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Engineering Synthetic Antibody for Prostate Cancer Detection

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ENTITLED

ENGINEERING SYNTHETIC ANTIBODY FOR PROSTATE CANCER DETECTION

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ENGINEERING SYNTHETIC ANTIBODY FOR PROSTATE CANCER DETECTION

By

Kimberley Gonzalez, Tatum Prosswimmer, Cassandra Stawicki

SENIOR DESIGN PROJECT REPORT

Submitted to
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Abstract

This Senior Design project seeks to manufacture an *E. Coli* based antibody for prostate cancer detection using unnatural amino acid incorporation. Current diagnostic techniques take advantage of the high binding specificity of monoclonal antibodies to detect the concentration of prostate specific antigen (PSA) in a blood sample. Production of this technology is lengthy and costly, while simultaneously incurring countless ethical problems related to animal welfare and accessibility. This project proposes a synthetic antibody that can be produced efficiently in single *E. Coli* cells and exhibits complementary binding to PSA. By incorporating an unnatural amino acid into a recombinant peptide sequence, binding affinity between substrate and antibody is substantially increased, effectively expanding the genetic code by introducing a novel function. Expression of the antibody is followed by extraction and purification of the synthetic antibody from cellular debris. Finally, the antibody's binding strength and specificity to PSA is tested with respect to the monoclonal antibody with complementary binding to PSA. Assuming the synthetic antibody has greater or equivalent binding affinity than the currently used protein, this project will replace ethically questionable diagnostic techniques with a cheaper alternative.

Acknowledgments

Dr. Zhiwen (Jonathan) Zhang

Santa Clara University, School of Engineering

Santa Clara University, Department of Bioengineering

Santa Clara University, Undergraduate Programs Senior Design Project Fund

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Abbreviations

E.coli: Escherichia coli

ELISA: Enzyme-linked Immunosorbent Assays

FPLC: Fast Protein Liquid Chromatography

GFP: Green Fluorescent Protein

6-His: 6 Poly-histidine

IMAC: Immobilized Metal Affinity Chromatography

IPTG: Isopropyl β-D-1-thiogalactopyranoside

ITC: Isothermal Titration Calorimetry

Kan: Kanamycin

K_d: Dissociation Equilibrium Constant

LB: Lysogeny Broth

L-DOPA: 1-3, 4-dihydroxyphenylalanine

mAb: Monoclonal Antibody

$NaIO_4$: Sodium Periodate

Ni-NTA: Nickel-charged Nitrilotriacetic Acid

OD_{600}: Optical Density at 600nm

PBS: Phosphate Buffered Saline

PSA: Prostate Specific Antigen

RPM: Revolutions Per Minute

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

TAG: Amber Stop Codon

Tet: Tetracycline

tRNA: Transfer Ribonucleic Acid

UV: Ultraviolet

Introduction

Background, Significance, and Motivation

Antibodies are natural glycoproteins produced by the immune system that neutralize or eliminate foreign invader molecules and organisms. Due to high affinity, specificity, and strength in binding to a specific antigen, antibodies enable immune cells to locate and terminate target cells. This binding capacity has resulted in the commercialized use of antibodies for a variety of research and medical practices, including qualitative and quantitative analyses, and mediating or modulating physiological effects. Diagnostic applications include Western Blotting, used to increase the sensitivity of an assay system, and ELISA, used to analyze soluble antigens.[1] In these processes, antibodies are manipulated to detect the presence of specific proteins, antibodies, or antigens in a sample. Therapeutic antibodies are currently used to treat asthma, autoimmune disorders, and cancers, but have also been tested for treatment of infectious diseases.[1]

Among a myriad of therapeutic and diagnostic applications is the detection of prostate cancer using monoclonal antibodies. Prostate cancer is the most common form of cancer among white, black, asian, and hispanic men, the second most common among American Indian/Alaska Native men, and the second leading cause of cancer death among white, black, American Indian/Alaska Native, and Hispanic men.[2]

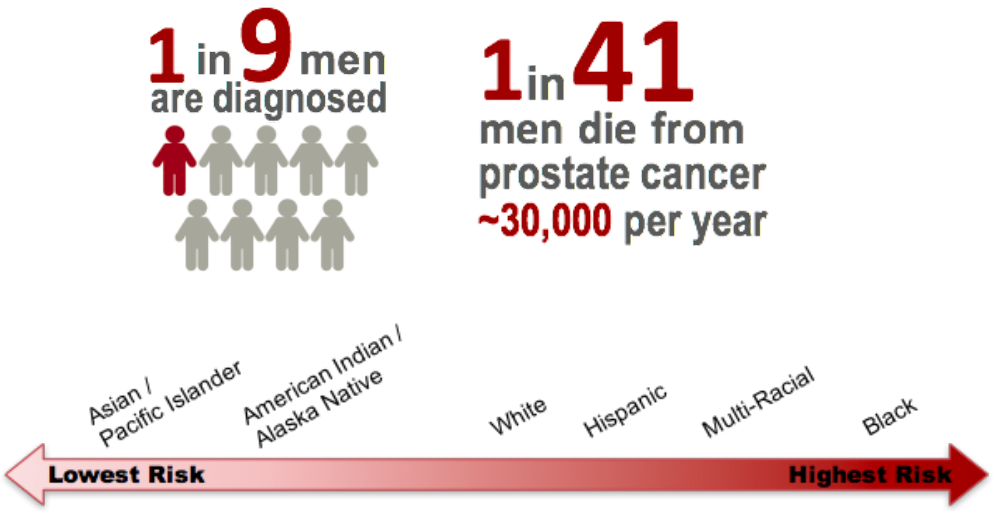

Figure 1. **Overview of prostate cancer statistics**

One in nine men will be diagnosed with prostate cancer within his lifetime, while one in forty-one men will die from it.[3] Early detection is crucial: the relative 5-year survival rate for the "local stage" in which the disease is contained within the prostate is nearly 100%. Meanwhile, survival is only about 29% for the "distant stage", which includes stage IV cancers.[4]

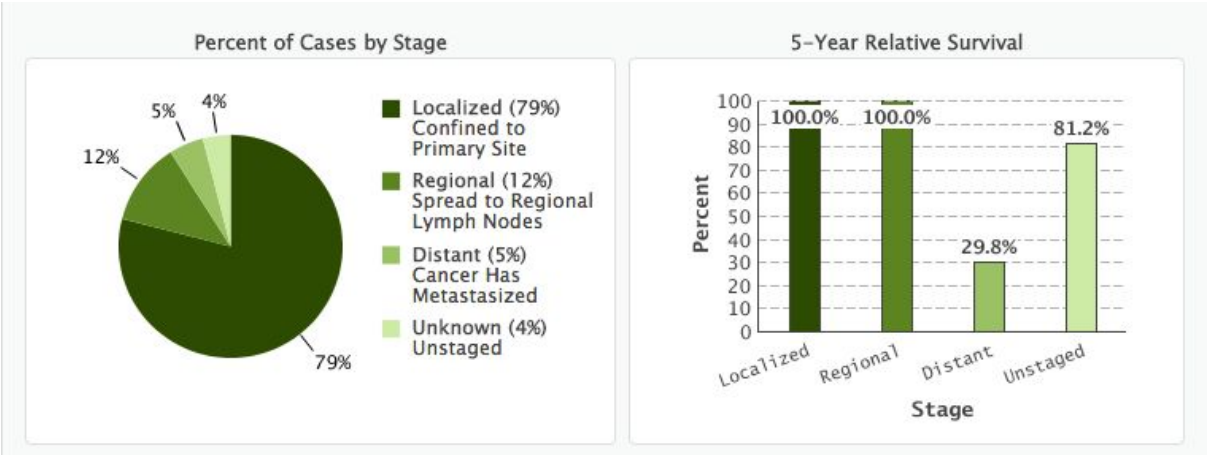

Figure 2. **Diagnosis statistics.** Distribution of prostate cancer diagnosis stages and relative survival rates 5 years post-diagnosis[4]

Detection is done either through a digital rectal exam or by measuring the concentration of PSA in the blood.[4] In the rectal exam, the prostate is assessed for enlarged or abnormal physiology. In the blood panel, the level of PSA in the blood is detected using a monoclonal antibody. As shown in Figure 1, a healthy prostate secretes a relatively small concentration of PSA, part of which is taken up into the bloodstream. However, in those with prostate cancer, an elevated level of PSA is secreted and absorbed into the blood.[4]

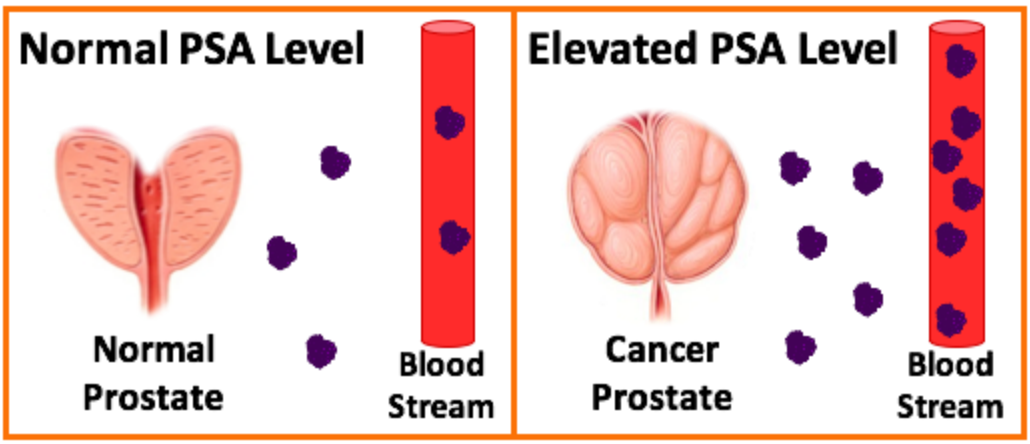

Figure 3. Healthy versus disease state related PSA levels. Comparison of PSA release from a healthy prostate and a prostate with cancer, as well as uptake into the bloodstream.

Monoclonal antibodies currently dominate both the research and medical spheres for countless diseases, including prostate cancer. Despite their unique specificity and uniformity, the use of monoclonal antibodies raises ethical concerns, presents high contamination risks, and requires a lengthy and expensive production process due to the sheer complexity of the molecule. While many of these challenges can be overcome by using multiple monoclonal antibodies, this route is difficult, expensive, and time consuming.[5]

Thus, there is a need for a similarly selective molecule which can be engineered to target specific antigens, without incurring these ethical, social, and economic challenges. Unnatural

amino acid incorporation is a promising new technology which will expand the genetic code and introduce novel functions into the biological world. By incorporating a non-biologically relevant amino acid into a recombinant peptide sequence, a "synthetic antibody" can be engineered with specific binding capabilities; and, by designing a synthetic antibody which can be produced in *E. Coli*, there is potential to circumvent the many disadvantages incurred through the traditional monoclonal antibody model.

Literature Review

Unnatural Amino Acid Incorporation

Unnatural amino acids contain functional groups that are uncommon to the 20 known biological amino acids. Bioengineers Wals and Ovaa have explored the various applications of unnatural amino acid incorporation through a mutated tRNA synthetase, allowing engineers to introduce novel function in proteins by taking advantage of the inherent relationship between protein structure and function.[6] Unnatural amino acid incorporation allows selective modification that can be used for therapeutic applications.

Design

A paper by Umeda et al. explores the possible applications of unnatural amino acid incorporation, such as for protein detection probes or therapeutic purposes.[7] The proposed design of the experimental synthetic antibody is partially derived from that of Umeda et al. paper. Their system describes the incorporation of an unnatural amino acid, 3,4-dihydroxy-L-phenylalanine (L-DOPA), to a peptide (TOP1), GFP reporter protein, and a 6-His purification tag. This project proposes a similar design, but includes a genetically engineered sequence specific for PSA, thus pioneering the use of unnatural amino acids for prostate cancer detection.

Specifically, this design replaces the TOP1 peptide used by Umeda et. al with the PSA-specific sequence; however, the principles of unnatural amino acid incorporation are generally the same as those outlined in the referenced literature.

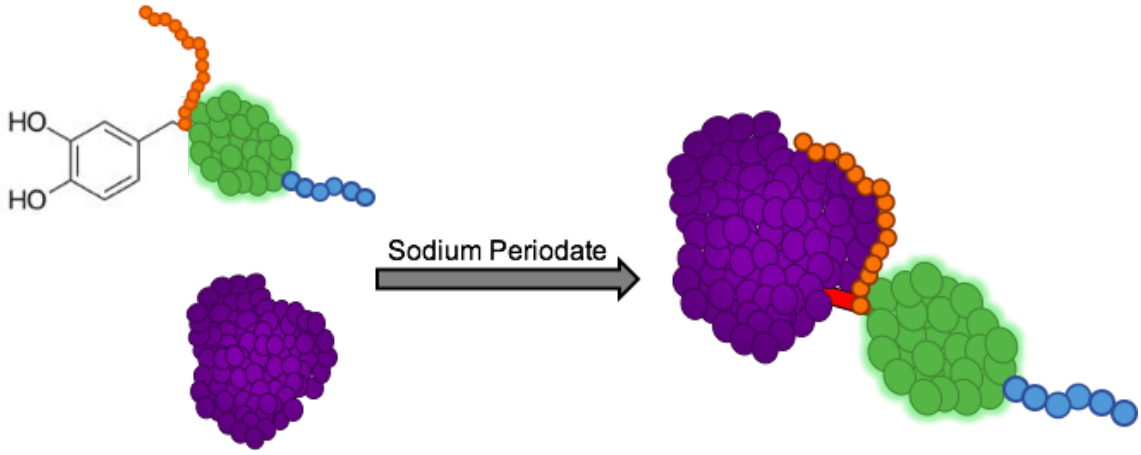

Figure 4. Synthetic antibody binding PSA. Design of the synthetic antibody includes 15 amino acid peptide sequence (orange), unnatural amino acid (L-DOPA, black), green fluorescent protein (green), and 6-His purification tag (blue). The addition of sodium periodate triggers the formation of a covalent bond (red) between the antibody and PSA (purple).

Critiques of Current Literature and Technologies

Monoclonal antibodies

Monoclonal antibodies are often used by researchers as drug targets and diagnostic tools because they can be used in most every stage when researching protein targets and characterization. However, the production of monoclonal antibodies incurs questionably ethical practices. To avoid jeopardizing human safety, production of monoclonal antibodies is done in animals, typically mice, using the hybridoma model. The hybridoma model involves injecting live animals which disease agents in order to induce an immune response, leading to antibody synthesis. The antibodies are then extracted through an extensive process which often causes excessive distress in the animal.[8] These practices present a clear need for a diagnostic and therapeutic replacement.

Polyclonal antibodies

One potential alternative is polyclonal antibodies, which uphold identical structure and specificity as the monoclonal counterpart, while requiring less time for production at a significantly lower cost. However, they maintain the same ethical pitfalls as monoclonal antibodies because they must also be produced in animals. Also, there is batch to batch variability in polyclonal antibody production, as well as potential for cross reactivity because the antibodies are able to recognize multiple epitopes, unlike monoclonal antibodies.[1]

Aptamers

Aptamers have also been considered as another potential alternative to monoclonal antibodies. They are short molecules of either RNA or single-stranded DNA that are capable of folding into 3-D conformations which can bind specific target proteins. Aptamers are much smaller than monoclonal antibodies and can be synthesized by simpler chemical mechanisms, but they are not a viable alternative to monoclonal antibodies because they lack stability over ranges of temperature and pH, and in the presence of commonly used buffers.[9]

Protein scaffolds

Protein scaffolds are formed from fragmented polypeptides and can also demonstrate similarly specific binding to target molecules. However, similarly to aptamers, protein scaffolds are not able to maintain a stable structure over a range of conditions. They also lack common functional modifications exhibited by monoclonal antibodies, such as disulfide bonding.[10]

Project Objectives

The goal of this project is to engineer a synthetic antibody for prostate cancer detection as a cost-effective and ethically sourced diagnostic alternative to monoclonal antibodies. Because

preliminary cloning and sequencing of the plasmid is complete, this project will focus on the production and testing of the synthetic antibody.

Toxicity screening must be done first in order to ensure that proper expression of the engineered plasmid in *E. Coli* is possible with the additional synthetic components. Next, the synthetic antibody is isolated through a protein purification method known as manual affinity chromatography. Finally, the protein will be analyzed for binding affinity, specificity, and selectivity toward PSA and statistically compared to mAb. This validation step is crucial to proving viability of the proposed synthetic antibody as an ethical and effective replacement of mAb; the former must have equivalent, or greater, diagnostic capabilities as the previously established standard method in order to be considered as a possible replacement.

This project focuses on the incorporation of an unnatural amino acid into a peptide which specifically codes for PSA binding. This amino acid and the PSA specific sequence are not part of the bacteria's biological code; thus, this molecule is considered a synthetic antibody. Because this system is produced in *E. Coli* and has potential to assist in prostate cancer detection, it serves as a model for the development of additional synthetic antibodies which may replace monoclonal antibodies as the gold standard in detection. This model will hopefully be able to overcome the challenges of mAb discussed previously as it will incur fewer costs, shorter production times, and greater scalability, while maintaining comparable functionality.

Overall Design

A synthetic antibody manufactured in *E. Coli* presents three main advantages which overcome the challenges associated with monoclonal antibody use and production. The proposed synthetic antibody will be more cost efficient than the monoclonal antibody counterpart, be ethically produced, and will contain no batch to batch variability between samples. There are four main components to the design of the antibody: a 'P2' peptide

sequence which is complementary to the PSA sequence, the replication machinery necessary for unnatural amino acid incorporation of L-DOPA, a GFP reporter protein, and a 6-His purification tag.

Peptide Sequence

The P2 peptide sequence was computationally designed by Jon Henry Therriault and Thomas Evans using an open source software called Chimera.[11] The peptide sequence was screened against anti-PSA monoclonal antibodies to have high binding affinity (3.5 μM) and specificity to PSA.[12]

L-DOPA Incorporation

The second component of the synthetic antibody is an unnatural amino acid called 3,4-dihydroxy-L-phenylalanine (L-DOPA). L-DOPA is a derivative of tyrosine and it has been demonstrated that it can be incorporated in recombinant proteins in *E. Coli* by suppressing the amber stop codon, which has the codon sequence TAG.[7] The unnatural amino acid is able to form a stable covalent bond with a protein of choice, in this case PSA, upon exposure to $NaIO_4$.[7] The L-DOPA is incorporated with a mutant tRNA and tRNA synthetase, which are present in the engineered plasmids, in order to have successful incorporation of the amino acid.[11]

GFP Reporter/Payload

Additionally, a GFP reporter protein is included in the gene sequence to visualize expression of the synthetic antibody using UV light. This method of visualization and validation is straightforward, simple to use, and a reliable, commonly used method to determine whether the transformation and expression are successful.

Purification Tag

Finally, a sequence of 6 consecutive histidines is placed in the gene sequence to aid in protein purification. The 6-His tag is used to facilitate the protein purification by using immobilized metal-affinity chromatography.[13] Histidine has previously been established as the amino acid with the most efficient retainment to the chromatography matrices.[13]

Plasmid Overview

In total there are two plasmids that are transformed into the *E. Coli* competent cells: pAC-DHPheRS-6TRN plasmid and pET28b plasmid. The first plasmid has the L-DOPA unnatural amino acid production sequence, including the mutant tRNA and tRNA synthetase, and a Tet resistance gene. The second plasmid contains the GFP, 6-His tag, the P2 peptide sequence, and a Kan resistance gene. The resistance genes are used as a verification for successful transformation of both plasmids into the *E. Coli* cells, which will be discussed further in the following sections.[11]

Milestones and Expected Results

The two plasmids were successfully designed, cloned, and transformed into *E. Coli* competent cells by a previous senior design team.[11] The first milestone of this project is to successfully express the synthetic antibody in *E. Coli* cells. Here, different growth conditions will need to be tested in order to optimize an adequate environment in which the cells will not recognize the unnatural amino acid as a foreign entity and commence an apoptotic sequence.

Once the expression is successful, the next step is to purify the protein from the *E. Coli*. The purification must be done multiple times using the gold standard of monoclonal antibody purification as a representative model. The process will need to highly effective and optimized to its highest potential in order to ensure the greatest level of purity.

The final step, once the expression and purification of the synthetic antibody is complete, is to test the binding capabilities of the engineered synthetic antibody. In order to prove the advantage of using a synthetic antibody over a monoclonal antibody, it must be tested for binding strength, specificity, and sensitivity for PSA. The standards for the anti-PSA monoclonal antibody are already well established and can be used as benchmark measurements for the synthetic antibody.

Team Management

To ensure continuous efficiency and culpability from each team member throughout the project, responsibilities will be distributed throughout the team. Cassandra is the primary liaison with the School of Engineering, manages the team finances, and ensures all deadlines are met. Kimberley coordinates meeting and laboratory scheduling for the team and facilitates communications with the advisor, Dr. Zhang. Tatum supervises all writing and editing for assignments and presentations, as well as the final report.

Budget

Through funding granted from the School of Engineering, the project has a $1,500 budget for the year. We have $300 allocated for ten reactions using TOP10 competent cells. A $400 budget for reagents including: buffers, media, our protein target, and monoclonal antibodies. To ensure that no genetic mutations occurred within our *E.coli* production system, we have $90 for DNA sequencing analysis of our 45 nucleotide synthetic antibody. For protein purifications, we have $500 allocated to cover the cost of protein gels and purification beads. Finally, we estimate our consumables to cost approximately $210, which will cover dialysis cassettes, filters, petri dishes, pipette tips, and other items needed for laboratory research. We do not anticipate any issues to arise within our budget as we have accounted for additional runs of sequencing and reactions using competent cells.

Timeline

During the Fall term, we initiated toxicity screening and *E. Coli* expression. However, due to the anticipated challenges of cell survival associated with the mutant tRNA synthetase, our toxicity screening will run into the next term as we attempt to narrow in on the ideal conditions to avoid cytotoxic responses. Additionally, within the Winter term, we will be running the protein purifications and validation screening. Finally, during the Spring term, we plan to finalize our results with quantitative analysis and any needed troubleshooting. We do not anticipate any major setbacks or challenges beyond the toxicity screening phase.

Chapter 1: Protein Expression

Introduction

The proposed synthetic antibody has been designed to make use of the highly conserved lac operon. In a biological system, the complexly regulated lac operon stimulates lactose metabolism. Because it is among the most well-understood operons, protein engineering techniques often make use of it to produce recombinant proteins. Production of the L-DOPA incorporated synthetic antibody in *E. Coli* first requires design of a plasmid which contains the desired sequence within the lac operon transcription region. By mimicking the biological system's promoter and repressor functions, expression of the desired sequence can be easily regulated. In this way, production of the proposed synthetic antibody can be closely controlled.

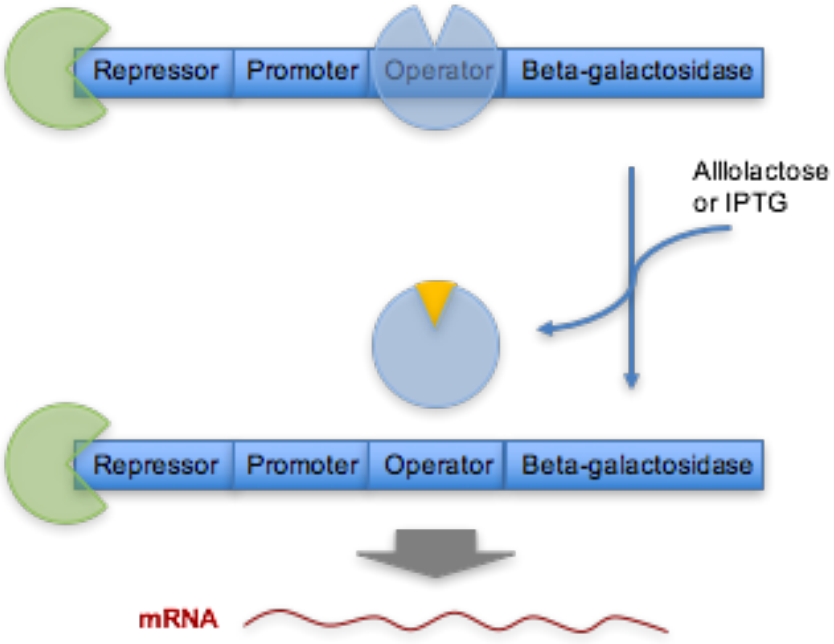

Figure 5. **Induction of lac operon with IPTG.** Release of inhibitor from operator site using IPTG, instead of allolactose, to induce transcription.

Back-up Plan

Although expression protocols are well established and documented, it is not a simple task to accomplish using unnatural amino acids and mutant translation machinery. The main concern at this critical step is the lack of *E. Coli* growth. In order to ensure optimal growth, multiple carbon sources including glycerol and glucose will be introduced at varying concentrations to optimize cell growth.

Materials and Methods

Plasmid Validation

The first step in this project is to validate that the experimental plasmids have been properly transformed into the *E. Coli* competent cells by plating the cells with specific antibiotics. The pAC-DHPheRS-6TRN plasmid contains the mutant tRNA and tRNA synthetase needed for unnatural amino acid incorporation as well as the tetracycline resistance gene. The pET28b-P1+TAG+GFP+His contains an enhanced GFP reporter protein, the specific sequence which will bind to PSA, a 6 Histidine purification tag, and kanamycin resistance genes. Cells were plated on a variety of antibiotics to ensure that the plasmids were properly transformed.

Antibiotic(s)	**Growth?**
Tetracycline	Yes
Kanamycin	Yes
Ampicillin	No
Tetracycline + Kanamycin	Yes

Table 1. Validation of plasmid transformation using antibiotic resistance genes.

Because the cells did not grow in the negative control (ampicillin) but did grow when plated with both conferred resistance genes, correct plasmid transformation into competent cells is verified.

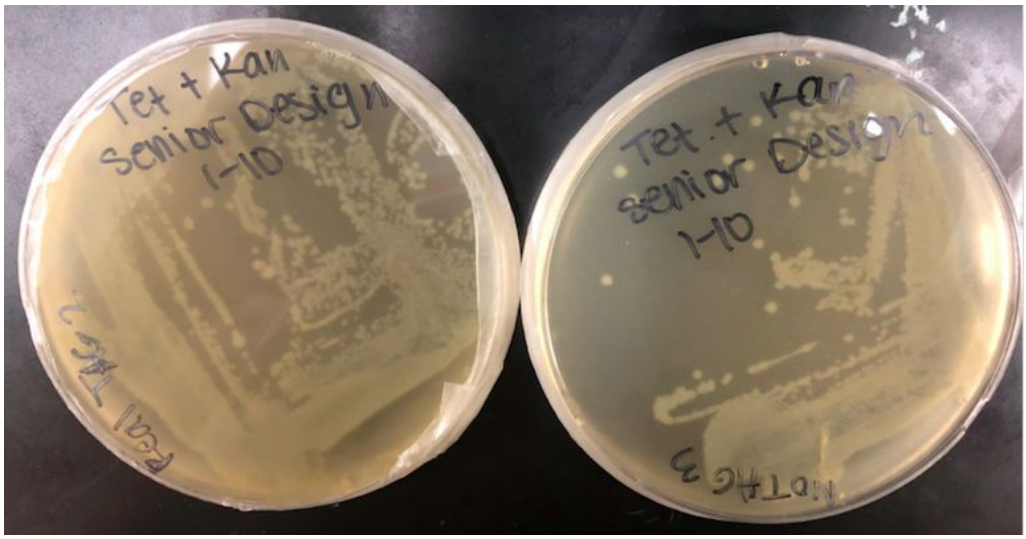

Figure 6. **Plated TOP10 competent cells.** Experimental cells containing plasmids pAC-DHPheRS-6TRN and pET28 (left) and control cells (right).

Toxicity Response

The plated cells above show the differences in colony growth between the experimental and control. The plated cells on the left contain both experimental plasmids, which include a TAG codon sequence which signals for the addition of the unnatural amino acid, L-DOPA, into the growing peptide sequence. The cells on the right contain a GCG codon sequence in place of the TAG, which codes for an alanine amino acid residue. Because the control contains only biologically relevant amino acids, there is no toxicity response evident in the size and shape of the cell colonies. By comparing the colonies on both plates, it is evident by the small and slightly misshapen colonies on the left that there is a toxicity response based on the synthetic nature of the experimental peptide sequence. This indicates that an optimized antibody expression protocol is necessary to ensure adequate cell growth and expression.

Optimized Antibody Expression Protocol

The optimized antibody expression protocol was developed from a similar design described in a paper by Umeda et. al. First, 500 microliters of glycerol stock containing the experimental competent cells were added to 250 milliliters of M9 salts with 5% glucose as the carbon source. Thirty and twenty-five micrograms per milliliter of Kanamycin and Tetracycline, respectively, were subsequently added. This culture was grown in a dark incubator at 37°C with agitation. Once an optimal OD_{600} is reached, 1 mM L-DOPA was added for unnatural amino acid incorporation. Agitation at 37°C continues for 40 minutes, at which point 1 mM IPTG is added. Once induction with IPTG begins, the temperature is lowered to 30°C, and shaking continues for an additional six hours. Finally, the cultures are viewed using a Transilluminator to visualize the fluorescence of both the experimental and control.

Results

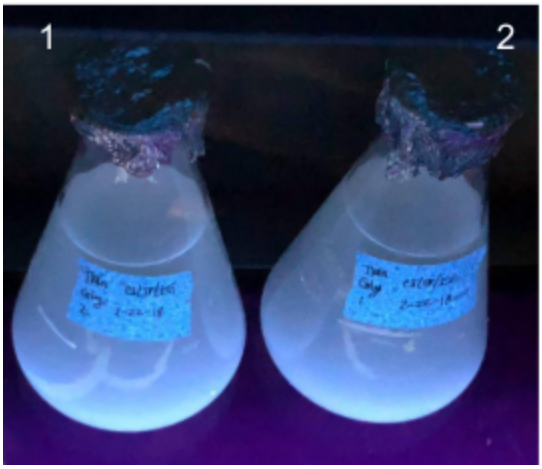

Figure 7. Expression visualization under UV light. Experimental cultures, with L-DOPA incorporation (1) and no L-DOPA (2), measured under UV light after 6 hours of incubation

Flasks 1 and 2 contain *E. Coli* competent cells transformed with the synthetic antibody experimental plasmid. Flask 1 contains L-DOPA, which was added once the cells reached OD_{600} of 0.4, while flask 2 does not contain L-DOPA. Both flasks 1 and 2 contain IPTG in order to induce antibody expression. Green fluorescence indicates successful expression of the synthetic antibody with unnatural amino acid incorporation due to the strategic placement of GFP at the end of the experimental plasmid. As may not be clear in Figure 7, flask 1 fluoresces brighter than flask 2. This is to be expected because flask 1 contains the experimental, and flask 2 the control.

Discussion

Plasmid Validation

The growth of colonies on both culture plates, each with Kanamycin and Tetracycline antibiotics, demonstrated the successful transformation of both pAC-DHPheRS-6TRN (containing a Tetracycline resistance gene) and pET28 (containing a Kanamycin resistance gene) plasmids. The visible variation in colony size suggests that the cultures with TAG are expressing an expected toxicity response. This toxicity response must be diligently addressed during the antibody expression step.

Toxicity Response

Due to the toxicity response caused by mutant tRNA and tRNA synthetase, cell growth and subsequent antibody expression proved challenging. Multiple expression protocols were executed to determine which protocol would optimize cell growth and expression.

First, a traditional growth protocol was followed in which 500 microliters of glycerol stock containing the competent cells of interest were grown in minimal media. Optimal growth was expected after approximately 24 hours. However, after incubation for 72 hours, the cells grew only to an OD_{600} of approximately 0.2; the desired OD_{600} reading is between 0.4-0.6.

Next, a similar protocol was conducted in which an additional carbon source, glycogen, was added to the culture during growth. Similar to the first protocol, optimal cell growth was not reached, even after multiple executions and extended incubation.

Following approximately 10 weeks of troubleshooting, the optimal protein expression protocol was reached. It was determined that adding glucose to the growth media, along with M9 salts, effectively disguised the mutant tRNA and tRNA synthetase, preventing the cells from signaling for apoptosis. Using this protocol, it was possible to grow the cells to optimal concentration, following which the antibody could be expressed using induction with IPTG. Thus, by adding an additional carbon source, glucose, the cells were able to bypass their foreign invader response and grow to an optimal optical density.

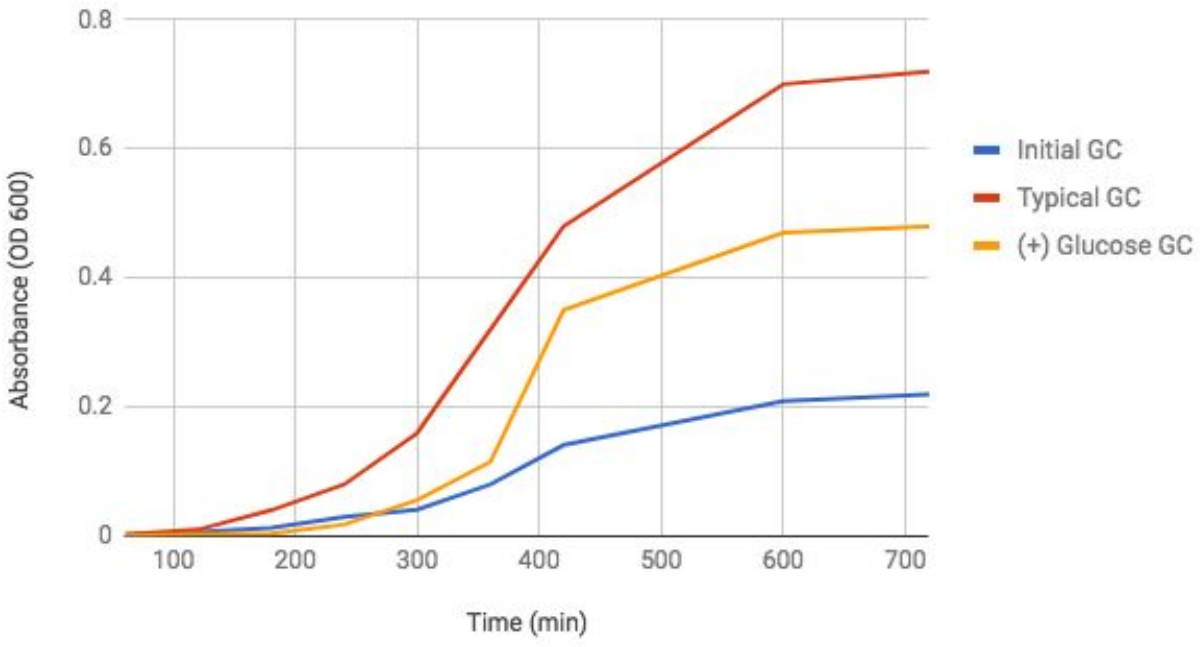

Figure 8. Bacterial growth curves. Comparison between initial growth curve (without added carbon source), the typical bacterial growth curve, and the growth curve following addition of glucose as a carbon source.

As shown in Figure 8, adding glucose as an additional carbon source did not entirely combat the toxicity response caused by the synthetic antibody. This can be determined by the fact that the cell's logarithmic growth phase ended prematurely, transitioning to the stationary phase before reaching OD_{600} of 0.6, which is the typically desired optical density. However, for the purposes of this project, an OD_{600} of 0.4 will suffice for subsequent antibody expression. Thus, the protocol containing glucose as an additional carbon source was determined to be the optimized protocol for cell growth and antibody expression.

Antibody Expression

The validation of a successful protein expression is the signature green illumination from GFP. Based on Figure 7, the *E. Coli* cells were able to successfully synthesize and express the synthetic antibody. The flask on the left has a high level of fluorescence, indicating that the plasmids have been fully expressed and that the synthetic antibody is being produced. The flask on the right does not contain L-DOPA, but still appears to fluorescence. However, this is because all living cells emit baseline fluorescence, so this is not of concern. Thus, from these figures, accurate transformation and expression is confirmed.

Chapter 2: Protein Purification

Introduction

In order to quantify the binding affinity and activity of the synthetic antibody, the protein must be extracted and purified. Immobilized Metal Affinity Chromatography (IMAC) will be used to purify the protein by taking advantage of the 6-His tag incorporated into the synthetic antibody. This technique will allow the purification tag to bind to Nickel-charged Nitrilotriacetic Acid (Ni-NTA) beads while other proteins are washed away. IMAC methods are well-established and widely used as a method of protein purification.

Back-up Plan

In case the standard protocol is not successful, fast protein liquid chromatography, or FPLC, will be used to purify the synthetic antibody. FPLC is an automated process which incorporates a mobile phase and a stationary phase to separate proteins based on affinity. Although this process is automated, it is typically used for large-scale purifications, and is complicated to program. Thus, IMAC is the preferred method.

Materials and Methods

Lysate Step

Purification was conducted individually based on cell type and stock media. The pellet was resuspended in 5 mL of lysis buffer, containing PBS, 1 mg/mL lysozyme, 0.5 mM PMSF, and 10 mM imidazole. Suspension was transferred to a metal centrifuge tube and incubated on ice for 20 minutes. The cells were sonicated at 30% alternating between 6 seconds on and 59 seconds off for 6 cycles. The lysate was then centrifuged at 4°C and 16,000 rpm for 40 minutes. 1 mL of the supernatant was stored at -20°C for gel analysis.

During centrifugation of the lysate, 250 μL of HisPur™ Ni-NTA beads (Thermo Fisher Scientific) were resuspended in 5 mL of wash buffer, containing PBS and 25 mM imidazole. This was done for each culture with a volume ratio of 1:1000 for the volume of beads to volume of initial culture. The suspension was centrifuged at 2,000 rpm for 4 minutes, following which the supernatant was discarded. This process was repeated 3 additional times.

Flow-Through Step

Using the supernatant of the centrifuged lysate solutions, the Ni-NTA beads were again resuspended. To allow the protein to bind to the 6-His tag specific beads, the suspension was rocked at 4°C for 1 hour. Centrifugation was conducted at 4°C and 2,000 rpm for 4 minutes. At the end, 1 mL of flow-through was saved and stored at -20°C.

Wash Step

The beads were then washed 5 times. In each wash, the beads were resuspended in wash buffer, containing PBS and 25 mM imidazole, rocked for 20 minutes at 4°C, and centrifuged at 4°C and 2,000 rpm for 4 minutes. After each wash, 1 mL of wash supernatant was stored at -20°C for gel analysis, while the remainder was discarded.

Elution Step

The elution process was conducted twice, each time resuspending the beads in 200 μL of elution buffer, containing PBS and 250 mM imidazole. The suspension was rocked for 20 minutes at 4°C, then centrifuged at at 4°C and 2,000 rpm for 4 minutes. After, supernatant was extracted and stored at -20°C.

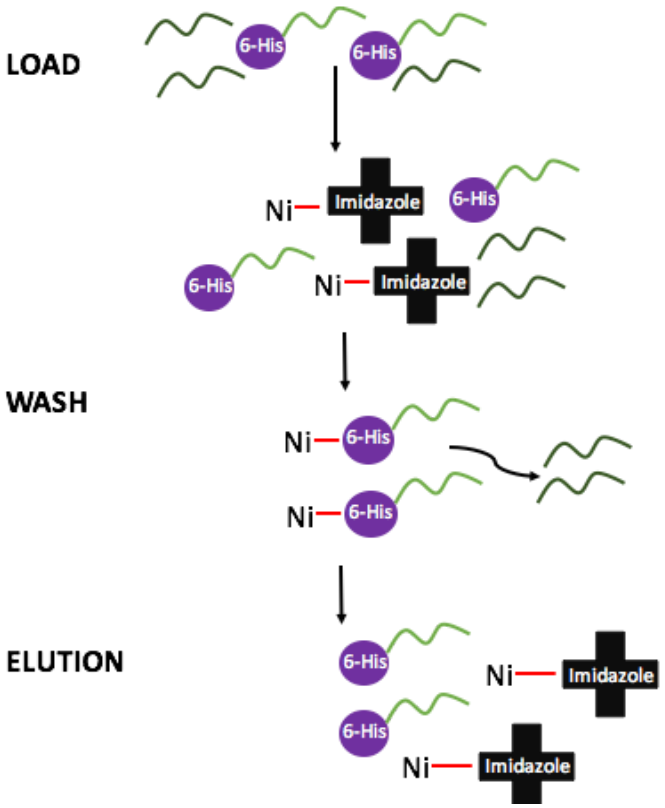

Figure 9. Representation of protein purification stages. Diagram of competitive binding between imidazole and 6-His tag to bind Ni-NTA beads

The eight stored samples (1 lysate, 1 flow-through, 5 wash steps, and 2 elutions) for each purification conducted were analyzed using SDS-PAGE (GenScript) at 150 volts for 40 minutes. Visualization was done using Coomassie Brilliant Blue Dye to identify the location of our protein throughout the purification and troubleshoot ways to keep our antibody in the solution.

Results

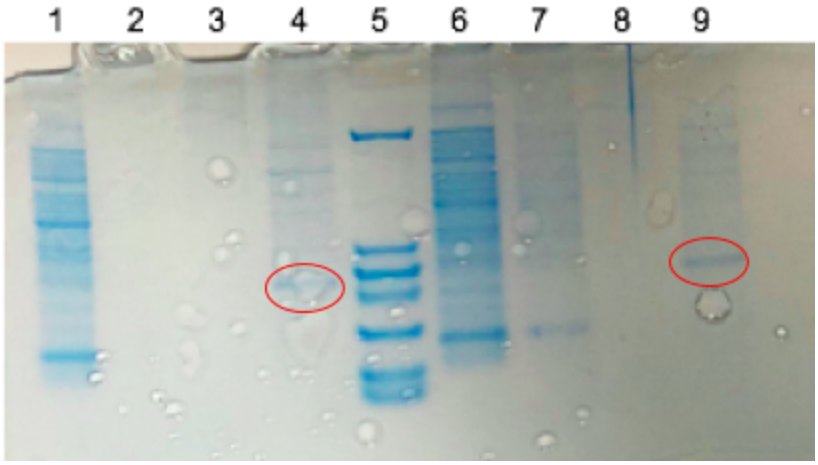

Figure 10a. Coomassie Brilliant Blue stained SDS-PAGE Gel. Gel visualization of eight samples from protein purification washing and elution steps, with synthetic antibody band (circled in red) between 25-30kD.

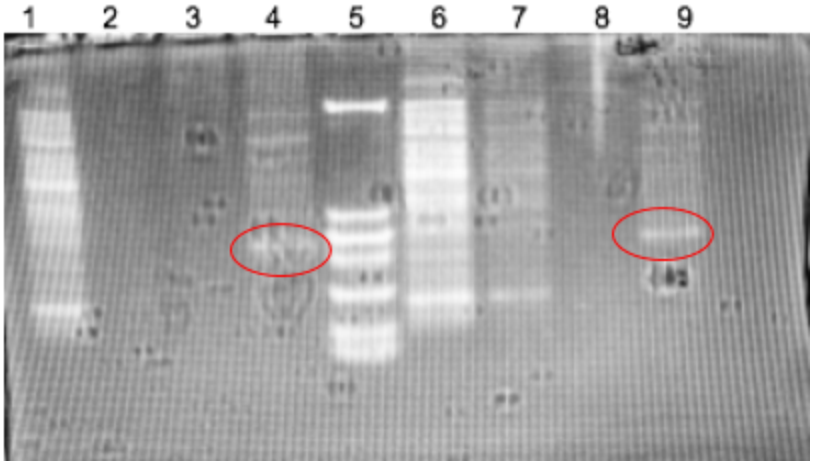

Figure 10b. Chemiluminescence image of SDS-PAGE Gel. Gel visualization of eight samples and protein ladder using chemiluminescence, with synthetic antibody band (circled in red) between 25-30kD.

Figures 10a and 10b show the results of the SDS-PAGE gel in both white light and chemiluminescence. Wells 1-4 are from the protein expression of the synthetic antibody while wells 6-9 are part of the control experiment that did not have L-DOPA. These wells correspond to the samples that were stored during the purification process. As expected, there

is no antibody in the wash steps because they are all bound to the Ni-NTA beads and it is until the elution in well 4 where we see the band at 27kD. This band is due to the increase in the imidazole concentration during elution which knocks the antibody off of the Ni-NTA and into the supernatant.

Discussion

The imidazole in the various buffers competes with the 6-His tag on our antibody to bind to the Ni-NTA beads due to the similarities in their molecular structure. The imidazole is bound to the beads during the initial suspension and competes with the 6-His tag during the various washes. The amount of imidazole added to the buffer solutions is inversely proportional to the amount of protein that is bound to the Ni-NTA beads. We are able to keep our antibody attached to the beads and wash out the impurities before we move on to the elution, which knocks the antibody off the bead and allows us to harvest them from the supernatant. We used native conditions to purify the synthetic antibody from the solution to keep the protein intact through the purification step. In order to limit unspecific binding, we washed the beads with wash buffer 5 times and eluted twice to unbind the synthetic antibody from the Ni-NTA beads.

The predicted size of our antibody is 27kD and we were able to get a clear band between 25-30 kD (shown in red circle on Figure 10a and 10b). However, there are still other molecules present in our elution which is evident from faint bands above and below our target band. This is not an issue because the validation testing that will be conducted is specific to the prostate specific antigen.

Chapter 3: Validation Testing

Introduction

Validation testing is required to analyze the functionality of the synthetic antibody to ensure it has equivalent or greater binding specificity and strength comparable to the current standard measure of monoclonal antibodies. Our engineered synthetic antibody must be tested to prove that proper incorporation of the unnatural amino acid is accomplished. To do this, the K_d of both the experimental and control synthetic antibodies to PSA will be measured using isothermal titration calorimetry (ITC). The K_d value will express the binding affinity of the synthetic antibody to PSA.

The specificity and sensitivity of the engineered synthetic antibody to PSA is crucial to establish a reliable system with both accuracy and precision. Each of these measures will be tested with the use of Western Blot on titrated PSA samples to analyze the experimental synthetic antibody compared to anti-PSA monoclonal antibodies.

Materials and Methods

Specificity Testing

In the first Western blot, the field will be divided into two sections (with PSA on both sides), inserting the monoclonal antibody on the left and the synthetic antibody on the right. Both are expected to bind to PSA, which will be visualized as a black band for the monoclonal antibody and as a green band for the synthetic antibody carrying GFP. This confirms the specific binding of the synthetic antibody to PSA.

Sensitivity Testing

A second Western blot will be used to verify the sensitivity of the synthetic antibody in comparison to the standard sensitivity of the monoclonal antibody. By adding an increased concentration of the synthetic antibody to each successive well, the minimum concentration of the synthetic antibody needed will be determined. Each successive well, beginning with the well containing the minimum concentration for binding, will fluoresce green, indicating the lowest concentration needed to bind PSA. Finding that the synthetic antibody has greater sensitivity than the monoclonal antibody will confirm that this new detection method is a successful replacement of the monoclonal antibody based diagnostic.

Discussion

It is expected that the binding strength of the experimental synthetic antibody will be significantly greater than the control synthetic antibody since the L-DOPA, in the presence of sodium periodate, triggers the formation of a covalent bond between the synthetic antibody and PSA. Once the K_d values of the synthetic antibody are compared to the known binding strength of the anti-PSA monoclonal antibodies, which is 1.1 ± 0.2 nM, the requirements of binding affinity are satisfied. The experimental synthetic antibody needs to have at least a comparable K_d in order to be a successful replacement technology for PSA detection.

Based on the current detection measures of PSA, normal conditions are considered to be below 2.5 ng/mL, while concentrations of PSA between 2.5 ng/mL and 4.0 ng/mL are indicative of prostate cancer. These ranges will be used as a standard in testing the functionality of the synthetic antibody.

Chapter 4: Conclusion

Summary

This project sought to engineer a synthetic antibody for prostate cancer detection using unnatural amino acid incorporation. By circumventing the challenges incurred through the use of monoclonal antibodies, this synthetic antibody presents a more efficient and cost-effective method to detect prostate cancer. The use of *E. Coli* cells as replication machinery also eliminates animal welfare concerns, as well as ensures there is no batch to batch variability of the synthetic antibodies. Validation testing is currently underway, and must be continued to validate that the designed antibody has equal or greater binding affinity to PSA. Once verified that the synthetic antibody can accurately and efficiently detect prostate cancer with reference to the current monoclonal antibody, this design promises to revolutionize prostate cancer diagnostics.

Future Work

In order to replace current diagnostic techniques with this newly engineered synthetic antibody, future work must be done to ensure that non-specific binding does not affect measurements of PSA concentrations. This will be done through various assays which will test the synthetic antibodies tendencies to bind to various molecules or surfaces. Issues caused by non-specific binding can also be ameliorated by introducing various constraints which will allow a signal to be emitted only when the synthetic antibody is bound to PSA.

Future Applications

Both the unnatural amino acid incorporation and synthetic antibody designs present models which can be used for a variety of medical and research applications. Unnatural amino acid incorporation is a promising new technology; by introducing non-biologically relevant amino acids into protein sequences, engineers are effectively expanding the genetic code. As shown

by L-DOPA's ability to greatly increase bond strength, expansion of the genetic code through this method introduces novel functions into the biological world. The synthetic antibody model can also be replicated for countless applications. By replacing monoclonal antibodies produced by the hybridoma model, these *E. Coli* based synthetic antibodies can ameliorate ethical concerns regarding animal welfare and accessibility in research and medical applications.

Chapter 5: Ethical Concerns

Traditional monoclonal antibody production is a well known process that has been used to create antibodies that have high specificity and binding strength. While these traditional production techniques are highly effective, they carry with them two main ethical concerns that synthetic antibodies work to address. The first concern centers around animal welfare, as the production process requires the antibodies to be produced *in vivo*. The second concern is the related expenses associated with the current production techniques that have contributed to the extremely high cost of traditional monoclonal antibody therapies.

Animals are required for the current production of monoclonal antibodies. While their treatment during incubation may be up to current ethical standards, much debate exists around the extraction process. Prior to harvesting monoclonal antibodies, the immune systems of the animals must be stimulated with a particular disease agent to saturate the blood with antibodies. This practice is life-threatening, incurring a high high mortality rate, and as such, creates a extensive ethical dilemma for the scientists and companies that employ it. As an alternative, synthetic antibodies are manufactured in *E. Coli* which removes the animal from the production cycle entirely, thereby eliminating the ethical dilemma surrounding production.

The use of animals for production also contributes to the extremely high costs associated with traditional monoclonal antibody production. These high costs severely limit access to these antibodies to only the select few who can pay the high price. These antibodies are an incredibly useful tool for researchers and doctors alike, but due to high costs, many institutions and research centers shy away from them. This creates the ethical dilemma because many people could be helped by this technology, but due to its high cost, very few people can afford to work with or utilize them, severely limiting the scope of research on the subject. With synthetic antibodies, the production systems have been validated through

literature and practice as being low cost, high quality systems. This would dramatically reduce the cost of monoclonal antibodies, allowing for an increase in the scope of research and usage.

Chapter 6: Engineering Standards and Realistic Constraints

Purpose

Engineering design requires that certain considerations be taken into account before the design process begins. The following factors will be discussed using a framework that analyzes the impact each factor has on the project and its stakeholders.

Economic

Despite this being a project and product focused on saving lives, the economic factors of this project must be taken into account when considering the realistic constraints of this project. The budget for this project takes into account the funding required to fully carry out all of the experiments. The economic advantages of designed synthetic antibodies are apparent when discussing large scale antibody production. Traditional antibody production requires large investments in resources and animal care facilities to make production viable, but the extensive costs associated with these resources and facilities are eventually passed on to the patient in the form of high drug costs. Synthetic antibody production, on the other hand, is done in *E. Coli*, which both reduces the cost of small scale testing and makes production easily scalable, significantly reducing the costs and resources necessary for production. This makes the synthetic antibody proposed in this paper a less expensive alternative to traditional monoclonal antibodies, allowing for greater patient access.

Ethical

There are two main analyses that must be conducted from an ethical standpoint for this project. The first is to perform a stakeholder analysis. Stakeholders for this project include the producers of the synthetic antibody, intermediate users of the antibody such as

researchers, industry experts and scientists, and academic institutions, and finally the end users of the synthetic antibody as patients and those who would be both directly and indirectly impacted by the use of the antibodies. The next, and far broader analysis is one that involves addressing the ethical concerns, dilemmas, and benefits of these synthetic antibodies. The largest benefits of synthetic antibody production come from the cost savings associated with a leaner production cycle, and the elimination of animal usage during production. These benefits eliminate the two largest ethical concerns for traditional monoclonal antibody production.

One of the largest ethical concerns associated with synthetic antibody production is the concern of the possibility of using all parts of the process in the production of biological weapons. Screening methods can be repurposed to identify peptides that cause disease or physiological disorders. These peptides could then be manufactured using the production methods outlined in this paper to produce a bioweapon either in a small, unregulated facility or on a large scale for a very low cost. This ethical concern should be understood and addressed, but the utilization of the techniques described in this paper to produce a bioweapon requires extensive and intimate knowledge of multiple disciplines. This knowledge requirement significantly reduces the risk of exploitation of the manufacturing methods identified in this paper; however, engineering ethical standards should be maintained so as not to cause harm to any of the stakeholders or other members of society identified in this section.

Social

As this project has health and medical implications, social responsibility as it relates to every stakeholder identified must be taken into consideration. The current high cost of monoclonal antibodies prevents those stakeholders who do not have the financial means to afford antibody treatment from seeking antibody treatment, as insurance companies continue to refuse to pay the incredibly high costs. Reducing the overall cost of antibody treatments

through the introduction of synthetic antibody production would open up access to previously expensive treatment regimens to a much larger portion of stakeholders than ever before.

Manufacturability

This project aimed to validate the manufacturing process outlined in this paper on a small scale, with the notion that successes at this scale could be applied to a industrial scale manufacturing. However, it is to be understood that small scale success does not imply that the jump to production will be simple. There are several challenges and complications that arise when attempting to scale up monoclonal antibody production. In the traditional manufacturing methods, the largest problem is scaling up the animal production facilities while maintaining the proper level of quality control in the produced antibodies. For synthetic antibody manufacturing, this quality control challenge is all but eliminated through the use of *E. Coli* as the production platform. Large scale bioreactor production of *E. Coli* has been thoroughly documented in literature, and has been proven in practice in various industrial settings. These previous successes significantly improve the possibility of a successful scale-up of the manufacturing procedures verified through this project.

Health and Safety

Ensuring the health and safety of all stakeholders involved in the project is of the utmost importance at all phases of the product. For the small scale production detailed in this paper, all team members passed the Santa Clara University Laboratory Safety Protocol and Procedures exam. This ensured the execution of the manufacturing plan in a proper lab setting where all risks associated with the materials and procedures used in this project were minimized. Members also had previous lab experience performing various laboratory procedures, and as such were well trained in the handling of hazardous materials.

To protect the health and safety of the stakeholders undergoing treatments using these synthetic antibodies, it is paramount that these antibodies only be used for their intended

purpose. In the case of the project described in this paper, the specific antibody being developed has the sole purpose of detecting the PSA biomarker. As such, the synthetic antibody outlined in this paper should not be used to detect other biomarkers possibly present in stakeholders undergoing treatment. Adherence to this principle will help ensure the health and safety of those undergoing treatment.

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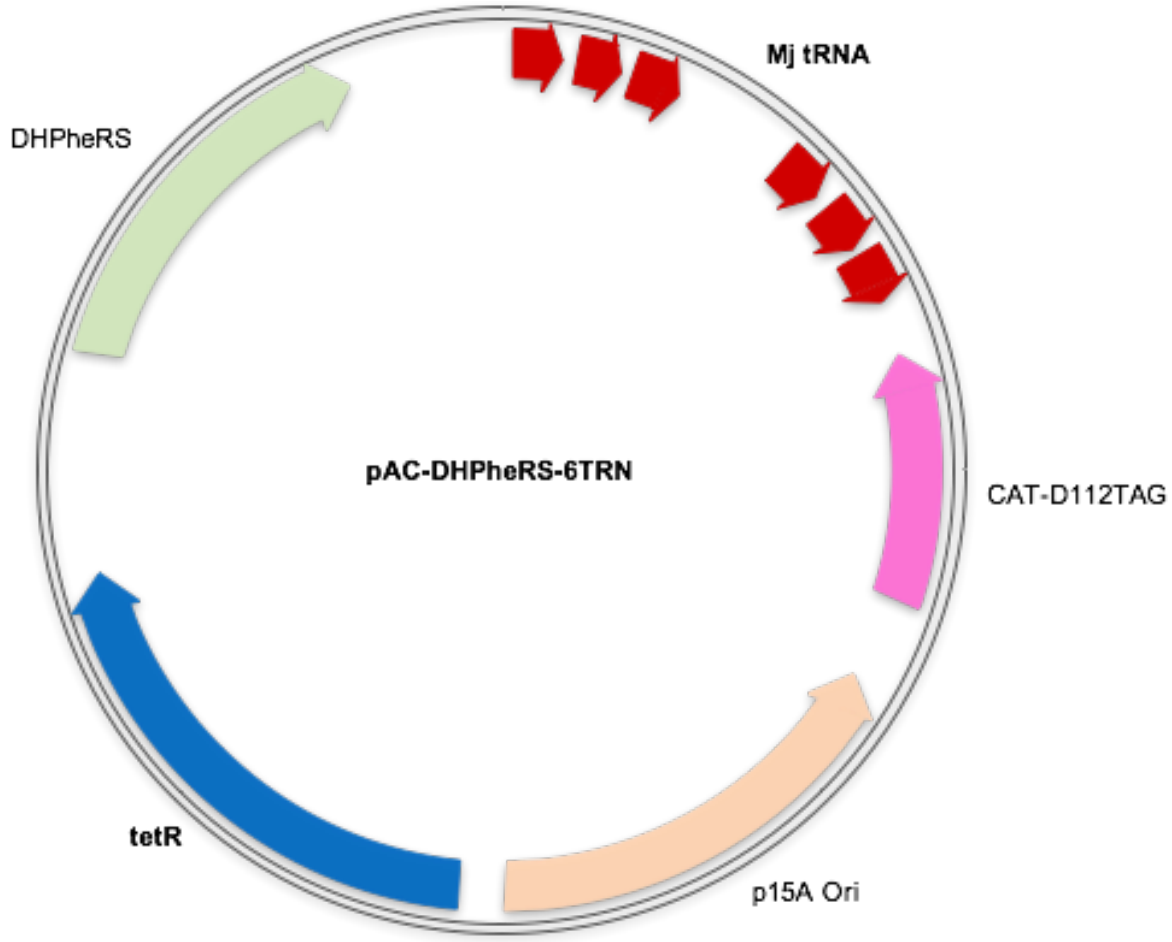
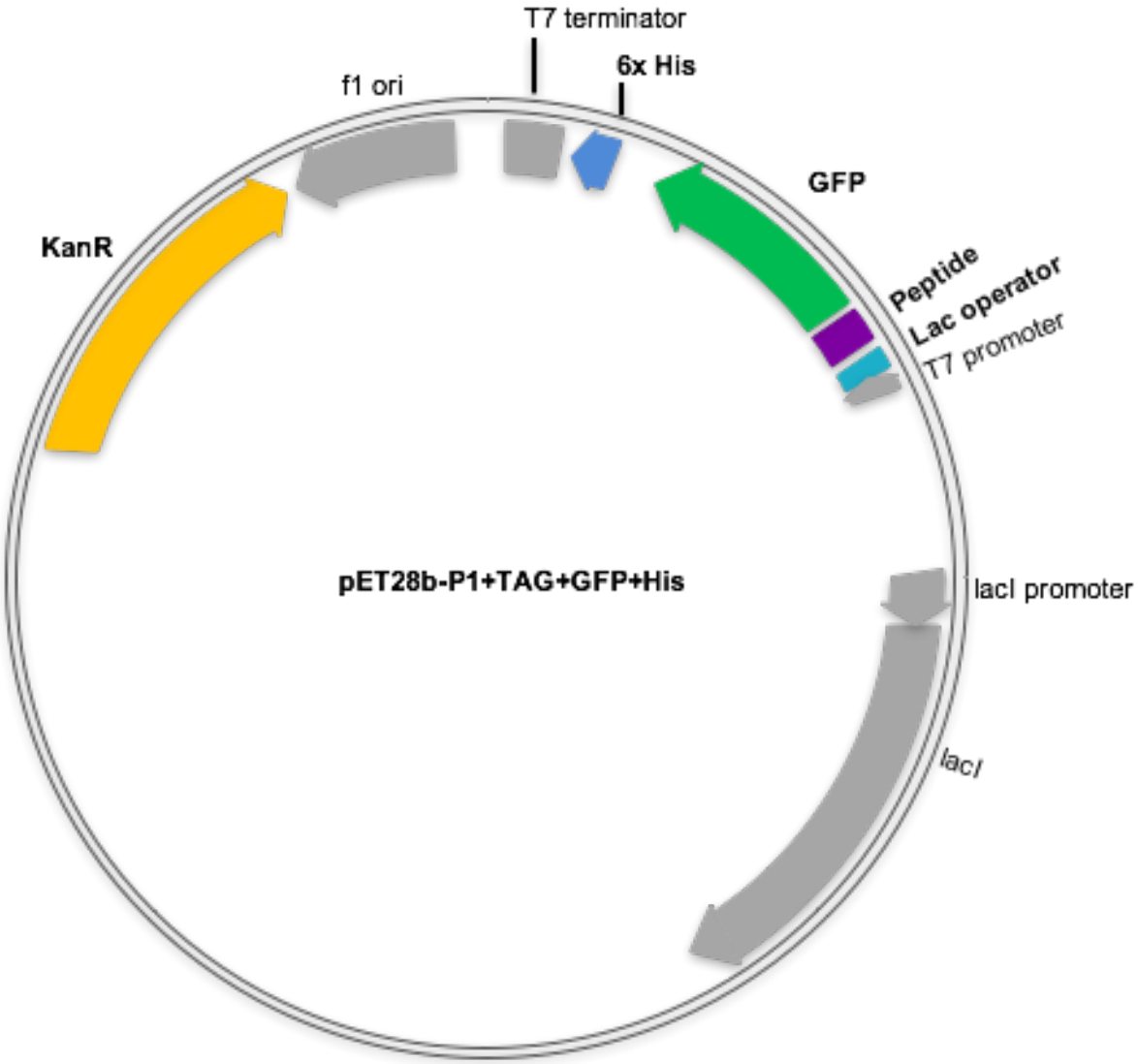
Appendix

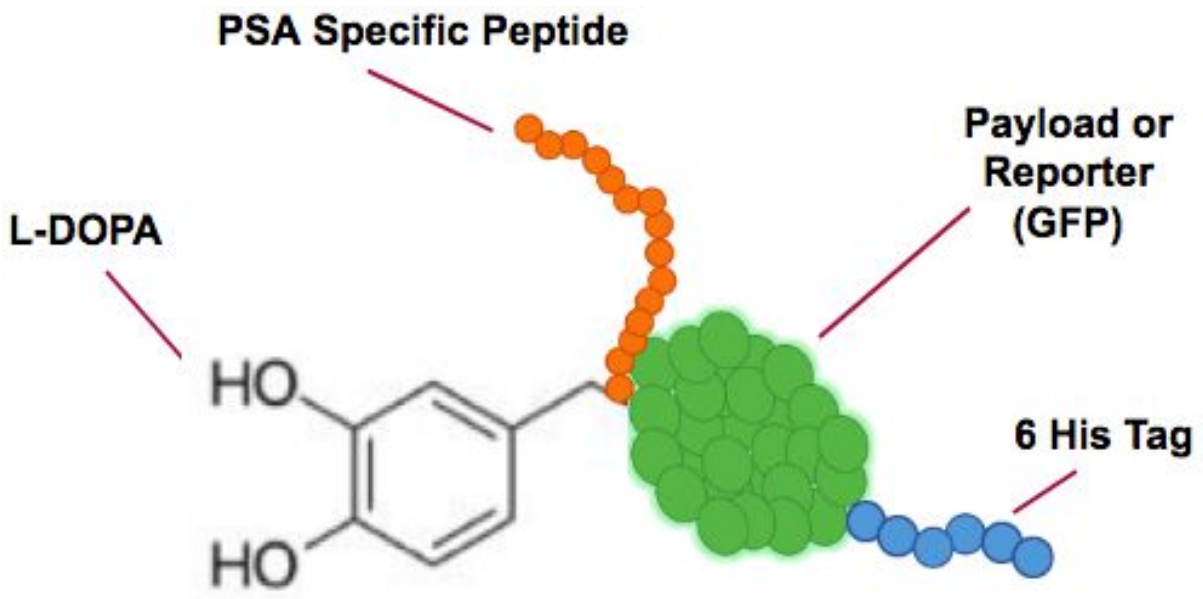
Appendix A. pAC DHPheRS-6TRN plasmid map



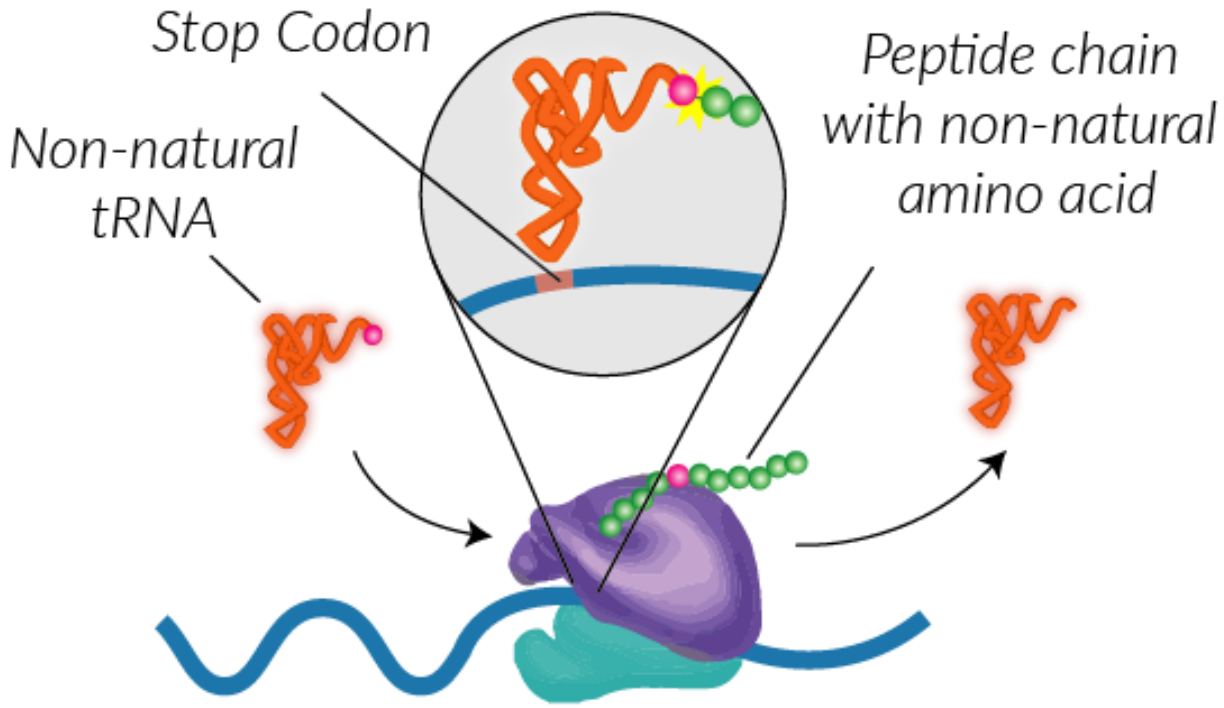
Appendix B. pET28b-P1+TAG+GFP+His plasmid map



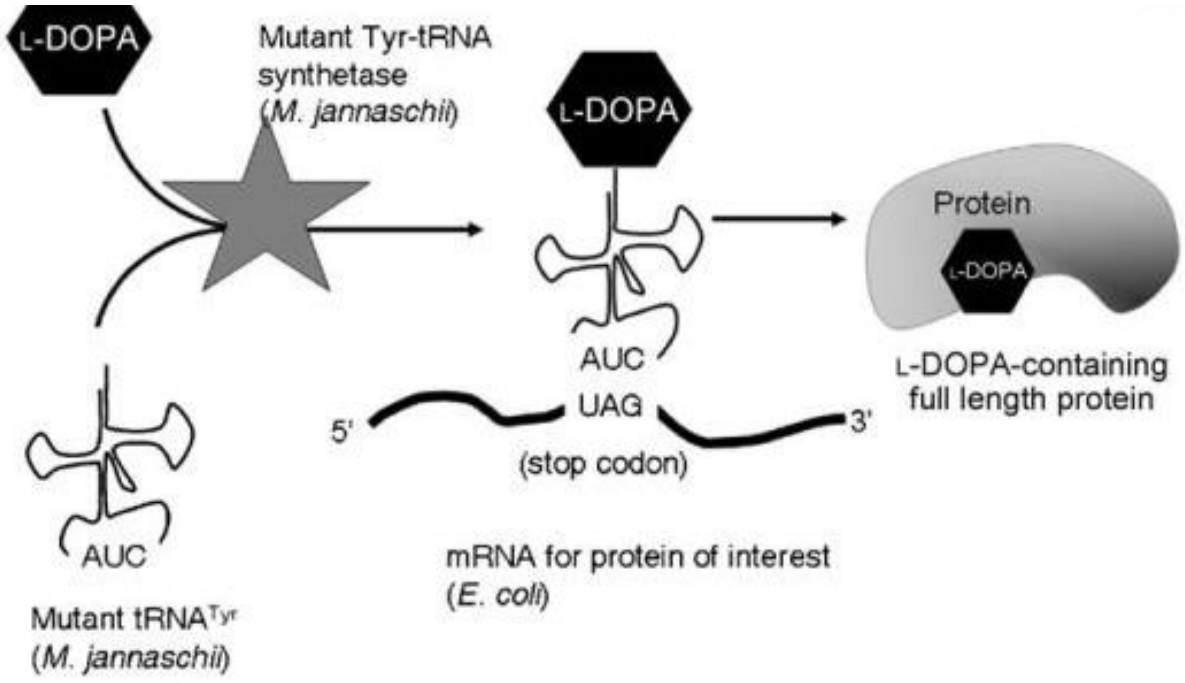
Appendix C. Synthetic Antibody Design



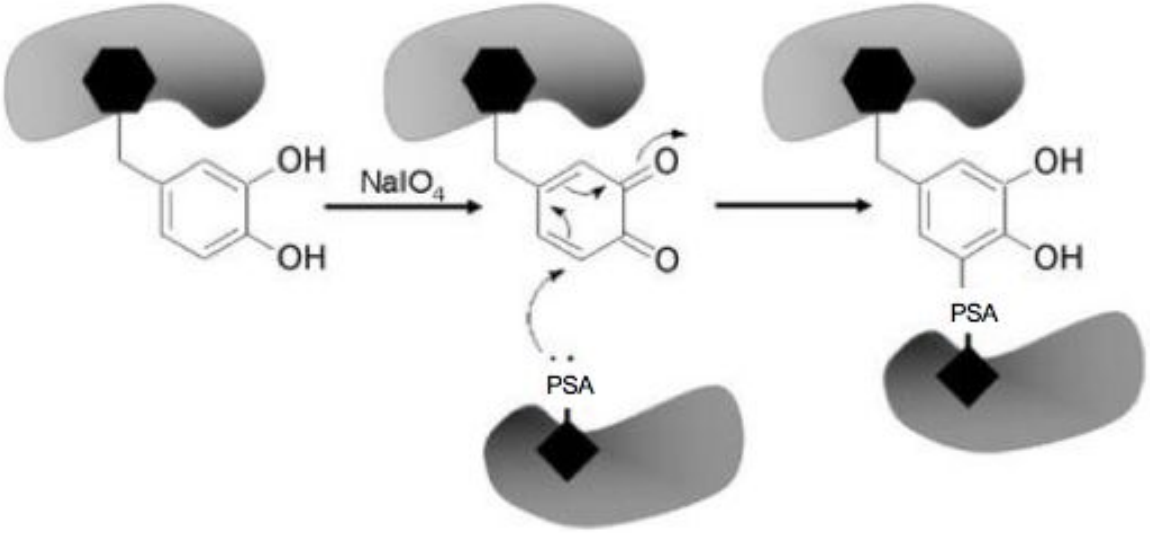
Appendix D. Unnatural Amino Acid Incorporation Schematic



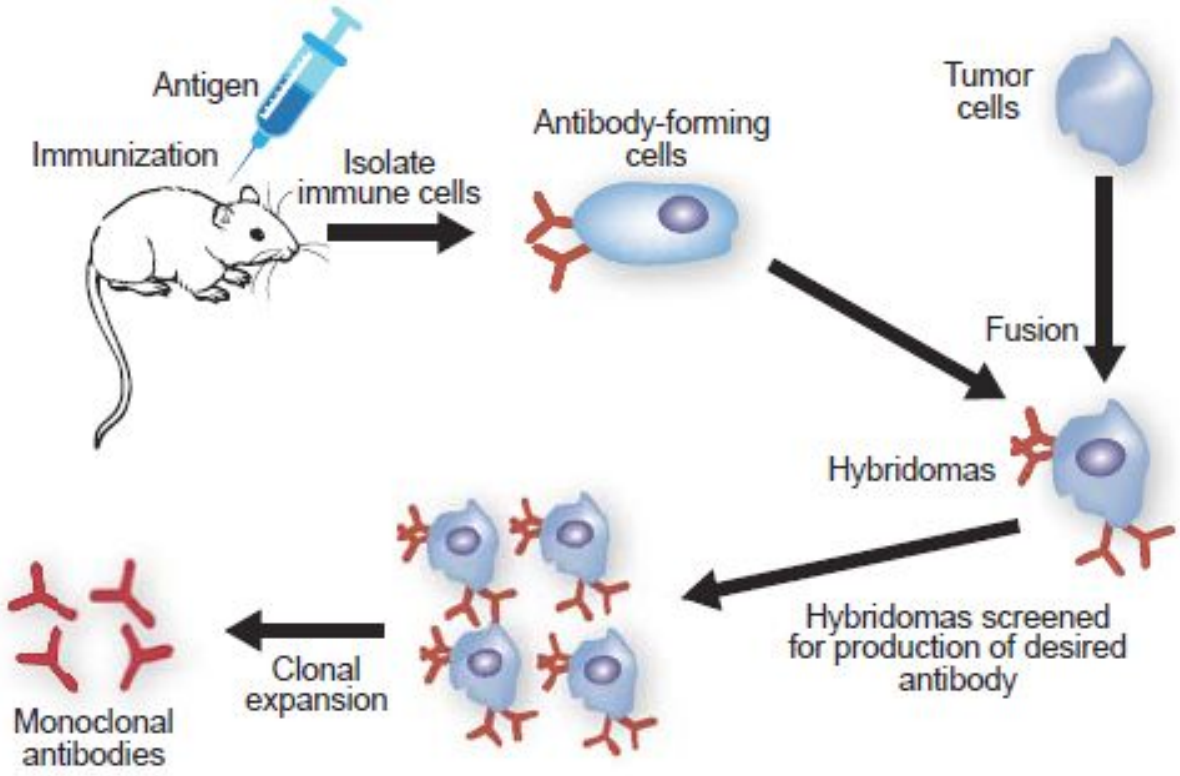
Appendix E. L-DOPA Incorporation



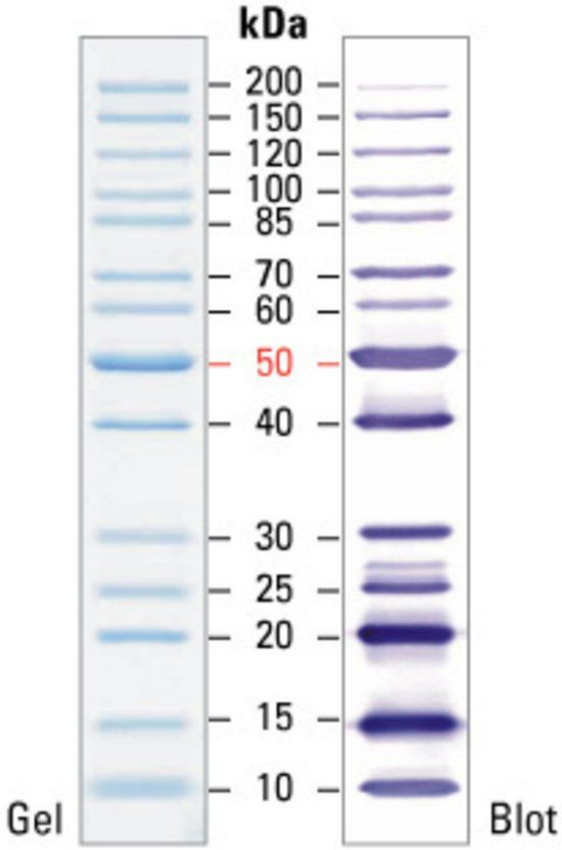
Appendix F. PSA Binding to Synthetic Antibody Schematic



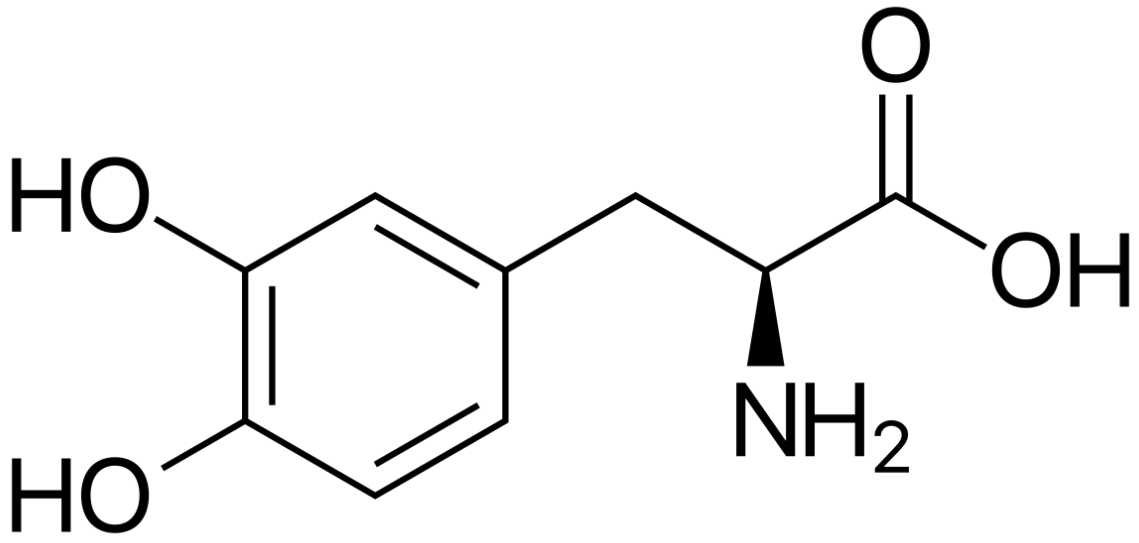
Appendix G. Hybridoma Model



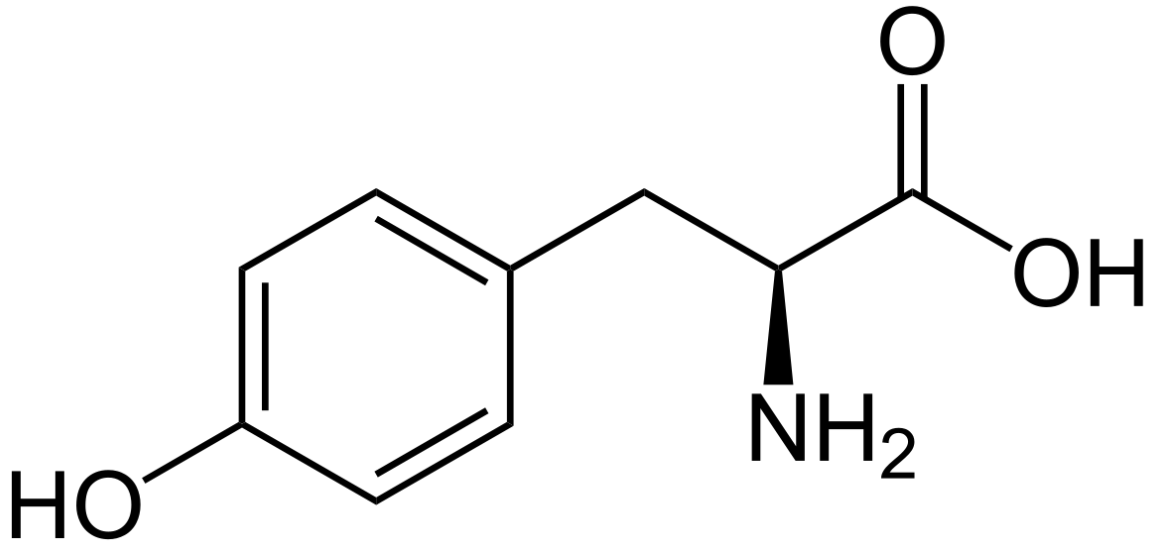
Appendix H. PageRuler Unstained Protein Ladder



Appendix I. L-DOPA and Tyrosine Structures



L-DOPA



Tyrosine