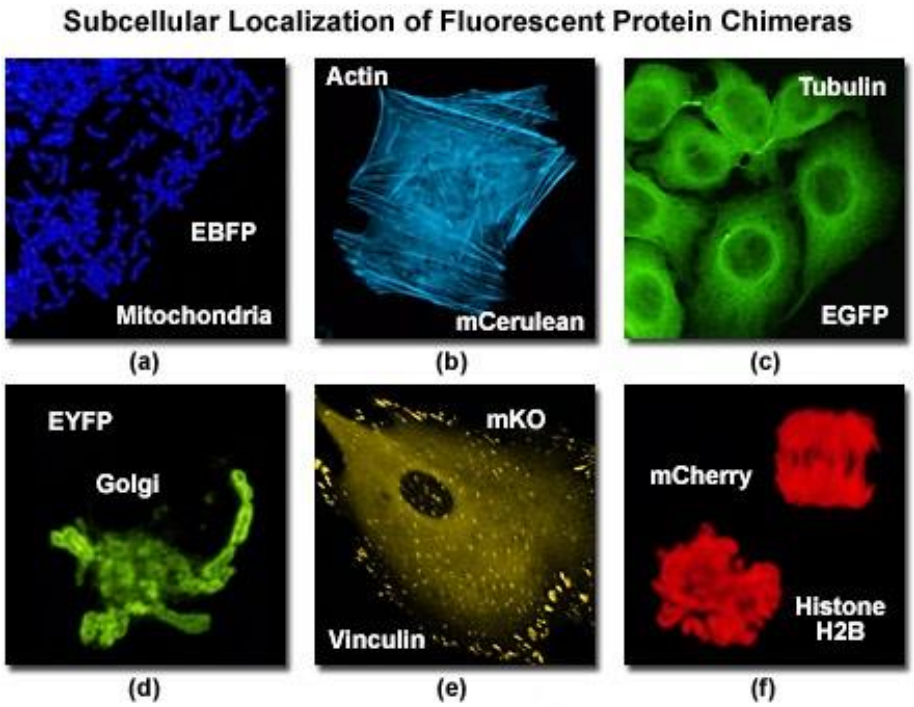

Figure 1.4 Fluorescent Protein/Intracellular Protein Chimera Expression.

Various cellular proteins tagged with a fluorescent protein within the cell. (**a**) A mitochondrial protein labeled with Enhanced Blue Fluorescent Protein (**b**) Actin filament proteins labeled with mCerulean fluorescent protein (**c**) Tubulin proteins tagged with Enhanced Green Fluorescent Protein (**d**) A protein from the Golgi complex tagged with Enhanced Yellow Fluorescent Protein (**e**) Vinculin cytoskeletal proteins tagged with monomeric Kusabira Orange fluorescent protein (**f**) Histone H2B proteins tagged with monomeric Cherry fluorescent protein.[21]

The fusion of the target protein and fluorescent protein to make a chimera is accomplished in a few different ways.[28] The simplest and most obvious method of protein fusion are the transposition of the gene that codes for the fluorescent protein on the N-terminal or C-terminal end of the gene encoding the protein to be tagged. This connection can include a floppy peptide linker sequence, usually of 4-30 amino acids, between them to allow space and eliminate disruptive interactions.[29][30] The success of either orientation is empirically determined with each fusion, although there is information available about the success and failure rate of N- or C-terminal fluorescent protein linkage with many of the available fluorescent proteins from past experimental attempts.[31][32] A unique facet of some fluorescent proteins, for example GFP, is exploited for a less common method of protein chimera synthesis called internal tagging. Because the C-terminal and N-terminal ends of the fluorescent protein are very close in the tertiary structure, GFP can be inserted in the middle of the protein sequence to be tagged, usually in a sequence in an external loop of the tertiary structure.[33] This technique allows the fluorescent protein to be inserted in the middle of the fusion partner, possibly alleviating any structure disrupting interactions that may have been problematic at the other fusion tag attachment points.[34][35] Figure 1.5 shows the relative proximity of amino termini on the tertiary structure of GFP and this property is shown in use for the internal tagging of a target protein with GFP in Figure 1.6.

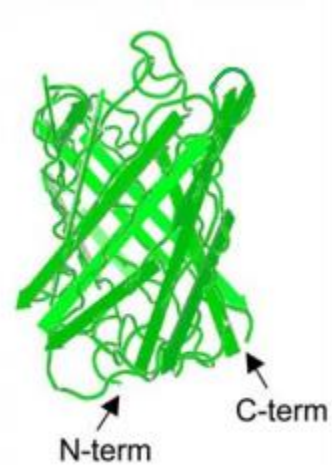

Figure 1.5 Location of Termini in Tertiary Structure of GFP.

Position of terminal amino acids in the fully folded structure of GFP. Termini are oriented close enough in the 3D structure to allow for internal tagging.[28]

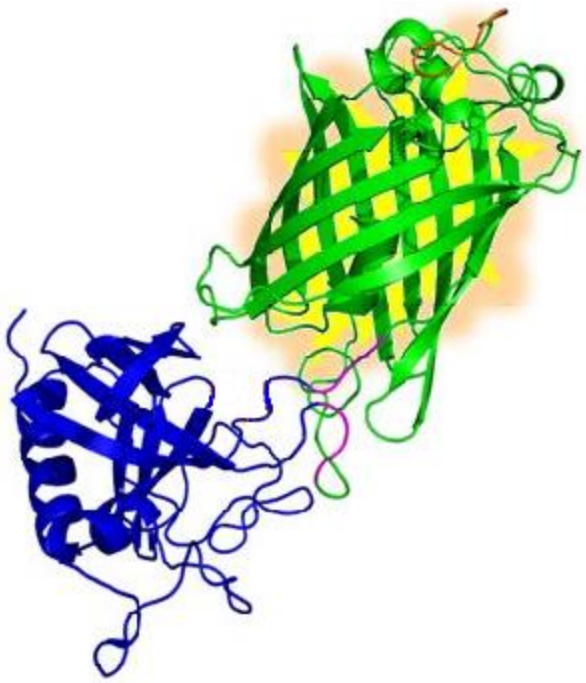

Figure 1.6 Internal Tagging with GFP.

Internal tagging of Green Fluorescent Protein (cpGFP) on a receptor target protein. Fluorescent protein is in green and the target protein is in blue. GFP amino termini are highlighted in purple connecting the two chimera domains. This demonstrates that although the fluorescent protein sequence has been inserted somewhere in the middle of the receptor protein sequence, both individual subunits retain their original functionality.[36]

CHAPTER 2 BACKGROUND

2.1 PLASMID CLONING

Plasmids are circular, double-stranded, extra-chromosomal DNA. Naturally occurring in bacteria and often containing some beneficial gene to the cell, plasmids are duplicated with each cell division along with chromosomal DNA.[34] Extracellular plasmids can also be introduced to bacterial cells, often a strain of *E.coli*, through heat shock/chemical, or electrophoretic treatment. A plasmid vector usually contains an antibiotic resistance gene which will select for cells containing that gene if in a growth environment where the antibiotic is present while any cells without the plasmid vector will not grow. This introduces the possibility of engineering a plasmid vector with a gene insert of interest and cloning a large number of easily purified copies. A generic plasmid vector can be seen in Figure 2.1.

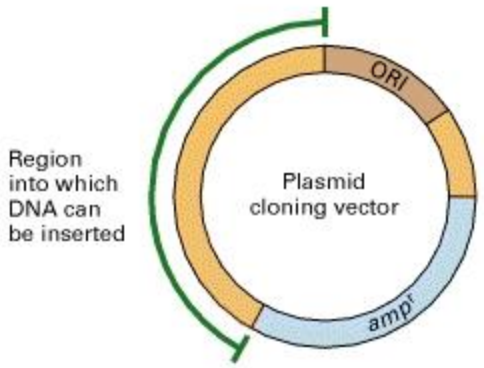

Figure 2.1 A Generic Plasmid Vector.

A plasmid cloning vector is shown containing an origin of replication region (ORI) and a β-lactamase gene (amp^r) which confers ampicillin resistance. Exogenous DNA can be inserted into the remaining region without disturbing the ability of the plasmid to grant drug resistance or be duplicated.[34]

Plasmid cloning vectors are usually engineered with the strategic placement of restriction sites. These sites are often palindromic DNA sequences that are 4-8 base pairs long and are cleaved by a corresponding restriction endonuclease. If the plasmid sequence is known, one can look for the location of enzyme sites and then choose the restriction enzyme that will cleave at the desired location. Depending on the enzyme used to split the DNA, the duplex is left with either blunt ends, meaning the double stranded sequence ends without either strand longer than the other, or with "sticky" ends. If the strand is left with a sticky end, the sequence will only reattach to its corresponding complementary sticky end. Blunt ends will ligate to other blunt ends. This provides a level of specificity during re-ligation. Once a plasmid has been cleaved and the digest fragment purified, a gene insert from a restriction digestion with the compatible cut ends is added, and the entire sequence re-circularized by the action of an enzyme called DNA ligase. Together, this can provide a means to insert a gene of interest into a plasmid. The plasmid is used to transform a strain of bacteria, and then the bacteria are grown in large quantities to acquire a sizeable amount of the plasmid and gene of interest. An overview of this chain of events can be seen in Figure 2.2.

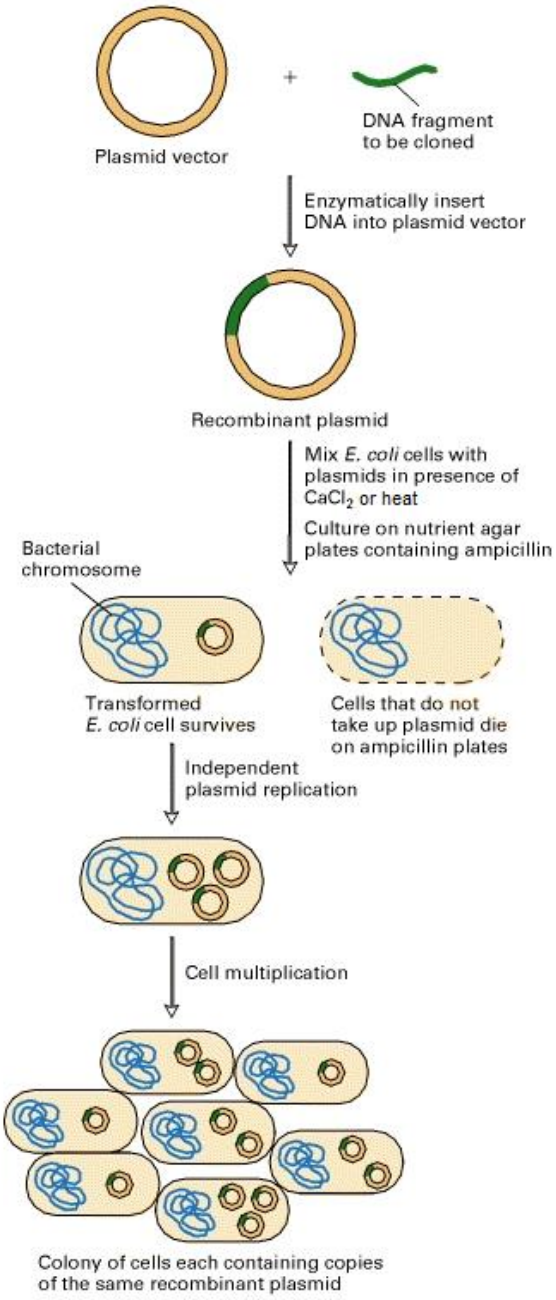

Figure 2.2 Overview of Plasmid Cloning.

A DNA fragment to be cloned is first inserted into the plasmid vector, which also contains a gene for drug resistance (not shown). Once the recombinant plasmid is re-circularized (green insert in the yellow circle), the plasmid vector is used to transform bacteria. Only those bacteria that contain at least one copy of the plasmid vector proliferate.[34]

2.2 ENZYME KINETICS

The kinetics of enzymes is an area of research which includes the study of PCR DNA polymerases.[37] Enzymes are molecular catalysts, usually protein, that facilitate and speed up a reaction while not being consumed themselves in the process. They achieve this by temporarily binding, usually non-covalently, to their substrate and lowering the activation energy required for the reaction. The rate of catalysis is influenced by several factors such as substrate concentrations, temperature, and pH.[38] A DNA polymerase enzyme is the molecule responsible for copying a single strand of DNA by polymerizing incoming single deoxynucleotide triphosphate molecules to the 3' hydroxyl end of the DNA strand. The accurate incorporation of incoming nucleotides onto the growing strand according to complementary base pairing is improved by a 3' to 5' proofreading exonuclease domain that is intrinsic to most DNA polymerases. An increased understanding of the polymerase catalyzed addition of nucleotides during DNA duplication would clarify the mechanisms in base misincorporation and proofreading. With a more detailed mechanism, the fidelity of DNA polymerase enzymes could be optimized, and the nature of some human diseases could be clarified. [39][40][41] Previous studies of DNA polymerase binding and catalysis mechanisms include crystal structure determination, varying reaction conditions, applying substrate analogues, or altering an enzymes active site through site-directed mutagenesis.[42][43]

In a literature search, PCR DNA polymerases have not been fluorescently tagged and studied by fluorescent resonance energy transfer (FRET). Fluorescence is the absorption of light or electromagnetic radiation energy by a substance and the subsequent emission of light, usually at a lower energy. FRET occurs when energy passes from one excited

chromophore molecule (the donor) to a second molecule (the acceptor) through long range dipole-dipole interactions, because the emission spectra of the donor overlaps with the excitation spectrum of the acceptor.[44][45] This transfer has been employed with the tagging of two molecules, one with a FRET donor fluorescent protein and the other with a FRET acceptor fluorescent protein both having known emission wavelengths, so that the molecular interactions of the two tagged molecules can be explored. FRET is a sensitive measurement of inter-fluorophore distance and orientation.[46] The efficiency of this reaction is very low unless the donor and acceptor are very close together (1-10 nm), and varies in proportion to the inverse sixth power of distance separating the donor and acceptor molecules. Molecular orientation also plays an important role in energy transfer efficiency because of the relationship between the emission transition dipole of the donor and the absorption dipole of the acceptor.[47] Fluorescent proteins exhibiting either thermostability or refolding properties could be tagged to molecules involved in the PCR DNA replication process. In this approach, FRET would illuminate protein-DNA interactions as well as conformational changes that go along with base pair addition in the polymerase molecule once associated to DNA. Therefore, FRET is a valuable tool for probing molecular interactions involved in the PCR DNA replication process.

2.3 REFOLDING PROPERTIES OF ZsGreen and tdTomato

The fluorescent proteins chosen for this experiment were tdTomato and ZsGreen. The tdTomato protein is the final product of many rounds of directed mutagenesis from the original tetrameric *Discosoma* fluorescent protein DsRed, and was at first a member of

the mFruits series as dTomato, which manifests itself as an obligate dimer. To exploit desirable characteristics while controlling dimerization, two dTomato units were joined with an optimized 12-amino acid linker.[48] This created a tandem dimer fluorescent protein, tdTomato, which is a very bright fluorescent protein having 283% the brightness of EGFP and an extinction coefficient of 1.38×10^5 $M^{-1}cm^{-1}$, as well as being one of the most photostable fluorescent proteins. Although this pseudo-monomeric protein configuration has some useful characteristics (extremely bright and photostable) the fused dimer also creates a larger fusion tag compared to monomeric fluorescent proteins as shown in Figure 2.3.

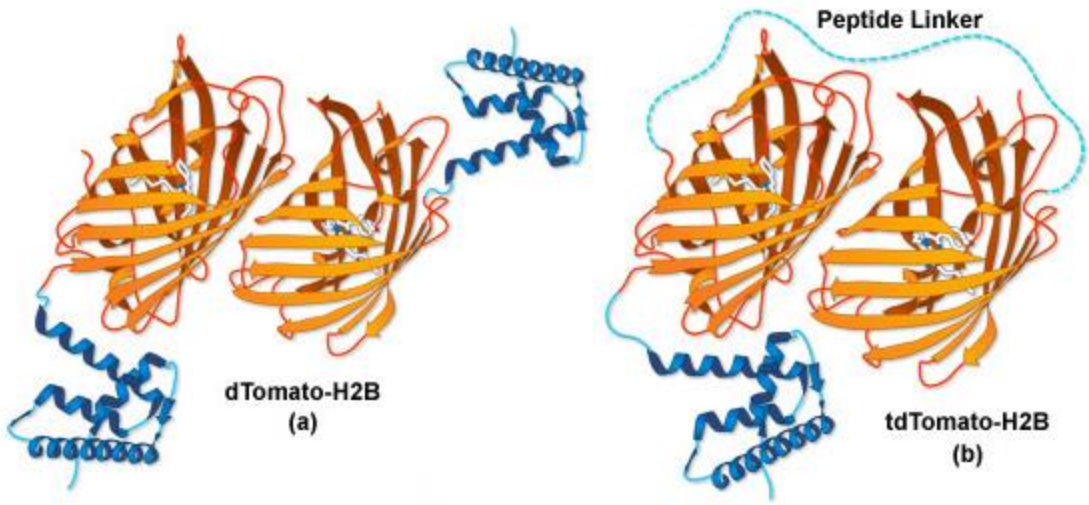

Figure 2.3 Tomato Protein in Dimer and tandem dimer Pseudomonomer Forms.

(a) dTomato fluorescent protein (orange b-barrel) in its obligate dimer form associating to another dTomato attached to a target protein (blue a-helical bundle). **(b)** tdTomato in tandem dimer form with the connecting amino acid bridge creating a pseudo-monomer. The a-helical bundles are monomeric proteins but when connected to dTomato, become dimers.[48]

The second fluorescent protein chosen for this experiment was ZsGreen (also known as zFP506). It is a tetrameric protein and one of the original fluorescent proteins from the *Zoanthus* genus of reef corals. The original protein was transformed into a commercial product by Clontech, who optimized it for expression in mammalian cells, and gave it the trade name ZsGreen.[49] The commercial protein is also one of the brightest green fluorescent proteins with 117% the brightness of EGFP and an extinction coefficient of $4.3x10^4$ $M^{-1}cm^{-1}$.

2.4 OBJECTIVES OF THE RESEARCH

The primary goal of this research work is to synthesize a chimera of a thermostable DNA polymerase fused to a fluorescent protein. Specifically, the tagging of the DNA polymerase enzyme with a fluorescent protein tag that exhibits either thermostability or refolding properties because otherwise the fluorescence would be irreversibly lost at elevated temperatures. Provided that both parts of the chimera retain some level of their original function once fused, this will allow for a study of the polymerase chain reaction. Another possible benefit is the creation of a highly visible PCR enzyme to increase confidence in properly combining various minute volumes of reagents and ensuring that they are adequately mixed before starting the thermocycle.

CHAPTER 3 MATERIALS AND METHODS

3.1 KITS

Plasmid purification was completed using QIAprep Spin Miniprep Kit by Qiagen, Valencia, CA, USA. The protocol included with the kit for plasmid purification was followed using a 2 mL volume of cell culture. Gel extraction DNA purification was completed using QIAquick Gel Extraction Kit by Qiagen, Valencia, CA, USA. Once the desired DNA band had been excised from agarose DNA gel, the protocol was followed according to the gel extraction kit protocol from the company.

3.2 CELL TRANSFORMATION

For all cell culture procedures unless otherwise stated, New England Biolabs 5α *E.coli* cells were used. For plasmid uptake procedures, a 1.5 mL microcentrifuge tube of cells was transferred from the -80°C freezer to ice with the plasmid solutions also stored on ice. In a pre-chilled, sterile 1.5 mL microcentrifuge tube, 20 µL of cells and 2 µL of plasmid DNA were combined. The mixture was allowed to sit in ice for 5 minutes, heat shocked at 42°C for 45 seconds, and returned to ice for 5 minutes. 180 µL LB or SOC media was added, and the tube was shaken at 37°C for 30-60 minutes before being plated and incubated overnight.

3.3 PLASMID AND PROTEIN PREP STARTER CULTURES

For cell volume cultures, 2 mL of LB or SOC media was introduced to a test tube with ampicillin for a final concentration of 1x (100 μg/mL). One colony of bacteria was introduced to the mixture, and the culture was grown overnight for ~18 hours at 37°C on a rotisserie. If being used for plasmid purification, the 2 mL of cell culture was centrifuged at 10,000 RPM in an Eppendorf Centrifuge 5417R for 10 minutes and then, subjected to the plasmid purification procedure. All microcentrifuge tube centrifugations were completed using this model. If being used for protein preparation, the 2 mL culture was introduced to a 50 mL centrifuge containing ampicillin and LB media for a total volume of 50 mL, and the protein purification procedure was followed after the cells had grown.

3.4 TAE BUFFER SOLUTION

To make the 1x TAE buffer a 50x stock solution of TAE was prepared by weighing out 242 g Tris base (FW=121.14 g/mol) and dissolving in approximately 750 mL dH_2O. To this was added 57.1 mL glacial acetic acid and 100 mL of 0.5 M EDTA (pH 8.0). The final volume was adjusted to 1 L by adding dH_2O (distilled and deionized water). This 50x stock solution can be diluted to 1x by adding dH_2O.

3.5 DNA GEL PROCEDURES

For the preparation of agarose DNA gels, a solution of 100 mL of 1x TAE and 0.8 g of agarose powder from Fischer Bioreagents was mixed to generate a 0.8% agarose gel. This was heated in a microwave until fully dissolved. Once cooled to near room temperature, 5 µL of 10 mg/mL ethidium bromide solution was added, and the mixture poured into a ThermoScientific gel system consisting of a gel tray and lane comb seated in an Owl Easycast B1 gel trough. This was allowed to cool until gel had congealed. The gel in its tray was turned and covered with 1x TAE solution also containing 5 µL ethidium bromide solution, and the lane comb was removed. The samples were loaded into individual lanes, the electrodes connected, and the samples electrophoresed at 60-120 V until band separation occurred. Lower voltage creates higher band definition while higher voltage causes faster band migration.

3.6 DIGEST PROCEDURES

DNA and protein concentrations after purification were determined using a ThermoScientific Nanodrop 2000 spectrophotometer. For restriction digest reactions, enzymes were selected according to desired cleavage sites on a plasmid map or primer design. Once enzymes had been selected, reagents were combined in a Eppendorf 1.5 mL microcentrifuge tube with amounts of: 200 ng DNA, 2 µL buffer, 2 µL 10x BSA, 0.5 µL of each enzyme, and filled to a total of 20 µL with dH_2O. The enzymes were added to the solution last and the reaction was incubated for ~1 hour at 37°C.

3.7 PCR PROCEDURE

The following reagents were mixed for PCR: 0.5 µL DNA template, 0.5 µL each of 10 µM forward and reverse oligonucleotide, 12.5 µL OneTaq Master Mix, and filled with dH_2O for a total volume of 25 µL for one reaction in a 200 µL PCR tube. 4-5 replicate tubes were prepared and then combined after the thermocycling in a BIO RAD MJ Mini Personal Thermal Cycler.

3.8 PROTEIN PURIFICATION

Purification of protein began with 50 mL volume of transformed cells containing the fluorescent protein gene. Once the cell culture had reached an optical density between 0.6 and 1.0, 50 µL of IPTG was added to a final concentration of 1 mM to induce gene expression. IPTG activates the transcription of T7 polymerase, which will transcribe the fluorescent protein gene. Four to six hours after induction by IPTG, cell cultures are removed from the incubator and transferred to a 50 mL conical tube. Cells were centrifuged for 10 minutes at 10,000 RPM, the supernatant was decanted and the cell pellet was frozen at -80°C. The cell pellet was resuspended in 10 mL of 20 mM Tris (pH 7.5) with 200 mM NaCl buffer and lysed by sonication using a XL2000 Misonix Branson sonifier. A one minute sonication at 15 Watts was performed every three minutes for a total of three cycles. Cells were placed on ice between each sonication. After sonication, cells were frozen at -80°C for approximately 1-2 hours. The lysate was clarified by centrifugation at 15,000 rpm for 5 minutes. This step separates insoluble cellular debris from soluble protein. The cell pellet was discarded, and the supernatant was purified by

running through a series of ammonium sulfate (AMS) cuts. Each AMS cut consisted of dissolving enough AMS in the protein solution to "salt out" different protein fractions, starting at 40% AMS (calculated using http://www.encorbio.com/protocols/AM-SO4.htm). The solution was dissolved completely at 4°C and centrifuged at 3000 RPM for 10 minutes with a Sorvall T 6000D centrifuge by DuPont. The pellet was kept, and in another centrifuge tube, more AMS was added to equal a 60% solution to the remaining liquid portion. After dissolution at 4°C, this was again centrifuged. The process was repeated for the final 80% AMS cut, and the 60% AMS cut pellet was used for further purification via a hydrophobic interaction chromatography column using butyl-sepharose resin as column packing. The 60% AMS cut was used because it was the most brightly colored implying the highest concentration of fluorescent protein. The pellet was reconstituted in 1 M AMS buffer and loaded into the column. Once protein was bound to the column, the protein was subjected to a series of 100%, 80%, and 60% 1M AMS elutions to purify the protein. The 100% AMS elution was the brightest, implying the highest fluorescent protein content, and so was used for the fluorimeter testing. A 20 μL sample was taken at each purification step for SDS-PAGE analysis.

3.9 FLUORIMETER

Fluorimeter readings were completed with a Horiba Jobin Yvon FluoroMax-4 Spectrofluorometer. A four-sided quartz cuvette containing a magnetic stir bar was filled with protein sample solution and placed into the peltier sample temperature regulator. The fluorimeter was programmed to collect a spectrum emission scan from 25°C to 85°C

with 2.5° increments and then once more after returning to 25°C. The temperature tolerance was set at 1°C, and 2 minutes of equilibration time were allowed before each scan.

3.10 SDS-PAGE PROCEDURES

Preparation of a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel involved the combination of 5 mL 12.5% EZ-Run Gel Solution, 20 µL tetramethylethylenediamine (TEMED), and 50 µL 10% ammonium persulfate (APS). The mixture begins to polymerize rapidly once combined, so the solution was quickly pipetted into a BIO RAD Mini-PROTEAN II glass plate sandwich to which a comb was added immediately after filling. The gel was allowed to polymerize for 30 minutes and loaded into a BIO RAD Mini-PROTEAN Tetra System. The reservoir was filled with 1x Ez-Run solution (proprietary solution from Fisher Scientific) until the gel was covered (approximately 600 mL). For each gel sample, 20 µL of the protein solution was combined with 10 µL SDS loading dye and boiled for one minute. Samples are then ready for loading into gel well lanes. The gels were electrophoresed at 75 mV until the dye front reached the bottom of the gels.

CHAPTER 4 RESULTS

First, fluorescent proteins were chosen according to ease of plasmid cloning, brightness, and availability. Each fluorescent protein was cloned and purified. After determining the excitation and emission maxima for each protein, experiments were conducted to determine the level of fluorescence retention at higher temperatures and after the return to ambient temperature. Once the fluorescent tags with heat resistance were identified, the goal was to create a functional fluorescent protein-PCR polymerase chimera. Plasmid vectors were designed to contain either the polymerase or the fluorescent protein and amplified using plasmid cloning techniques outlined in Chapter 3. Gene inserts were created using PCR with oligonucleotides engineered to include restriction sites for insertion into the plasmid vector. The plasmid and gene insert were each treated with the selected restriction enzymes, gel purified, and combined in a ligation reaction to complete the plasmid. The cloning products were checked using digest reactions and a DNA gel, sequenced, and the genes on each plasmid were expressed in bacteria and the bacteria were checked for fluorescence.

4.1 FLUORESCENCE CHARACTERIZATION

The first experiments were the purification and fluorescence characterization of the fluorescent proteins. Each protein was purified according to the protocol detailed in section 3.8, and the fluorescence was measured according to section 3.9 of Materials & Methods. The polyacrylamide gel of tdTomato protein purification is shown in Figure 4.1. The excitation and emission maxima graphs for tdTomato are shown in Figures 4.2 and 4.3 respectively. For tdTomato, maximum excitation was 535 nm and maximum

emission was 591 nm. These values will be used to set scan parameters in the temperature varying fluorescence spectroscopy portion of the experiment.

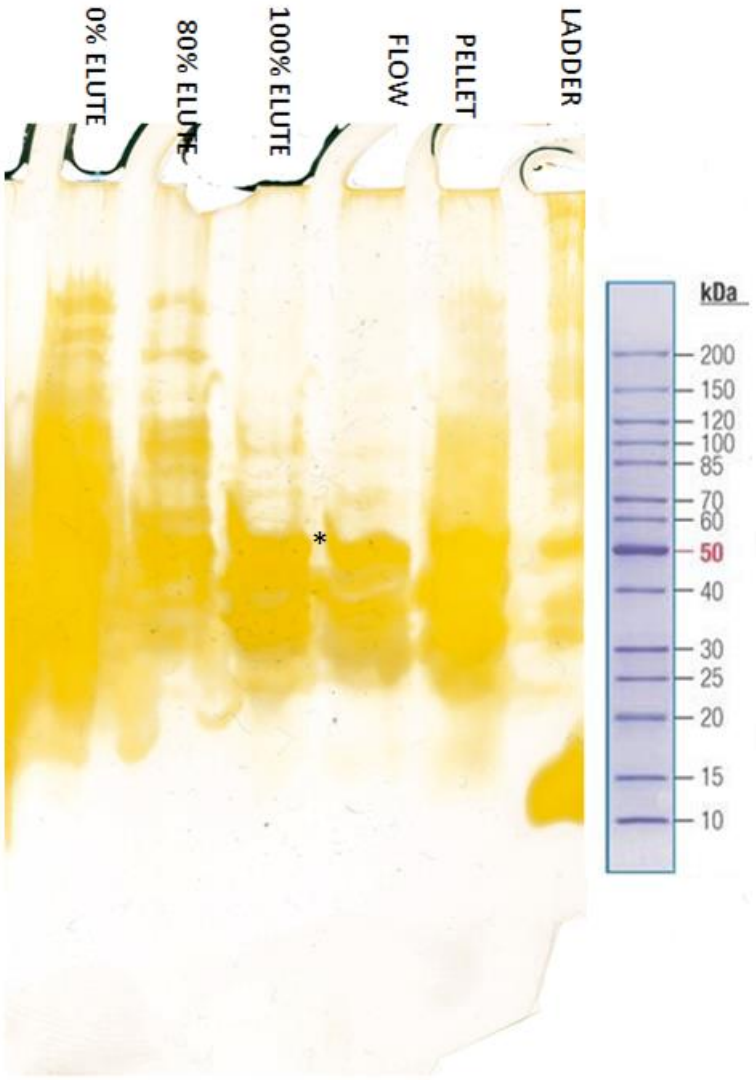

Figure 4.1 SDS-PAGE gel of tdTomato Purification.

The ladder used for this gel was EZ-Run Rec protein ladder, and the labeled band sizes are shown in kDa (kiloDaltons) to the right. The cell lysate before pelleting is shown in the pellet lane. The flow-through from the HIC column is the "FLOW" lane, and the 100%, 80%, and 0% AMS elutions are shown. An asterisk is placed by the anticipated 57 kDa size band for tdTomato.

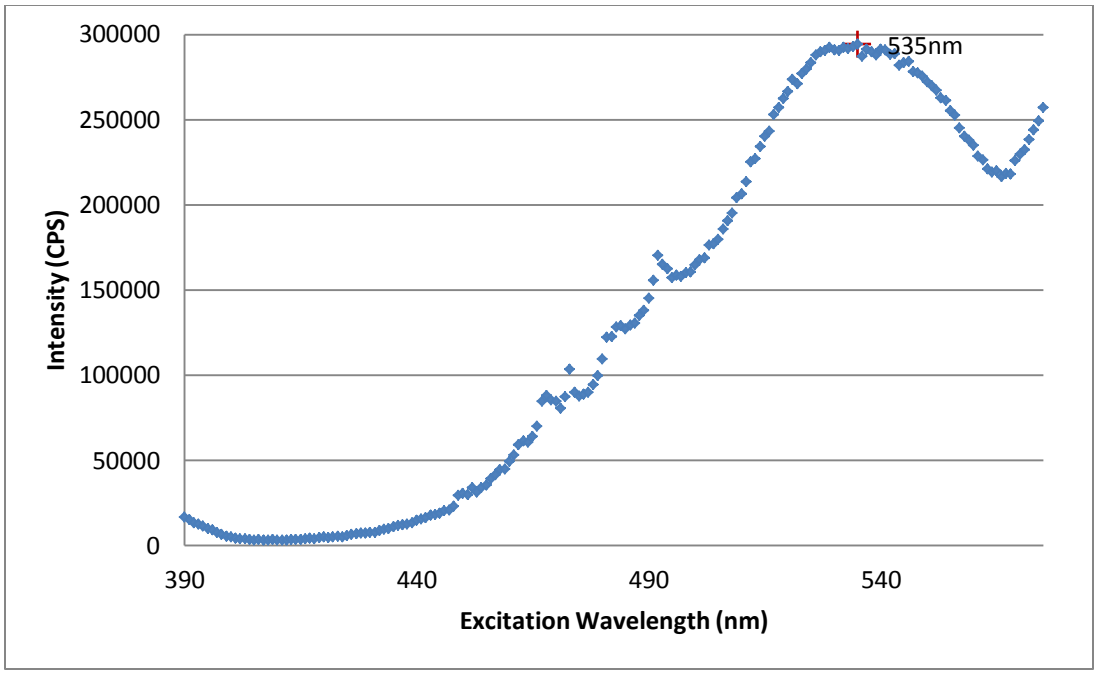

Figure 4.2 tdTomato Excitation Scan.

Excitation scan, from 390 to 575 nm, measured in counts per second (CPS). The excitation maximum at 535 nm for tdTomato is indicated by the red plus sign.

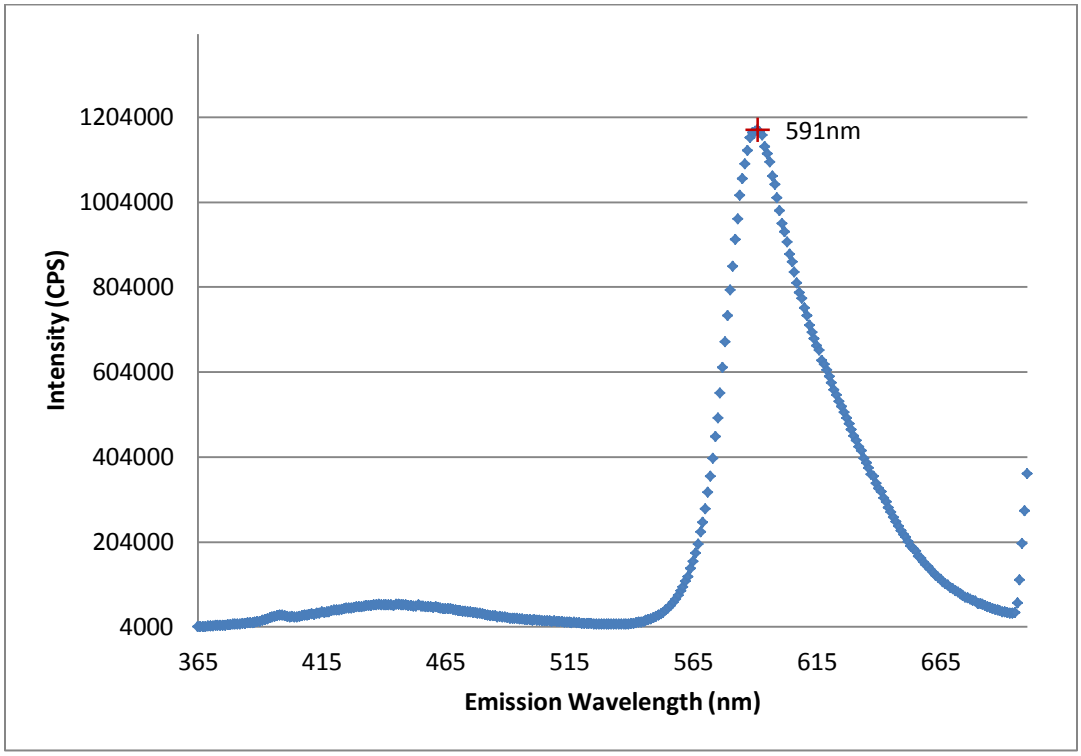

Figure 4.3 tdTomato Emission Scan.

Emission scan, from 365 to 700 nm, measured in counts per second (CPS). The emission maximum at 591 nm for tdTomato is indicated by the red plus sign.

DsRed2, tdTomato, and ZsGreen fluorescent proteins were analyzed at increasing temperatures using fluorescence spectroscopy. The fluorescence *versus* temperature plot for DsRed2 (Figure 4.4) showed 8.4% fluorescence return after the temperature change. The tdTomato protein showed 46.4% fluorescence return after the temperature change (Figure 4.5). ZsGreen showed 57.3% fluorescence at 85°C and 95.3% of initial fluorescence retention at 25°C after exposure to higher temperatures (percentages compared to initial 25°C reading, Figure 4.6).

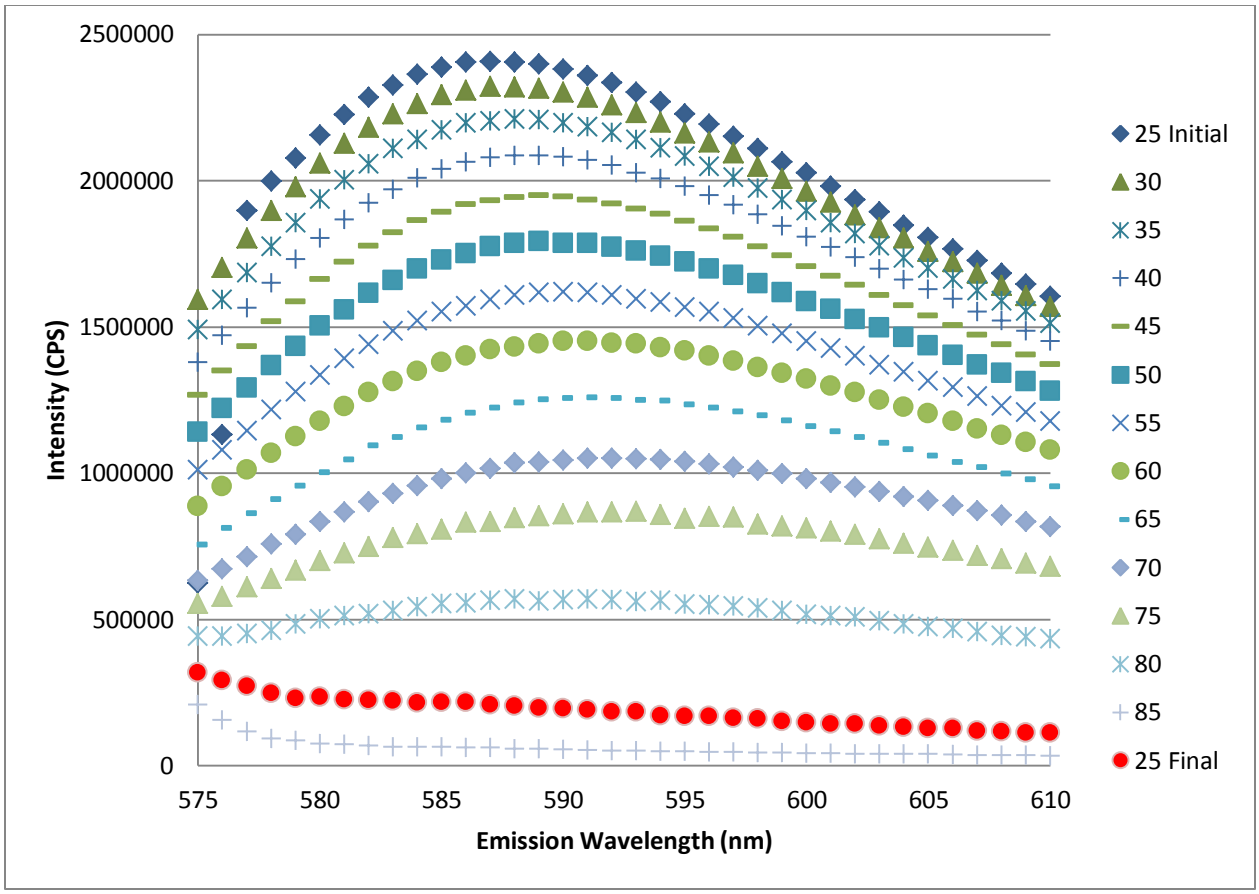

Figure 4.4 DsRed2 Fluorescence Intensity at Various Temperatures.

Emission scans, from 575 to 610 nm, measured in counts per second (CPS) with an excitation wavelength at 563 nm. Each scan is measured at a different, increasing temperature from 25°C up to 85°C with 2.5° increments between each scan. Only 5° increments are shown. The final scan (seen in red) is at 25°C again after the increasing temperature scans have been completed.

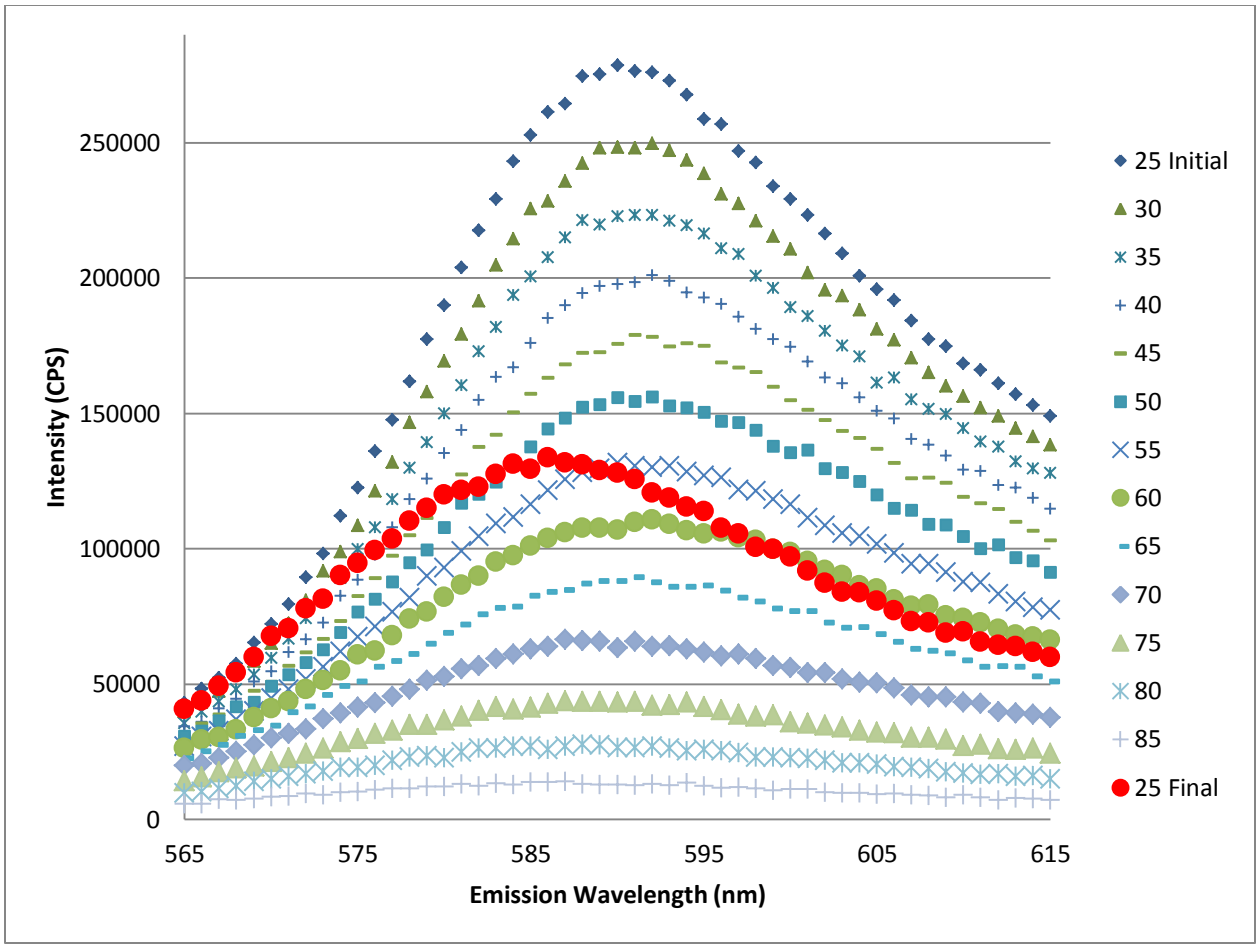

Figure 4.5 tdTomato Fluorescence Intensity at Various Temperatures.

Emission scans from, 565 to 615 nm, measured in counts per second (CPS). The experiment was conducted the same as in Figure 4.4.

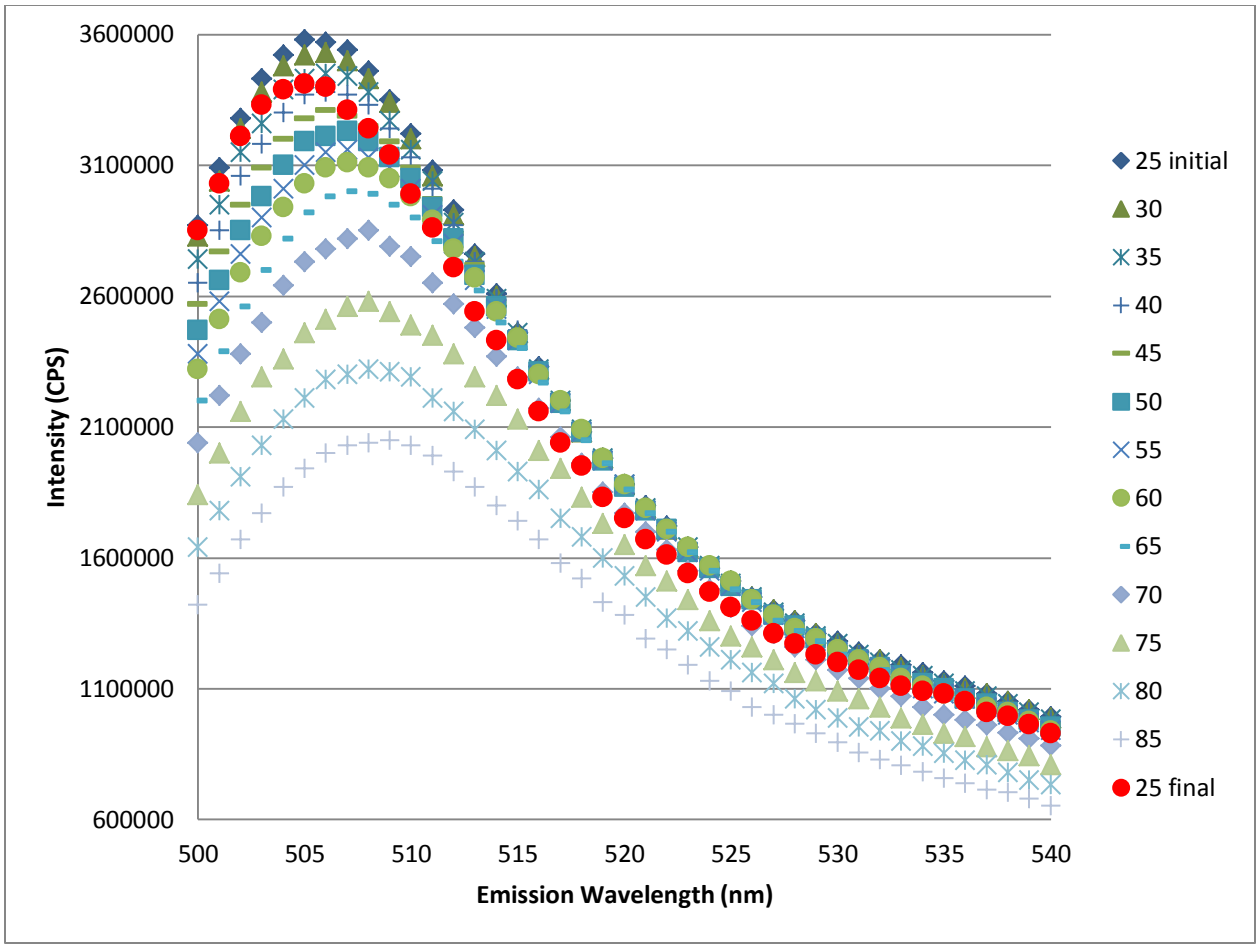

Figure 4.6 ZsGreen Fluorescence Intensity at Various Temperatures.

Spectrum scans from, 500 to 540 nm, of emission intensity measured in CPS. The experiment was conducted the same as in Figure 4.4.

4.2 CHIMERA PRODUCTS

The first chimeras completed were the tdTomato fluorescent protein gene attached to the N- and C-terminal ends of the *Pfu* DNA polymerase gene. For the N-terminal tdTomato fusion, the tdTomato gene insert was created using PCR with primers engineered to include restriction sites for BspHI and NdeI. Once the insert was digested using BspHI and NdeI restriction enzymes, the pET16b-Pfu plasmid was cleaved with restriction enzymes NcoI and NdeI in another tube. BspHI and NcoI cleavage ends are compatible for re-ligation, but the restriction sites are destroyed when ligated. The plasmid was then combined with the insert and ligase to complete the plasmid vector construct. Plasmid maps individually containing the *Pfu* polymerase and the fusion construct are shown in Figure 4.7.

For the C-terminal tdTomato fusion, the *Pfu* DNA polymerase gene insert was created using PCR with primers engineered to include restriction sites NheI and NdeI. After the pRSET plasmid was cleaved with restriction enzymes NheI and NdeI, the plasmid was combined with the insert and ligase to complete the plasmid vector construct. The pRSET plasmid containing the tdTomato gene is shown in Figure 4.8**(a)**, and the plasmid after addition of *Pfu* polymerase gene insert is shown in Figure 4.8**(b)**.

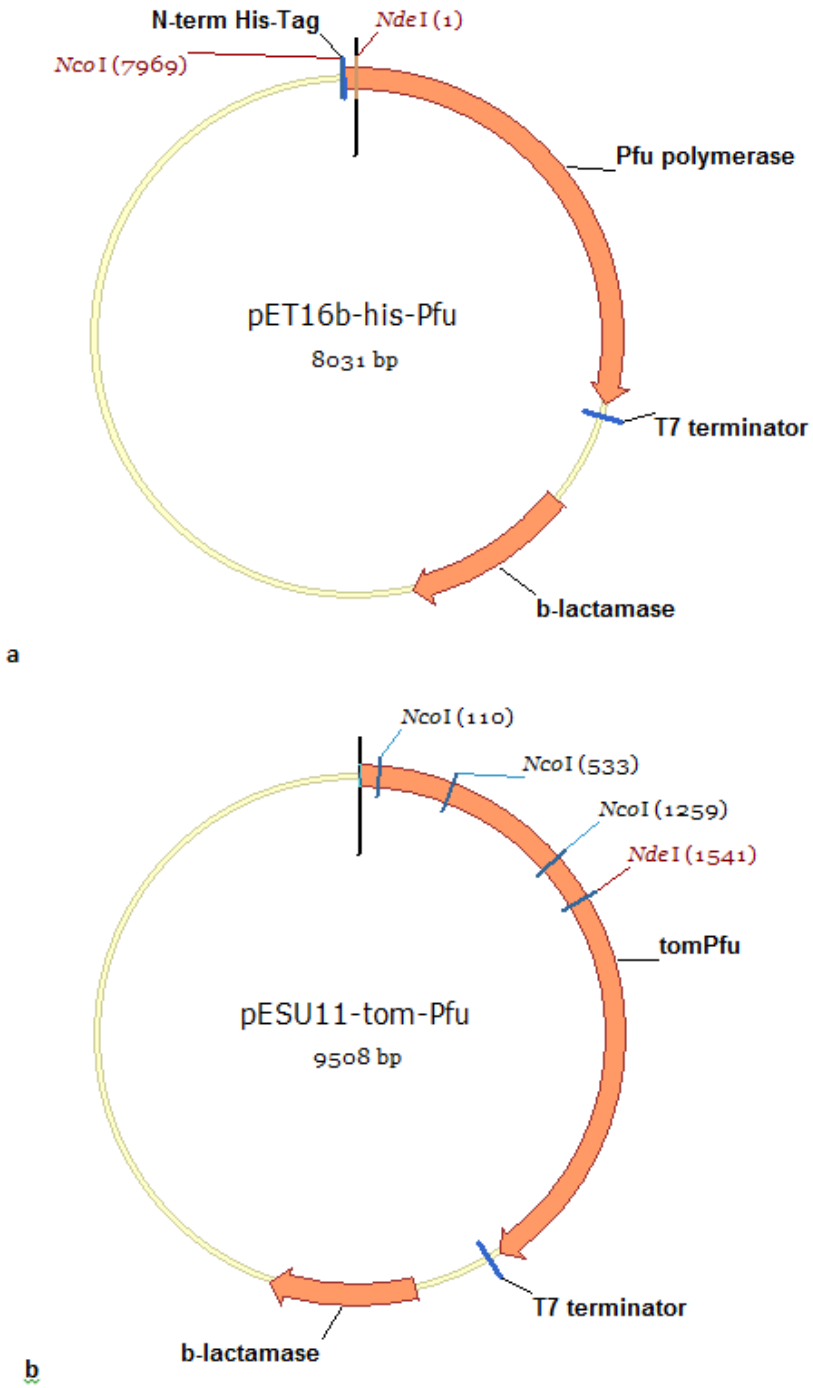

Figure 4.7 Plasmid Maps Containing *Pfu* Polymerase and the Chimera Construction.

(a) pET plasmid map with *Pfu* DNA polymerase gene. Sites printed in red are unique, and names printed in black have more than one site. Open reading frames are shown with orange arrows. **(b)** pET plasmid map with N-terminal tdTomato gene insert on *Pfu* DNA polymerase gene (tomPfu). The b-lactamase gene is also visible and confers ampicillin resistance when expressed.

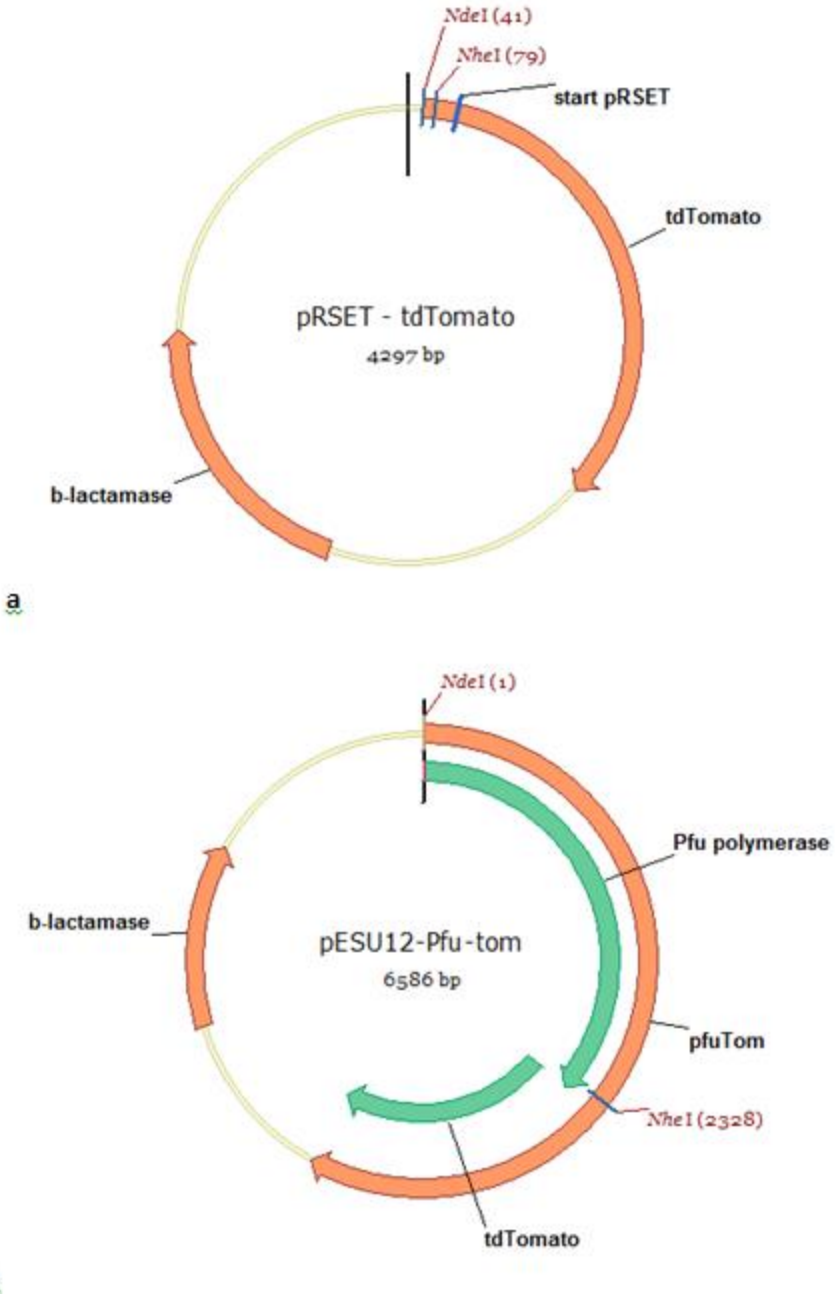

Figure 4.8 C-terminal tdTomato on *Pfu* Polymerase Plasmid.

(a) pRSET plasmid map with tdTomato gene. **(b)** pRSET plasmid map with C-terminal tdTomato gene after *Pfu* DNA polymerase gene insert (Pfutom). Individual *Pfu* polymerase and tdTomato genes are indicated with green arrows.

Each DNA segment after being treated with restriction enzymes was analyzed using an agarose gel to ensure correct band sizes. This gel can be seen in Figure 4.9 after the DNA bands had been excised for gel purification. Once both chimera plasmids had been created, they were purified and digested to check for correct band sizes. A DNA gel of the C- and N-terminal chimera plasmid digests can be seen in Figure 4.10. Figure 4.11 shows a DNA gel of various restriction digest reactions to verify the identity of C-terminal tdTomato on *Pfu* polymerase chimera gene construct.

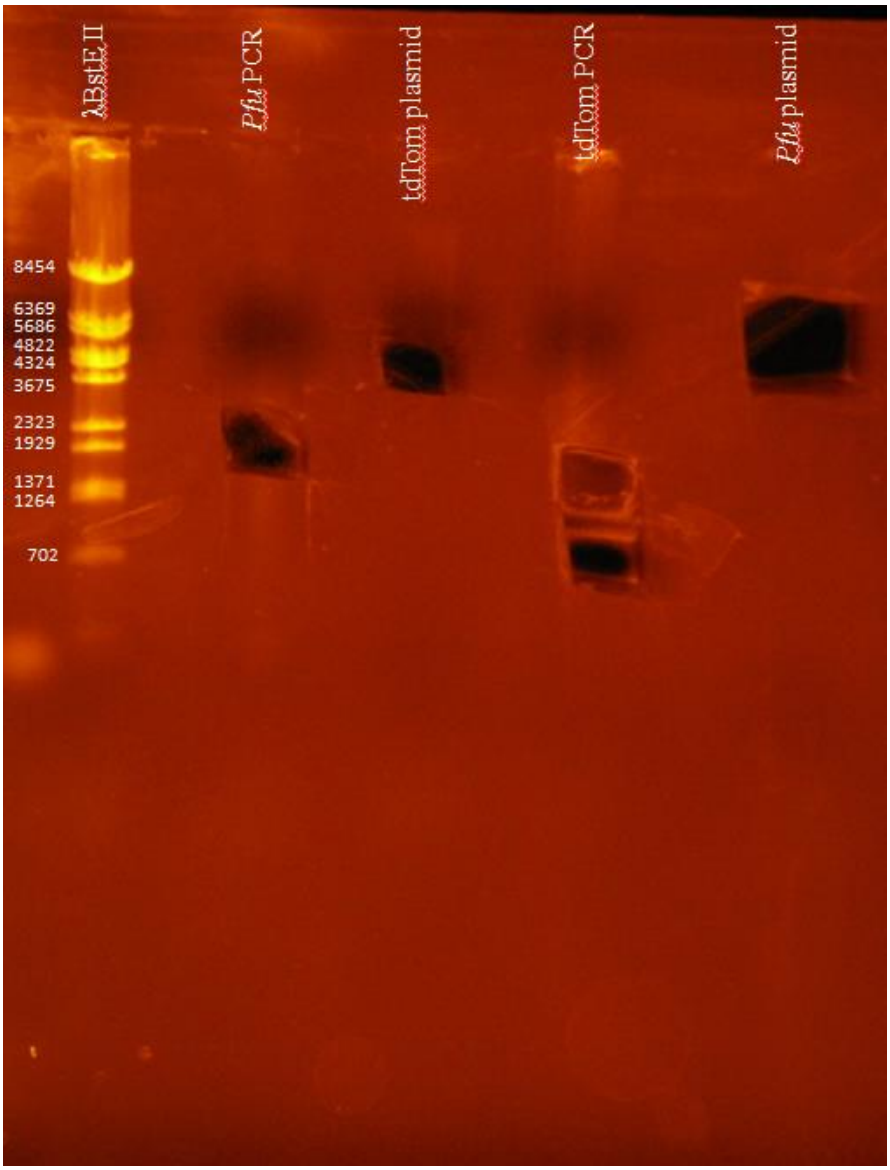

Figure 4.9 DNA Gel of Plasmid Components for Chimera Construction.

The ladder used was Lambda-DNA BstE II digest by New England Biolabs and is on the left. To the right of the ladder was the *Pfu* PCR product band at 2289 bp, the pRSET plasmid containing tdTomato band at 4297 bp, the tdTomato PCR product bands at 1477 bp and around half that size for the non-tandem dimer PCR byproduct. The lane farthest right contained the pET plasmid containing the *Pfu* polymerase gene band at 8031 bp.

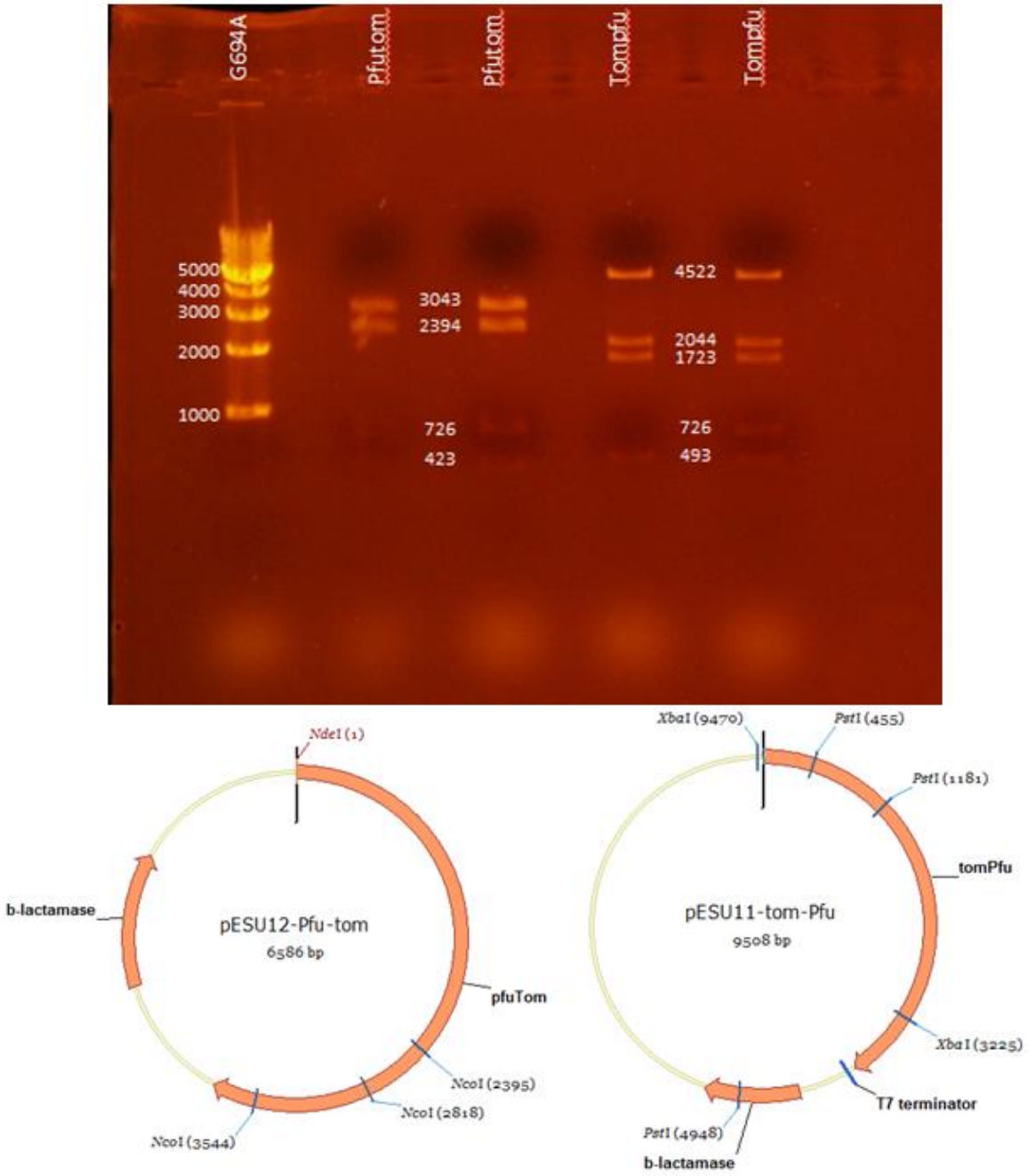

Figure 4.10 DNA Gel of Plasmid Digests.

The G694A 1 kbp step ladder by Promega is farthest left. The next occupied lanes to the right are both pRSET plasmids with C-terminal tdTomato on *Pfu* polymerase digested with NcoI and NdeI. The two right-most lanes are both pET plasmids with N-terminal tdTomato on *Pfu* polymerase digested with XbaI and PstI. The sizes listed are the expected band sizes if the ligation was successful. Digest sites NdeI and NcoI have been included on the pESU12-Pfu-tom plasmid map to show sequence locations. Digest sites PstI and XbaI have been included on the pESU11-tom-Pfu plasmid map to show sequence locations.

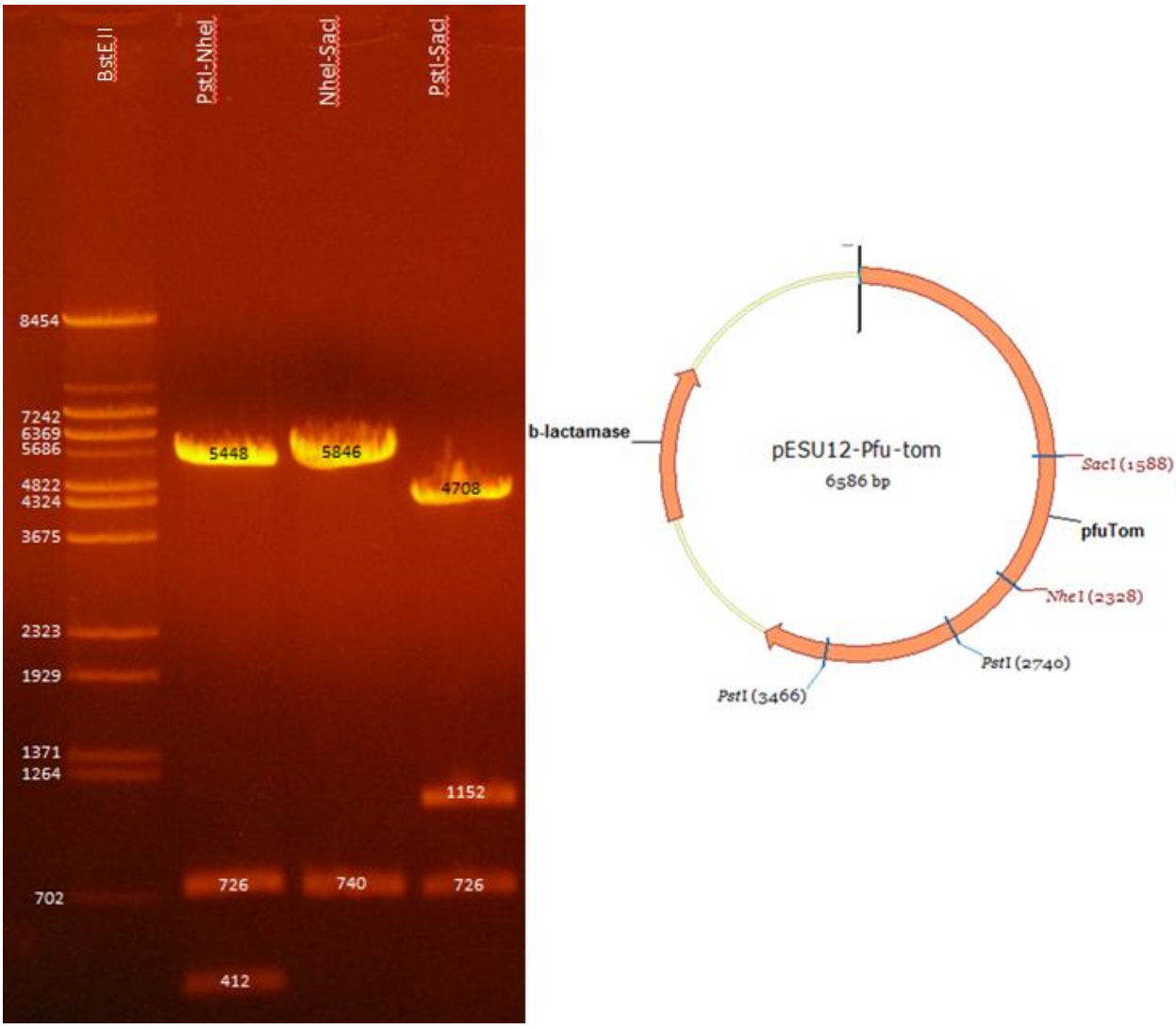

Figure 4.11 DNA Gel of Pfu-tom Plasmid Digests.

The Lambda-DNA BstE II digest can be seen on the left side of the gel. All other lanes are the Pfu-tom plasmid after digestion by the restriction enzymes indicated at the top of each lane. Expected band sizes are labeled for each band. The pESU12-Pfu-tom plasmid map is shown on the right with restriction sites to show sequence locations.

Once both chimera constructs had been created, they were used to transform cells, and the cells were plated on ampicillin plates. The N-terminal tdTomato construct conferred antibiotic resistance when compared against the negative control plates; however the colonies were not fluorescent red. The C-terminal tdTomato gene construct showed plasmid expression when compared against the negative control and had red colonies implying a functional fluorescent protein (Figure 4.13).

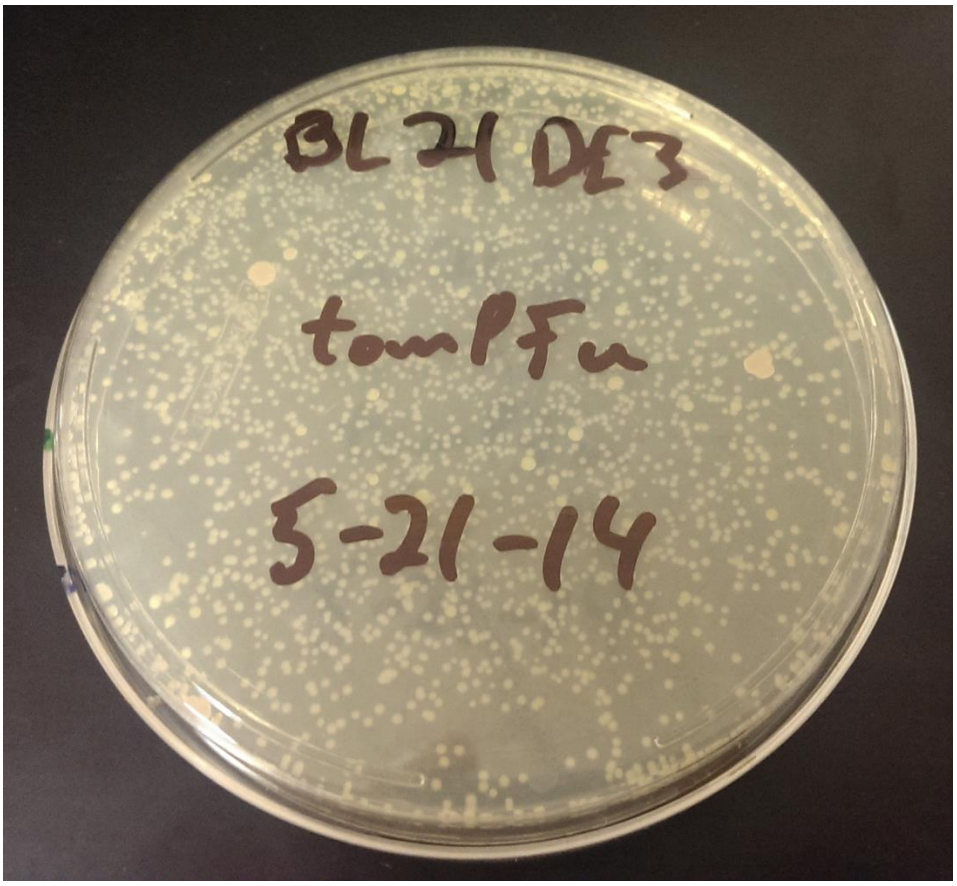

Figure 4.12 Colonies with N-terminal tdTomato on *Pfu* Polymerase.

Most colonies contain the N-terminal tdTomato and *Pfu* polymerase plasmid (data not shown). Colonies were the normal color of bacteria instead of fluorescent red.

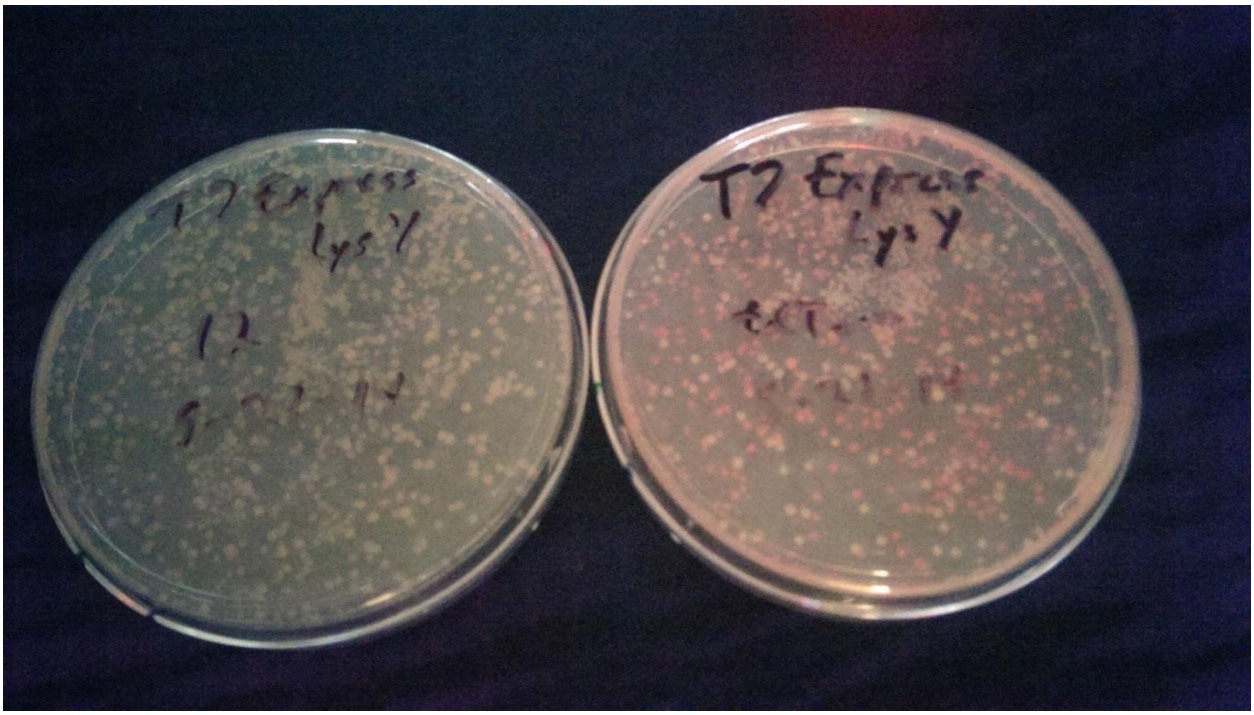

Figure 4.13 tdTomato and C-terminal tdTomato Plasmid Colonies.

On the right, colonies containing the tdTomato gene can be seen. On the left, colonies containing the C-terminal tdTomato on *Pfu* polymerase plasmid can be seen. Plasmid colonies were fluorescent red, although less bright than the tdTomato colonies.

The fusion construct of C-terminal tdTomato on the *Pfu* polymerase gene plasmid was sequenced by SeqWright DNA Technology Services. Sequence analysis revealed that the fluorescent gene had no mutations, but there was an Y217H mutation in the DNA polymerase gene compared against the wild-type sequence. Attempts were made to excise the mutated portion of the *Pfu* polymerase gene and replace it with a non-mutant section from another *Pfu* polymerase gene, but no simple replacement was available. This approach was put on hold for the pursuit of another strategy.

The next attempt involved using ZsGreen and ZsMutant as a C-terminal tag on *Pfu* polymerase. A gene insert of *Pfu* DNA polymerase was created using PCR with restriction sites for XhoI and BamHI engineered into the oligonucleotide primers. The pQE-30 plasmid containing the ZsGreen or ZsMutant gene was digested with XhoI and BamHI. Because there is an internal BamHI site on the *Pfu* polymerase gene, EcoRI was used to separate sections of the insert. The insert was digested in two parts with 1) EcoRI and XhoI to isolate a 1263 bp segment and 2) EcoRI and BamHI for a 1353 bp segment. Figure 4.14 shows the *Pfu* polymerase digest DNA gel after excision of the 1263 bp band.

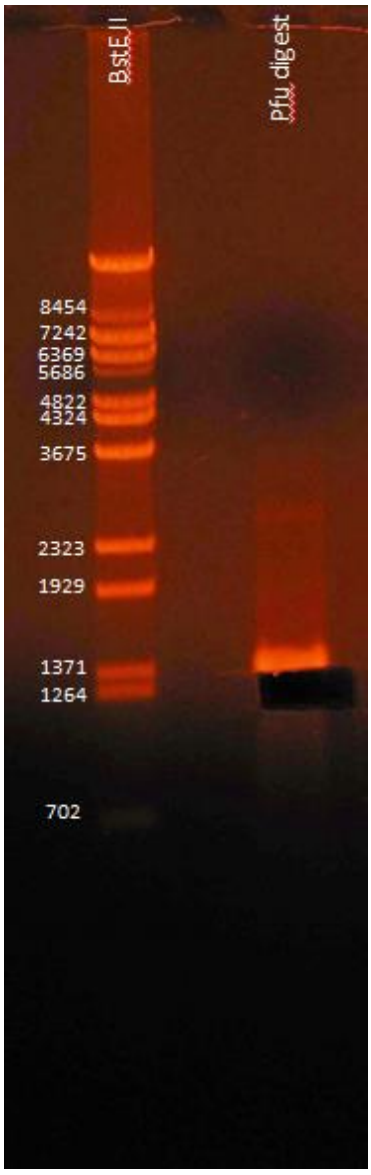

Figure 4.14 DNA Gel of *Pfu* Polymerase Digest.

The left lane Lambda-DNA BstE II digest ladder from New England Biolabs. The right lane is the *Pfu* polymerase gene after digestion with XhoI and EcoRI. The larger band is 1353 bp. The excised portion is 1263 bp.

The plasmid vector was combined with the two sections of the insert (XhoI to EcoRI and EcoRI to BamHI) and ligated to close the plasmid.

The C-terminal ZsMutant on *Pfu* polymerase plasmid was created according to the same procedure used with ZsGreen except using the pQE-30 ZsMutant plasmid as shown in Figure 4.15. Both the C-terminal ZsGreen and the C-terminal ZsMutant on *Pfu* polymerase were used to transform bacteria. Both plasmid constructs conferred antibiotic resistance when compared against the negative control plates; however, the colonies were not fluorescent green (data not shown).

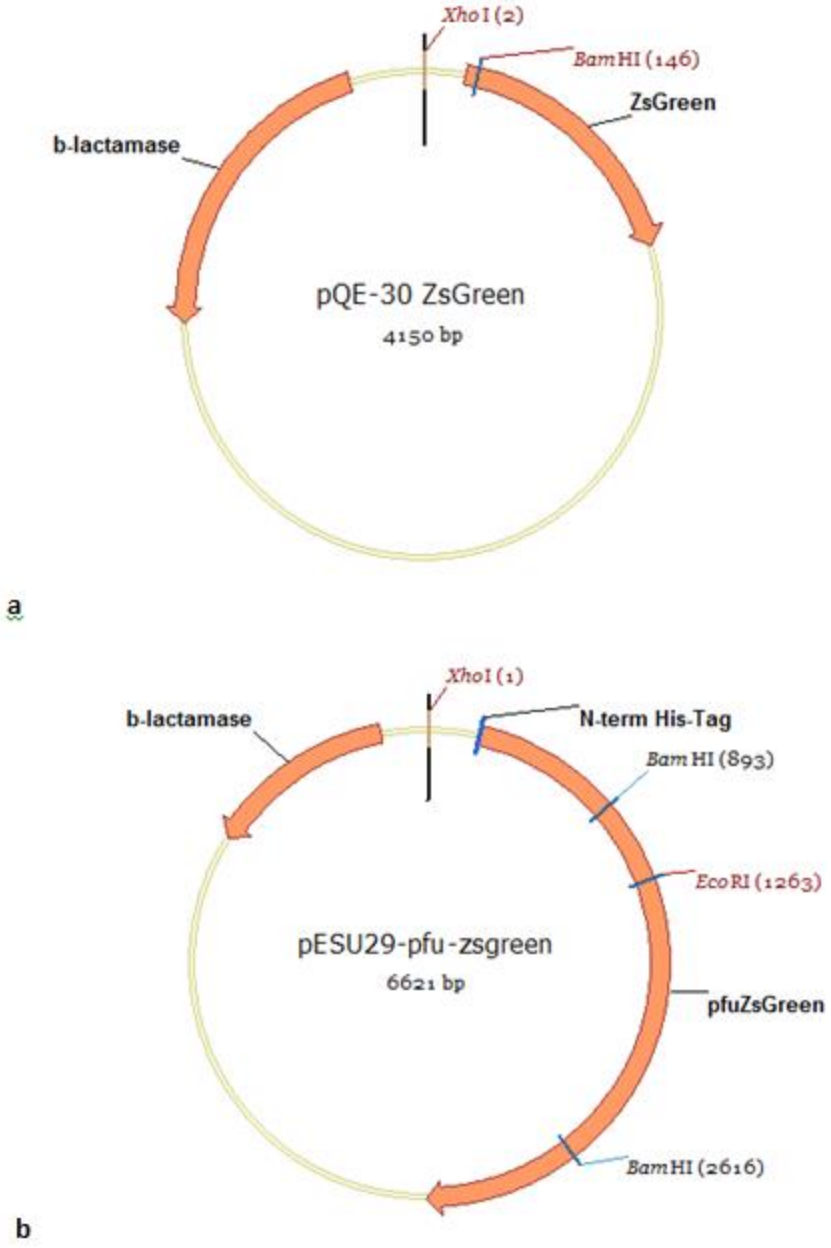

Figure 4.15 C-terminal ZsGreen on Pfu Polymerase Plasmid.

(a) Restriction sites XhoI and BamHI are visible on the pQE-30 plasmid. Replacement of the section between XhoI and BamHI removes the original pQE-30 promoter to be replaced by the pET-Pfu plasmid promoter which has become part of the PCR product insert. **(b)** Restriction sites XhoI, BamHI, and EcoRI are visible after the insertion of the *Pfu* polymerase gene. The three part ligation is required because the BamHI site at 893 bp is internal to the *Pfu* polymerase gene.

CHAPTER 5 DISCUSSION

5.1 FLUORESCENCE CHARACTERIZATION

The fluorescent proteins DsRed2, tdTomato, and ZsGreen were produced recombinantly, purified, and analyzed. The λ_{max} for excitation and emission was determined for each as well as an assessment of fluorescence retention at, and return from, high temperature. The experimentally determined excitation and emission maxima as well as the literature values for DsRed2[50], tdTomato[29], and ZsGreen[51] are given in Table I.

Table I. Excitation and Emission Maxima

	Experimental Excitation (nm)	Experimental Emission (nm)	Literature Excitation (nm)	Literature Emission (nm)
DsRed2	547	586	563	582
tdTomato	535	591	554	581
ZsGreen	491	505	493	505

Comparison of experimentally determined and literature values for excitation and emission maxima of DsRed2, tdTomato, and ZsGreen.

Discrepancies between the experimental and literature maxima may be the result of using different solution conditions for the purified protein. For example, different elution fractions from the HIC were used with each fluorescent protein for the measurements; each may have been collected in a different AMS concentration possibly affecting the readings. It is known that pH, ionic strength, and protein concentration affect the fluorescence maxima.[52]

Each protein was subjected to increasing temperature fluorescence scans and a final scan once returned to ambient temperatures. The fluorescence *versus* temperature plot for DsRed2 (Figure 4.4) is presented as an example of a fluorescent protein that does not exhibit a return of fluorescence and is considered the norm for fluorescent proteins. Of the 12 fluorescent proteins tested only mTFP1 (data not shown), tdTomato and ZsGreen exhibited fluorescence return, and tdTomato and ZsGreen were the most resistant to high-temperature inactivation; most exhibited behavior similar to DsRed2.[53] Fluorescence *versus* temperature plots for tdTomato (Figure 4.5) and ZsGreen (Figure 4.6) are presented as fluorescent proteins that do have a resistance to high-temperature inactivation. As a side note, the fluorescent proteins that were identified as being resistant to high-temperature inactivation used in these studies were both not monomeric. ZsGreen exists as a tetramer as wild type and has been engineered through directed mutagenesis to reduce aggregation tendencies (this engineered construct was not used in these studies). tdTomato is a connected repeat of a monomeric protein making it a tandem dimer. The precursor to tdTomato, dTomato, is a dimer. Perhaps this original

aggregation tendency is a reason for the resistance to high-temperature inactivation, although the proteins have been engineered to be monomeric.[52]

5.2 CHIMERA PRODUCTS

Once tdTomato and ZsGreen had been identified as having a resistance to high-temperature inactivation, several protein chimeras with the polymerases were synthesized using cloning techniques. After the chimera gene had been created, the correct plasmid construction was confirmed by restriction digests. The gel showing the digestions of N-terminal tdTomato on *Pfu* polymerase is in Figure 4.10. Expected band sizes should be 493, 726, 1723, 2044, and 4522 bp, all adding up to the expected total size of 9508 bp. Analysis of the gel indicates that the digest is consistent with the expected map. The digestions of C-terminal tdTomato on *Pfu* polymerase are on the gels in Figures 4.10 and 4.11. Expected band sizes for Figure 4.10 should be 423, 726, 2394, and 3043 bp, all adding up to the expected total size of 6586 bp. Band sizes for Figure 4.11 should be 412, 726, and 5448 bp in the PstI-NheI lane; 740 and 5846 bp in the NheI-SacI lane; and 726, 1152, and 4708 bp in the PstI-SacI lane. Each of these lanes adds up to the expected total plasmid size of 6586 bp, indicating a digest consistent with a successful ligation.

Each plasmid construct was used for a bacterial transformation, and the transformed bacteria were plated. After using a few different *E. coli* cell strains including NEB T7 Express, Genlantis SoluBL21, and Stratagene Gold pLysS, it was empirically determined that the NEB 5α T7 Express LysY strain had the highest fluorescent gene expression so this strain was used for nearly all transformations. The colonies containing the N-

terminal tdTomato on *Pfu* polymerase plasmid were ampicillin resistant but were not fluorescent red indicating a non-functional fluorescent protein or lack of expression (Figure 4.12). The C-terminal tdTomato on *Pfu* polymerase colonies also were ampicillin resistant, and most were fluorescent red, although less bright than tdTomato alone, indicating a functional fluorescent protein (Figure 4.13). Both tdTomato plasmid constructs were sequenced. The N-terminal tdTomato on *Pfu* polymerase chimera gene contained several mutations causing F10S in tdTomato, and Y217H and V308L (both on internal alpha helices) in *Pfu* polymerase. These could be possible reasons for lack of fluorescence. Structure analysis also revealed that the N-terminal end of *Pfu* polymerase appears to be more internal to the tertiary structure of the protein, possibly causing problems for tagging at this end as seen in Figure 5.1.

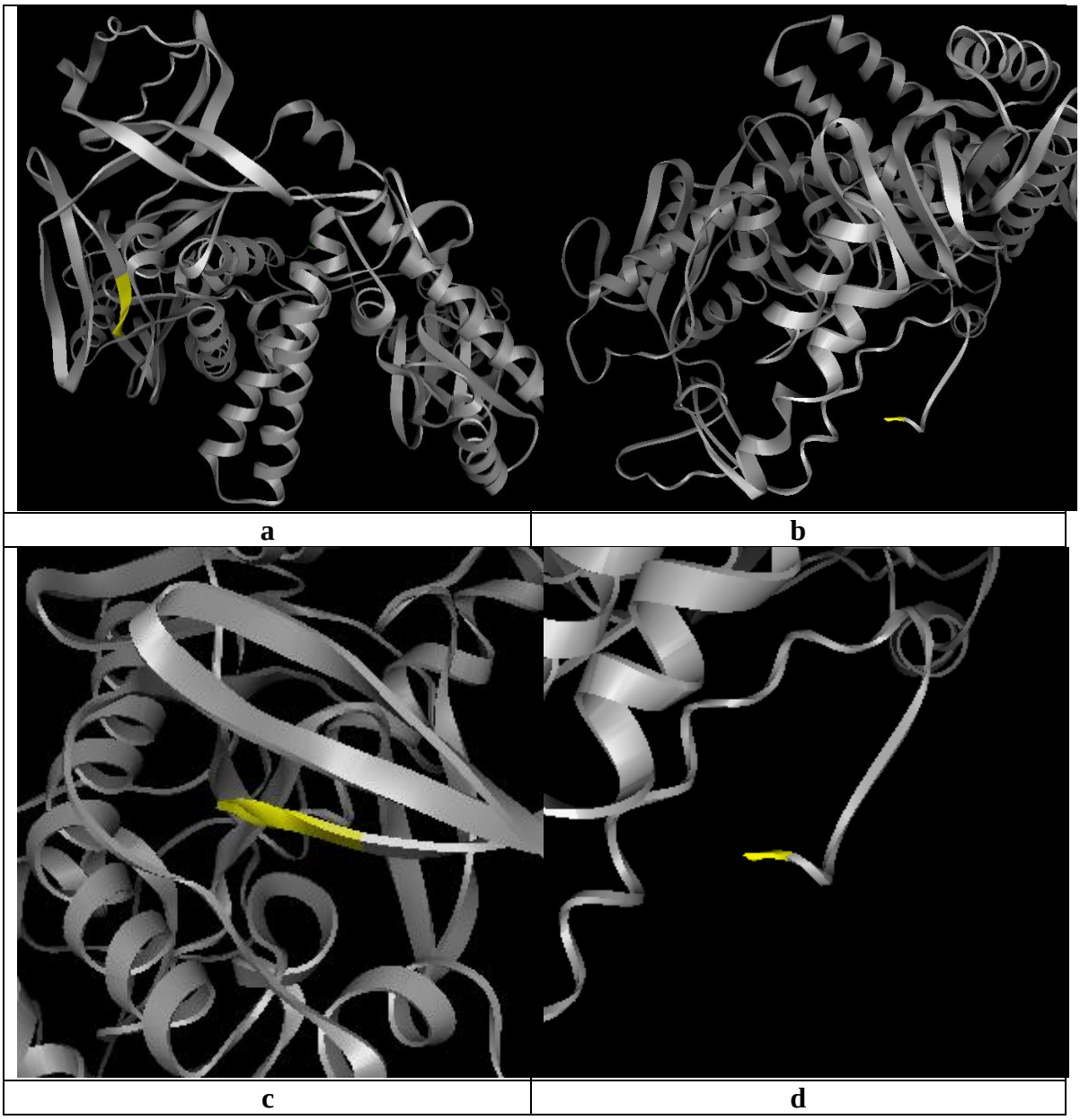

Figure 5.1 Sequence Termini of *Pfu* Polymerase

(a) The three N-terminal amino acids are highlighted in yellow **(b)** The three C-terminal amino acids are highlighted in yellow **(c)** Close up of highlighted N-terminal end **(d)** Close up of highlighted C-terminal end. Images created using crystal structure (2JGU)[54] and NCBI 3D molecule viewer, a component of Vector NTI Suite 8.0.

Sequence analysis of the C-terminal tdTomato on *Pfu* polymerase chimera revealed a Y217H mutation caused by a single nucleotide change in the polymerase gene sequence. This mutation is internal to the *Pfu* polymerase protein structure (Figure 5.2) and could be a cause of altered enzyme function or inactivation. A solution is to use site-directed mutagenesis to fix the Y217H mutation and to restore it to wild-type sequence.[55]

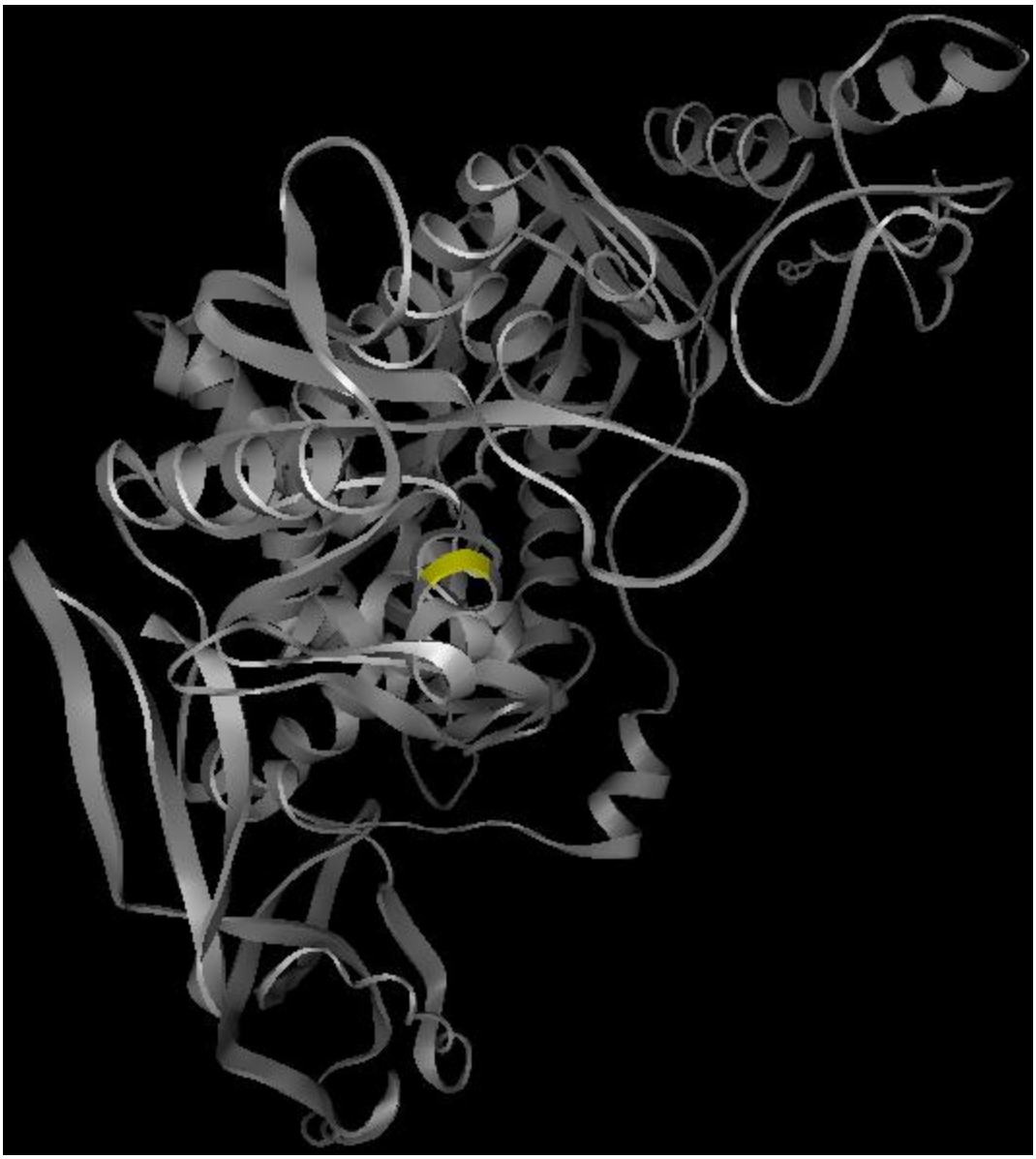

Figure 5.2 Pfu Polymerase Y217H Mutation Site

Position 217 on *Pfu* polymerase has been highlighted in yellow using the same structure as in Figure 5.1.

The next chimeras created include a C-terminal ZsGreen on *Pfu* polymerase and a C-terminal ZsMutant on *Pfu* polymerase. Both were created by inserting the *Pfu* polymerase gene into the pQE-30 plasmid containing either ZsGreen or ZsMutant (Figure 4.15). Both transformations showed successful plasmid uptake and ampicillin resistance; however, neither plates colonies were fluorescent green. The lack of fluorescence emission by all but one chimera product may be due to two reasons. (1) PCR DNA polymerase enzymes may be a difficult type of protein to tag while retaining function because of the relative dexterity required for the protein to serve its normal function. This required dexterity may compromise the folding integrity of one or both chimera domains if there is not enough separation between each. For the N-terminal tdTomato on *Pfu* polymerase chimera, there is no linker sequence between the domains, and for the C-terminal tdTomato on *Pfu* polymerase, the linker sequence was SMTGGQQMGRDLYDDDDKDPAT. This linker is reasonably long and hydrophilic. For both C-terminal ZsGreen protein chimeras there was no linker sequence separating the chimera domains. The addition of a floppy peptide linker between the two proteins of between 5 and 30 amino acids with over 40% serine and high amounts of glycine and threonine would confer a greater degree of separation with relatively small and uncharged amino acids.[30] This will hopefully ameliorate any inter-domain structure disruptions or hydrophilic/hydrophobic region offsets. For example, if a relatively hydrophilic region of one chimera domain is oriented facing a relatively hydrophobic region of another chimera domain this positioning may disrupt the structure and function of one or both. Greater separation with a somewhat hydrophilic linker sequence between the regions could allow for proper folding. Another strategy would be internal tagging if a suitably

innocuous position on the polymerase's tertiary structure can be found to insert the fluorescent protein. (2) Similar to other proteins isolated from corals, ZsGreen exists as a tetramer in the wild-type state, and this interferes with its use as a fusion partner.[48] Tendency to aggregate due to oligomerization may be another possible reason for the lack of protein chimera function. One possible solution to the aggregation problem could be to acquire a newer version of ZsGreen that has been engineered to be monomeric. Another solution could be to use mTFP (monomeric teal fluorescent protein), which has shown resistance to high-temperature inactivation.

Additionally, other PCR DNA polymerases can be tried as a fusion partner. *Taq* polymerase was a contender for the polymerase of choice to be tagged in our early designs, but after a few unsuccessful PCR, *Taq* was abandoned in favor of *Pfu*. Furthermore, the C-terminal tdTomato on *Pfu* polymerase chimera with the Y217H mutation could still be tested for PCR function, although a direct comparison between the mutated chimera and the un-mutated polymerase for function would be less than optimal. In closing, fluorescent proteins were characterized for excitation, emission, and high-temperature inactivation. Proteins with suitable high-temperature fluorescence retention were chosen and used to create several PCR DNA polymerase + fluorescent protein chimeras. Each chimera was assessed for fluorescence activity and sequenced. The most successful appeared to be C-terminal tdTomato on *Pfu* polymerase. However, this product contains a single Y217H mutation on *Pfu* polymerase. This chimera could still be purified and used for the next phase of experimentation comparing its activity to that of *Pfu* polymerase itself. Another option is to repair the mutation using site-directed

mutagenesis to return the mutated domain to the original polymerase sequence. Overall, the project was successful in creating a polymerase + fluorescent protein chimera.

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