

# Enzyme Purification and Plasmid Transformation in *E. coli*



By  
Scott Bedle

## Table of Contents:

List of Figures.....	1
Executive Statement.....	2
Introduction.....	3-5
Method.....	5-6
Results.....	7-15
Discussion.....	16-17
Conclusion.....	17
Citations.....	18

## List of Figures

Figure 1 – Class Gel electrophoresis.....	7
Figure 2 – Bacteria grown on +/- amp petri dishes.....	7
Figure 3 – Single and Double digest of certain Enzymes.....	8
Figure 4 – APE XhoI Digestion.....	8
Figure 5 – APE SacI Digestion.....	9
Figure 6 – APE EcoRV Digestion.....	9
Figure 7 – APE SacI and XhoI Digested .....	10
Figure 8 – APE SacI and XhoI digested.....	10
Figure 9 – APE EcoRV and XhoI Digested.....	11
Figure 10 – Hypothetical Gel.....	11
Figure 11 – SDS-Page.....	12
Graph 1 – Absorbance vs. Protein Concentration.....	12
Graph 2- Dilution Series.....	13
Graph 3 - Ave for ENZ $10^{-1}$ .....	13
Graph 4 - .3 ONPG sub series.....	14
Graph 5 - .6 ONPG sub series.....	14
Graph 6 – Average of all OPNG variations.....	15

**Executive Summary:**

Enzyme purification and ingesting it into other DNA can revolutionize modern medicine, farming, and life in general. Learning about how certain enzyme effect protein production can be done using many methods. The purpose of these labs is to obtain an amount of an enzyme that is ampicillin resistant, check its purity and make *E. coli* ingest this enzyme. Using current techniques to do all of this *E. coli* can be made resistant to ampicillin. The enzyme during these labs to do this is Beta-Galactosidase. Using modern methods the enzyme was purified, checked for purification, and ingested into *E. coli*. The enzyme activity was also looked at during these labs to look at the relation of concentration of the enzyme and how is effects activity.

Throughout the following pages one will find the information about each lab in the introduction. In methods section one will find a brief description of the methods used during each lab. In the results section one will find the results from each lab. In the last section, discussion a discussion of the results and what the results mean can be found.

## Introduction:

The objective of these labs is to learn about the preparation and plasmid transformation of *E. coli*, the expression and production of the enzyme Beta-galactosidase, protein purification and analysis of enzyme activity. During these experiments and a lot of other research experiments dealing with proteins, *Escherichia coli* (*E. coli*) is used as the bacteria. *E. coli* makes good models for most proteins and the one used in these labs is non pathogenic. A specific strain of DH5-alpha *E. coli* was used during these labs. DH5α is used for a lot of engineering DNA strands. To produce proteins the plasmid DNA must be changed. Plasmids are small, circular DNA chromosome elements that have a part of their replication component. Plasmids can be easily purified from bacteria and transformed into other bacteria. They also can be visualized by gel electrophoresis. Gel electrophoresis is a method for sorting proteins, in this case, for their size (Dickey 228). The plasmid used during these labs was transformed in DH5α *E. coli*. The plasmid used encodes an ampicillin-resistant gene, which will show the presence of the plasmid in the *E. coli* when placed on petri dishes with and without ampicillin. A control will also be used during the plating of *E. coli*. To obtain a DNA restriction digest map, the plasmid must be digested with a series of restriction enzymes. Restriction enzymes are the scissors used by biologist to cut DNA strands (Dickey 224). These enzymes cut certain sections of DNA by needing an exact strand of DNA to bind to it. Using different restriction enzymes and doing either a double digest or a single digest a digest map can be created. To create the map the resulted DNA digest is put into agarose gel, contain a DNA binding dye, which will allow visualization of the DNA in the gel. The map can be used to compare size and molecular weight. The restriction enzymes used during the mapping of the plasmid are EcoRV, XhoI, and SacI. A ladder to help determine molecular weight and size was also used to help with Gel analysis. These can be used to make a relationship between movement and size on the Gel.

Plasmid can be obtained by extracting it from bacteria. To do this a form of Alkaline lysis is used. Alkaline lysis is a method of obtaining plasmid out of bacteria and can be analyzed easily by Gel electrophoresis. To obtain the plasmid the pH of the solution is changed to denature the DNA and eventually after many pH change renature the plasmid wanted (Brinboim 1). Alkaline lysis produces very pure plasmid DNA that can be used for, sequencing, mutagenesis and restriction digestion. The plasmid produced (pET-ELP(11)-Intein(MtuΔCM)-lacZ) by the alkaline lysis can be test for purity and can also be ingest into other bacteria.

The plasmid (pET-ELP(11)-Intein(Mtu $\Delta$ CM)-lacZ) extracted from the DH5 $\alpha$  *E. coli* was ingested by a different bacteria, *E. coli* protein expression strain (BLR). These *E. coli* were also grown in rich media to an optical density (OD<sub>600</sub>) of 0.7-0.9, which make the cells grow faster and exponential (late-log phase). The bacterium was then cooled to help with the correct folding of the protein being over expressed by the plasmid. Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) was introduced to the *E. coli* to induce the production of protein. IPTG is a lactose analog that releases the LacI repressor to activate the Plac-promoter transcription. Plac-promoter catalyzes the *E. coli* to express the bacteriophage T7 RNA polymerase (RNAP), which is encoded on the bacteria's chromosomes. IPTG is introduced to the bacteria to amplify the RNAP expression via the release of LacI repressor. This is done via a signal cascade. A signal cascade is a chain reaction of signaling usually between cells to amplify production of a certain enzymes (Diwan). IPTG amplifies the production of T7 RNA polymerase, which transcribes beta-galactosidase. T7 RNAP is a very active polymerase that only produces the target protein that is encoded on the plasmid with a T7 promoter. Since IPTG produced T7 RNAP and T7 RNAP only produced the desired protein most of the protein produced by the bacterium will be the plasmid-encoded protein.

In order to purify the specific protein elastin-like peptide tagging (ELP) reverse phase transition (RPT) must take place. A sample of cells that have beta-galactosidase fused with a bi-functional protein-purification tag sequence engineered onto the N-terminus is needed to purify the beta-galactosidase. ELP tagging is done with transition of temperature. At higher temperature the ELP tagged protein falls out of the solution. After the protein falls out of the solution the tag must be dropped. In order to do this the protein is suspended by changing the pH to 6.0 with salts. The intein on the protein has been engineered to help with the protein purification. With another round of RPT the ELP tag can be dropped and a pure protein is formed. The ELP and intein are both controlled by temperature and pH but the ELP effects a physical transformation while the intein effects the enzymatic separation. Samples during the following steps can be taken and purification of the protein can be seen with SDS-Polyacrylamide gel electrophoresis (PAGE). PAGE uses a hydrophilic plastic matrix made of polyacrylamide in order to separate proteins based on their molecular weight. Sodium dodecyl sulfate (SDS) is used as the detergent to unfold the proteins and give the chain a constant charge/mass ration for separation. After the gel has been run (loading dye off runs off gel) it

must be stained in order to see the bands of protein. Coomassie Blue is the dye used to stain the protein.

There are many ways to test for protein purification. Bradford Assay can be used to test the concentration of the protein. Bradford Assay uses dye to determine the protein concentration. Coomassie Brilliant Blue G-250 the dye used in this experiment. In order to form a relationship between the absorbance and protein concentration a graph must be made. The standard curve uses know concentration of a know protein, Bovine Serum Albumin, in order to make this relationship for beta-galactosidase and the dye used. A direct spectrophotometric measurement of protein can be taken using a nanodrop spectrophotometer. Beers law must then be used to calculate the concentration. Proteins have an average absorbance peak at around 280 nm and this must be used with the nanodrop spectrophotometer. Nanodrop spectrophotometer can as test for how pure the protein is by comparing it to the absorbance of DNA. To test this, the protein and DNA are test at both 260 nm and 280 nm. These absorbances are graphed and the percentage difference between the two determines how pure the protein is.

Enzyme activity can be test using the spectrophotometer. O-nitrophenyl beta-D-galactoside (ONPG) can be used to test activity of beta-galactose in a solution. ONPG turns into galactose and a yellow o-nitrophenol. The yellow o-nitrophenol is what the spectrometer picks up on during the reaction causing an increased absorbance reading. This increase in absorbance reading can measure the activity of enzymes. Different concentration of both ONPG and the enzyme can be tested to find the best reaction concentrations for an enzyme.

### **Methods:**

#### **Gel Electrophoresis:**

A plasmid of DNA sample is loaded into the Gel and electricity is run through the Gel allowing the DNA because it is negative to run down the Gel. A UV light camera can take a picture of the Gel (Dickey 229)

#### **Modified Alkaline Lysis:**

The modified Alkaline Lysis that is used is done by changes of temperature and pH to purify the protein. This makes the ELP tagged protein suspend and resuspend. The intein then drops off the protein leaving just the Beta-Gal. During each step the solute becomes more pure than the last until just the Beat-Gal remains.

#### **PAGE:**

To run the digests each one was loaded into a gel with a buffer and an electric current was run through them. After the gel has been completely run the Gel is heated and the dye is added. After the dye is heated in a microwave the Gel is then clean by heating it in water to get excess dye out. A normal photograph can be taken of this gel.

#### Mini Prep:

The Qiagen Mini Prep was used during this experiment. This is used to extract a bacterial plasmid DNA. First the cells must be lysed to denature then neutralized to renature DNA but denatures and helps unfold the protein. Potassium-SDS must be used to precipitate DNA and some protein but not plasmid DNA. Once the plasmid DNA is caught in a spin column it is washed and then removed from the column as a pure plasmid DNA.

#### Transforming Bacteria:

To get bacteria to take in a selected plasmid DNA the cells must be heat shocked. This will cause the cells to ingest the plasmid DNA. The mini prep above can produce the plasmid for the bacteria to ingest.

#### Nanodrop Spectrophotometer:

The nanodrop spectrophotometer needs a small amount of protein to test the concentration of the protein and purity. It does this by using Beer law and graphing the results.

#### APE Software:

The APE software used has an coded plasmids (Beta-Gal) and allows one to added different enzyme to cut the plasmid and shows when the plasmid will be cut and can graph it to show what it would look like when run on a gel.

#### Absorbance Spectrometer:

The machines tests for the absorbance of during enzyme reactivity and the date from this can be plotted to find the concentration of the protein being tested. The machine picks up on the yellow color produced during the reactivity of the enzyme and ONPG.

#### Procedure for all methods:

The procedure of all of these methods can be found either online, in the sources at the end of the report or the lab protocol given.

## Results:

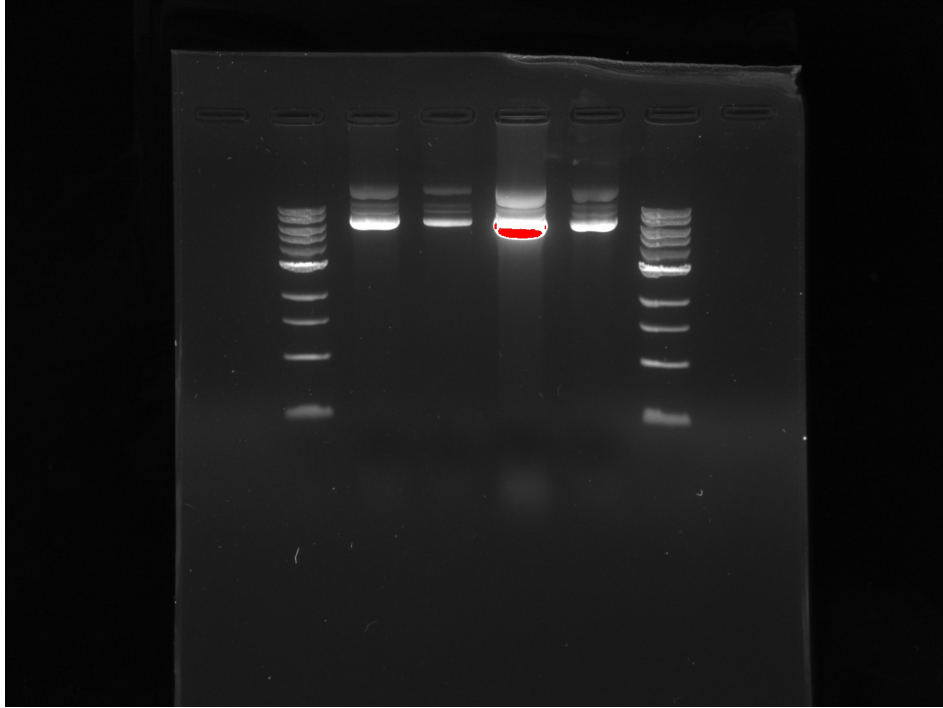


Figure 1 – Class Gel electrophoresis

The figure above shows the run for the uncut plasmid DNA for the entire class. The ladder DNA sample is on the side to compare with the uncut plasmid DNA

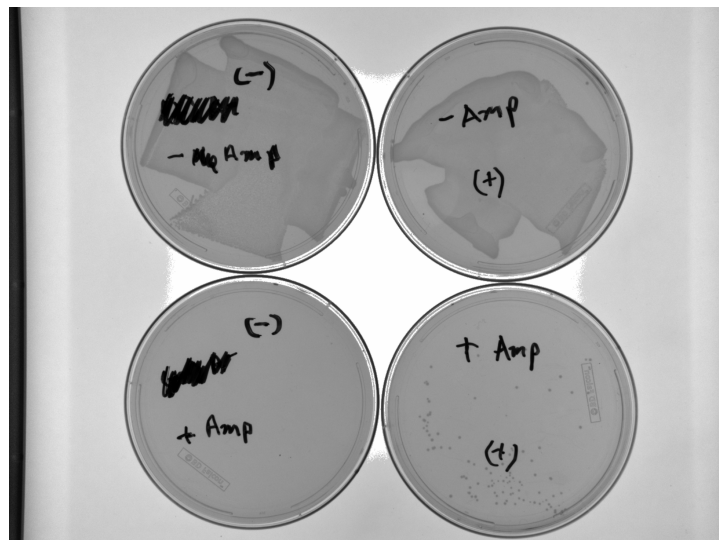


Figure 2 – Bacteria grown on +/- amp petri dishes

The figure above shows E. Coli with the plasmid in it and with out the plasmid in it. They were both put on petri dishes with ampicillin and without ampicillin.

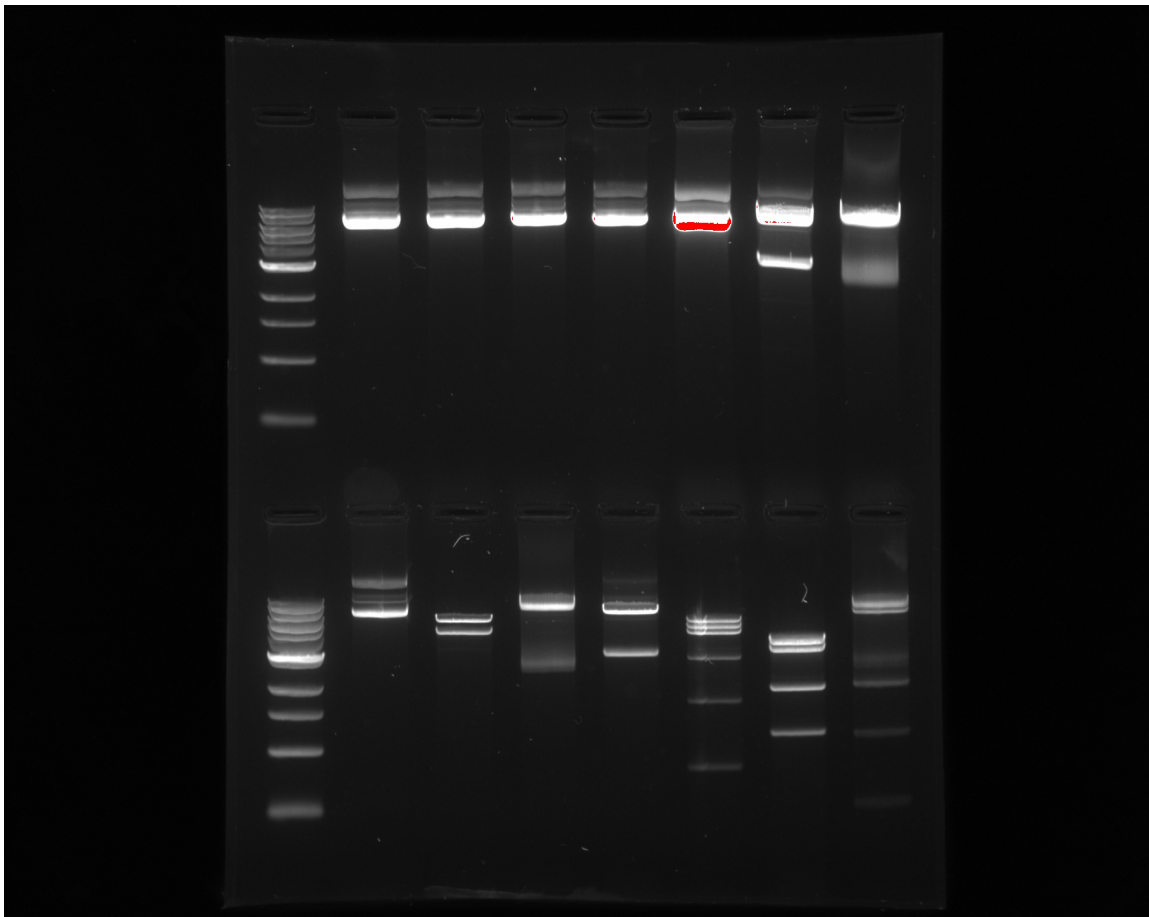


Figure 3 – Single and Double digest of certain Enzymes

The above figure shows plasmid DNA with cuts from certain enzymes. The upper one is the sample being looked at for this experiment.

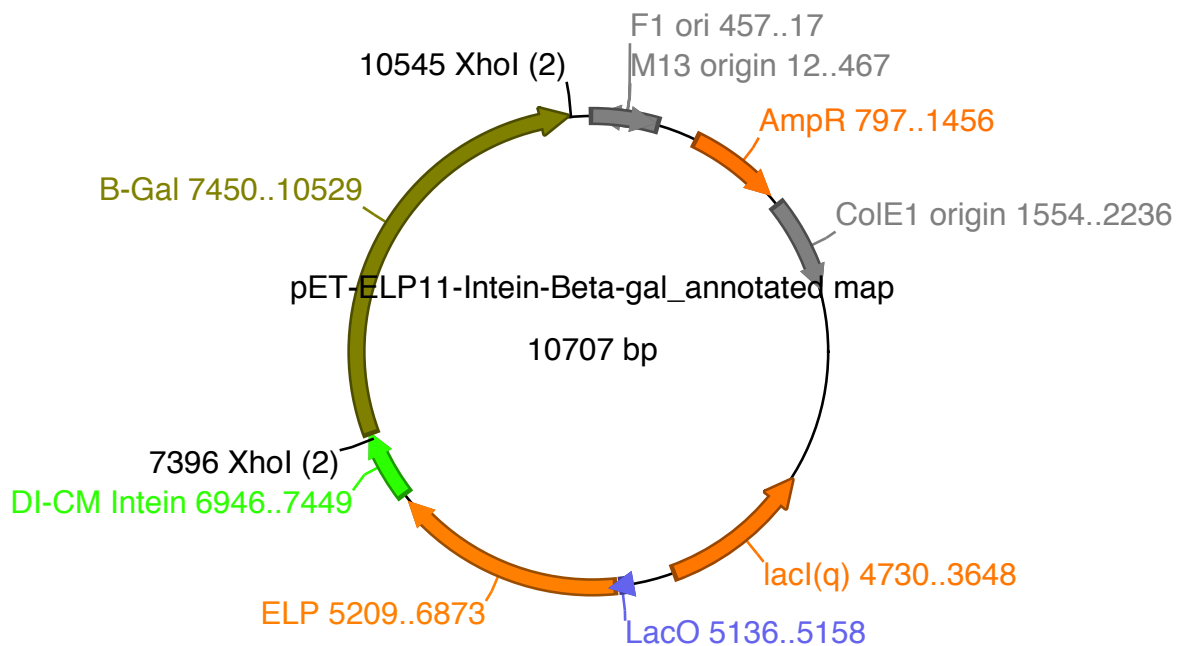


Figure 4 – APE XhoI Digestion

The above figure shows the digestion of XhoI enzyme in the Beta-Gal plasmid on APE software.

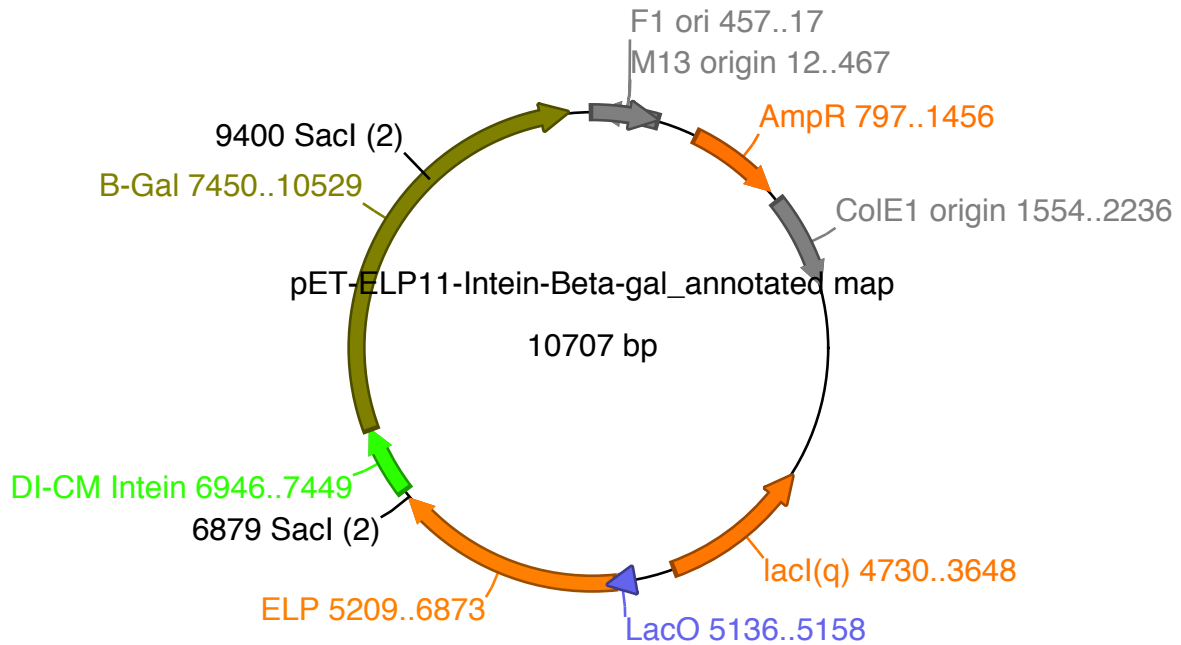


Figure 5 – APE SacI Digestion

The above figure shows SacI digested into the plasmid on APE software

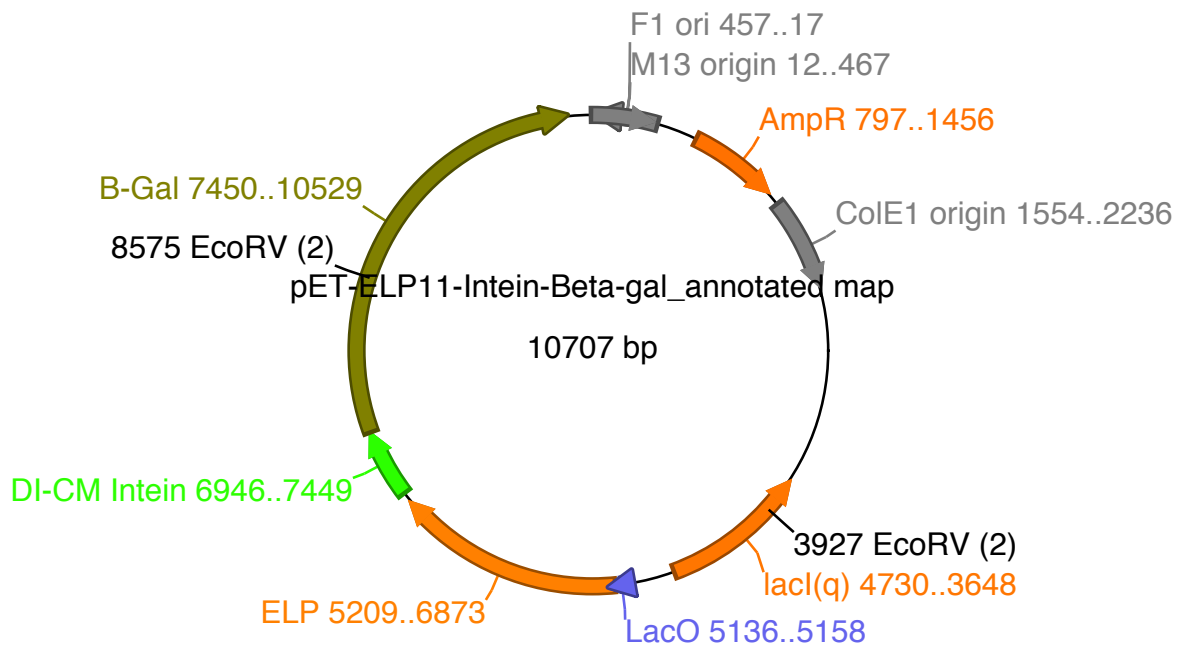


Figure 6 – APE EcoRV Digestion

The above figure shows EcoRV digested in the Beta-Gal plasmid on APE software.

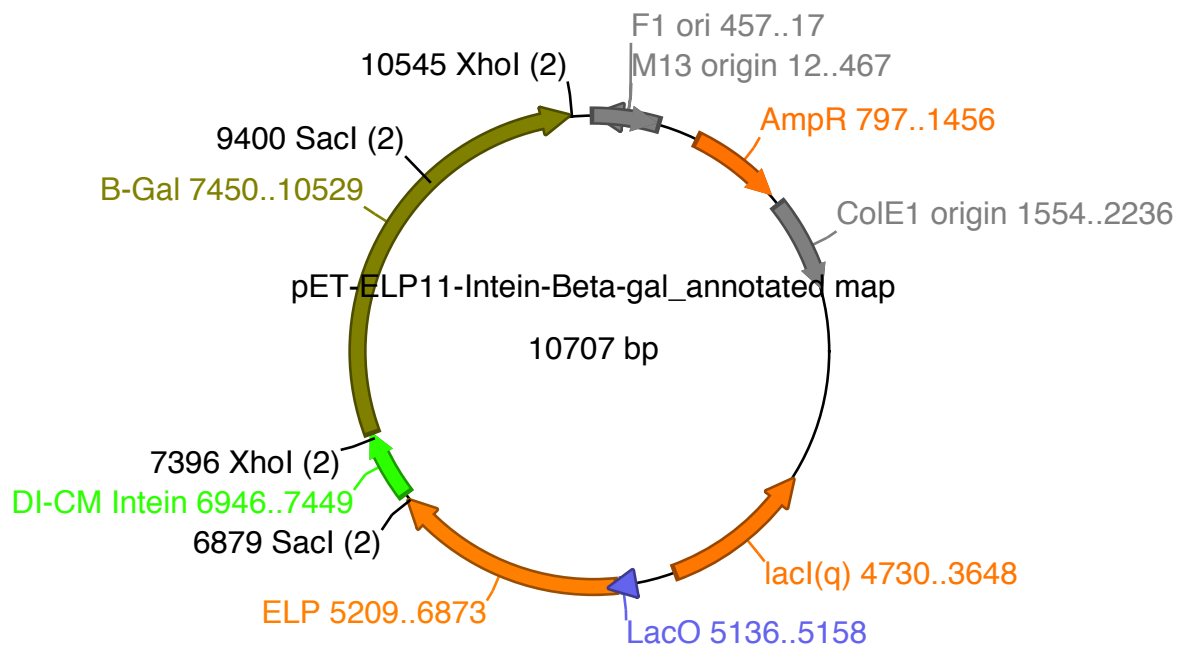


Figure 7 – APE SacI and XhoI Digested

The figure above shows the double digest of both SacI and XhoI in the Beta-Gal plasmid on the APE software.

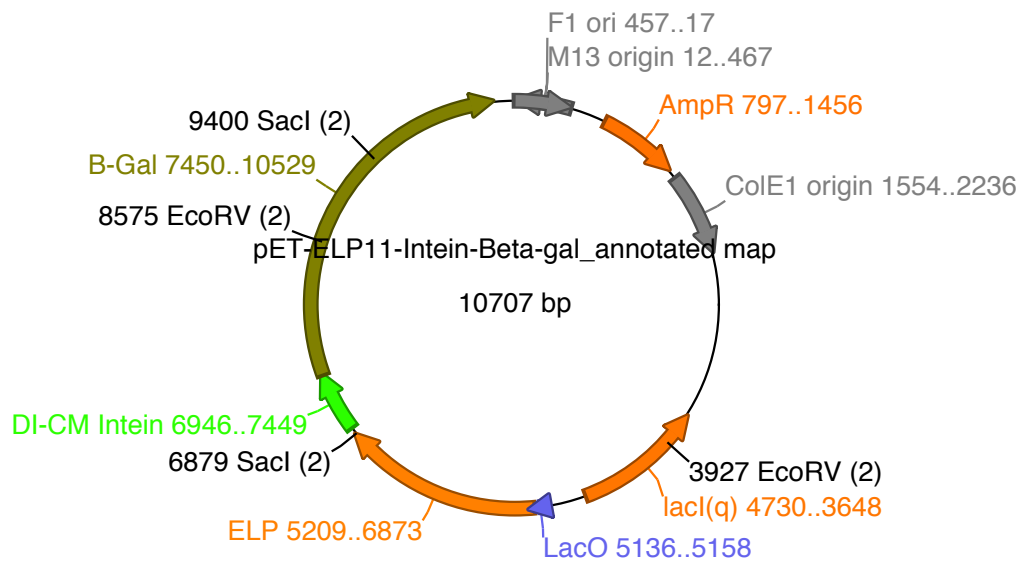


Figure 8 – APE SacI and XhoI digested

The figure above shows the double digest of both SacI and XhoI digested in the Beta-Gal plasmid on the APE Software

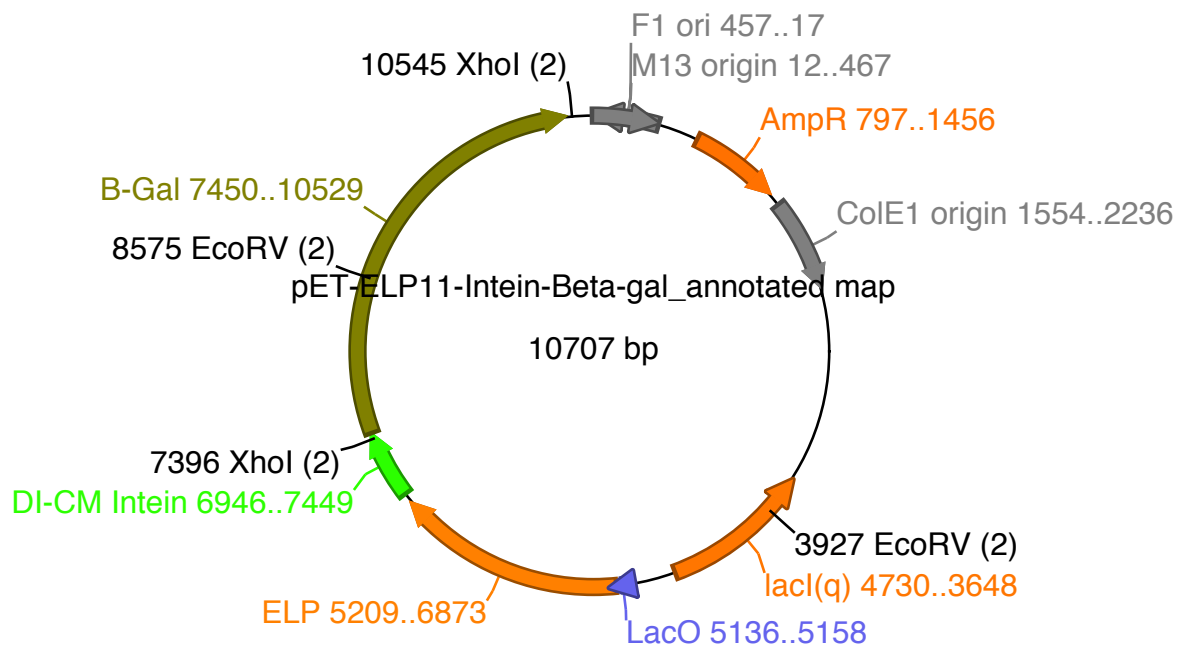


Figure 9 – APE EcoRV and XhoI Digested

The figure above shows the double digest of EcoRV and XhoI digested in the Beta-gal plasmid on the APE software.

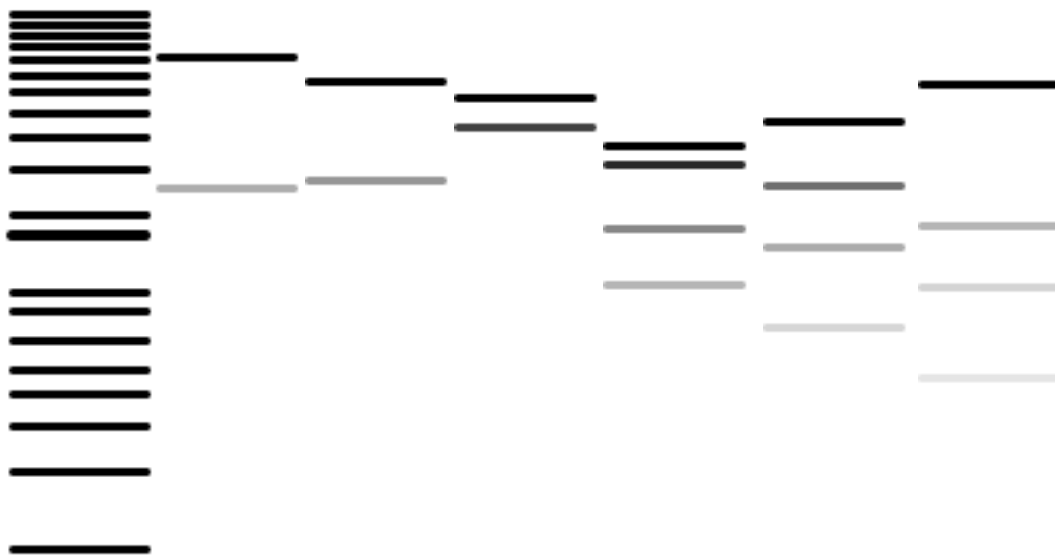


Figure 10 – Hypothetical Gel

Figure 10 shows what Figure 3 should look like.

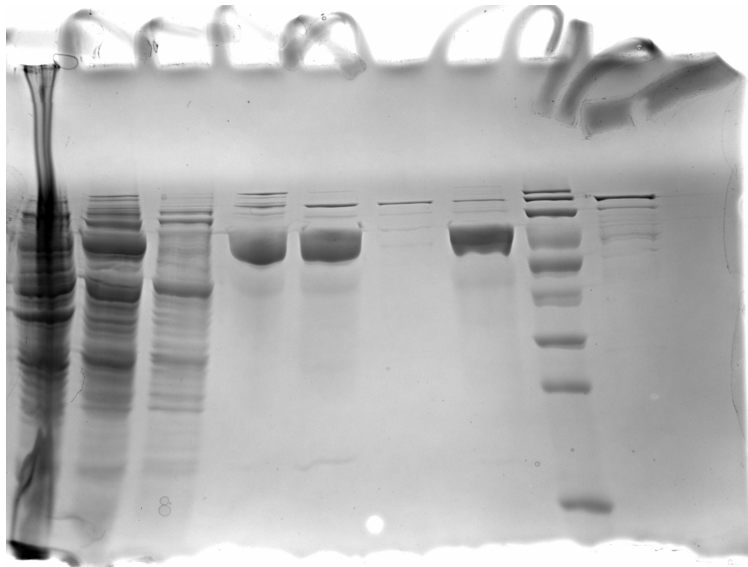
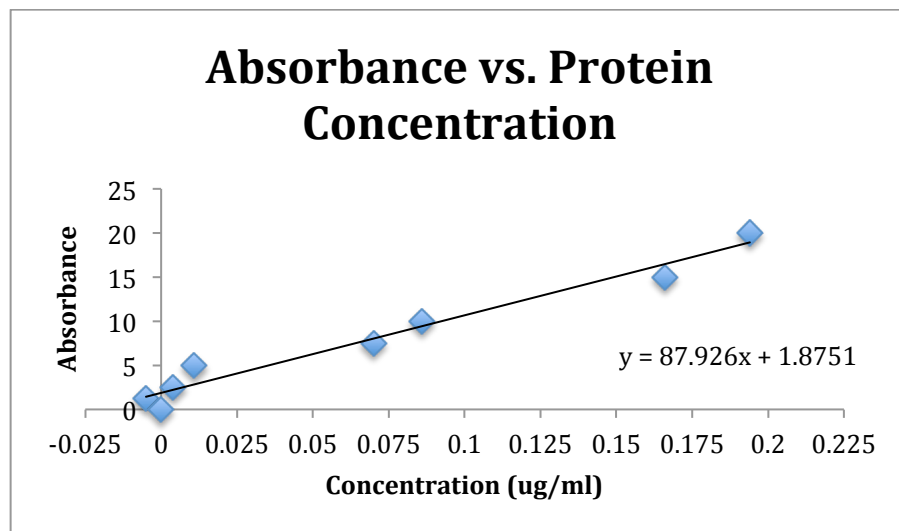


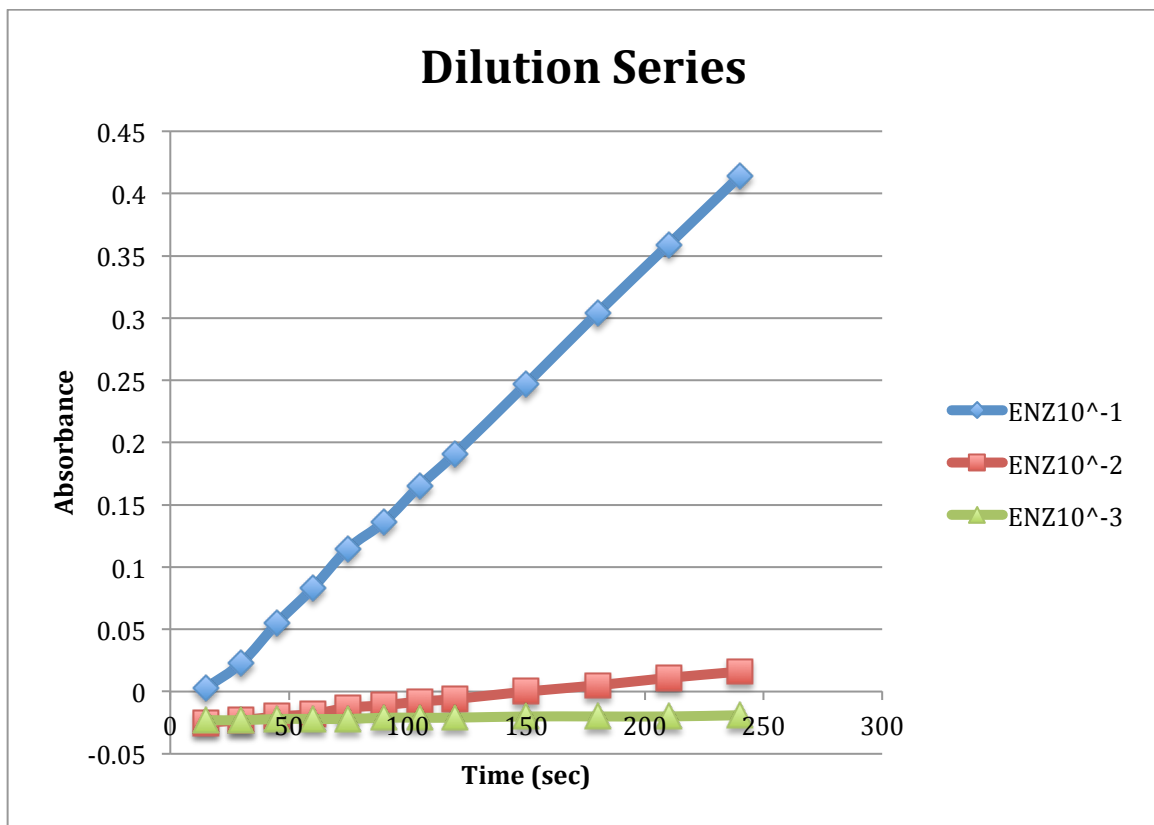
Figure 11 – SDS-Page

The order of the samples run goes whole lysate, clarified lysate, soluble contaminants, purified precursor, post-cleaving proteins, purified protein, precipitated tag, beta-gal, a purified protein, a ladder, and a control.



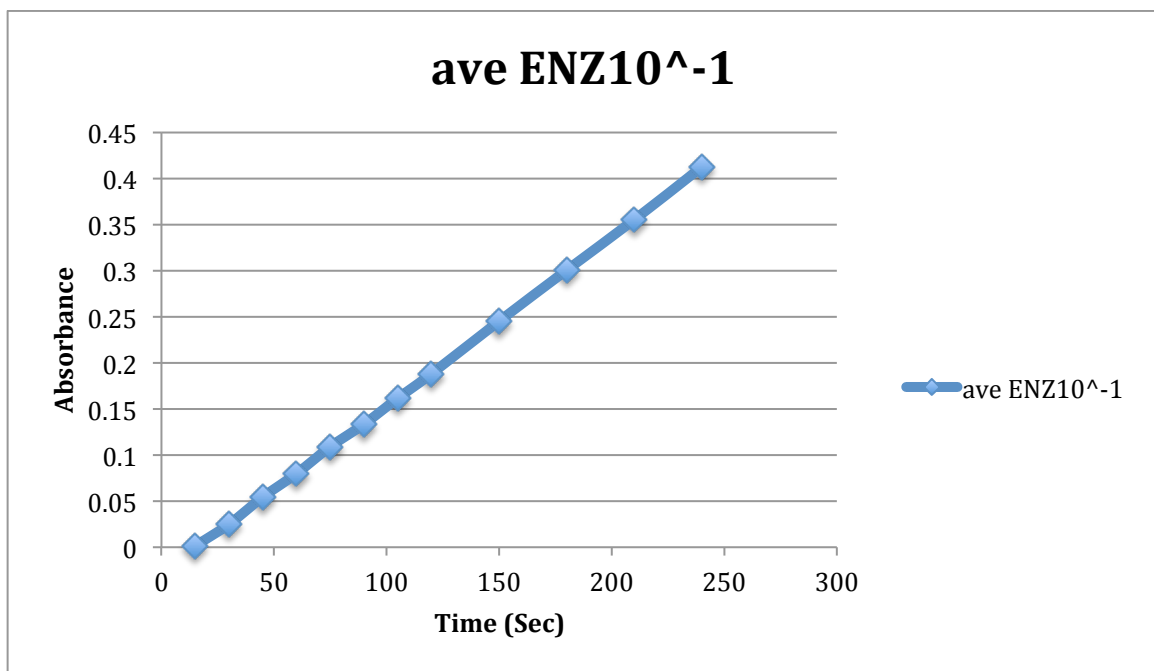
Graph 1 – Absorbance vs. Protein Concentration

The graph above shows the Absorbance vs. the concentration.



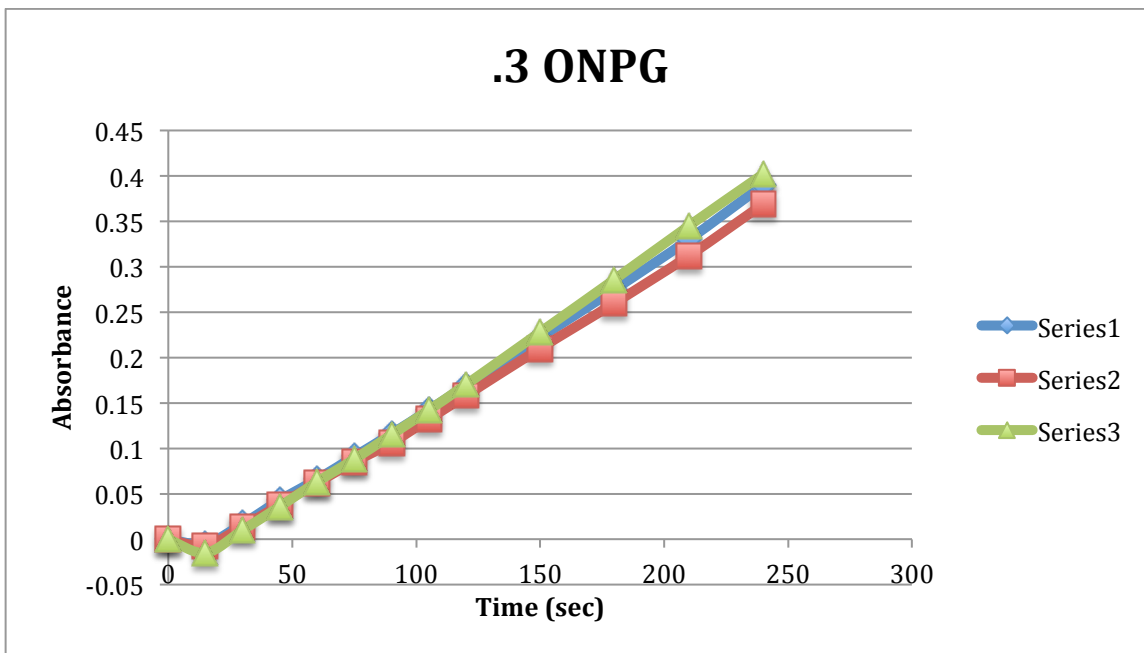
**Graph 2- Dilution Series**

The graph above shows the enzyme that was least diluted had the most activity while the other two dilutions did not react as much.



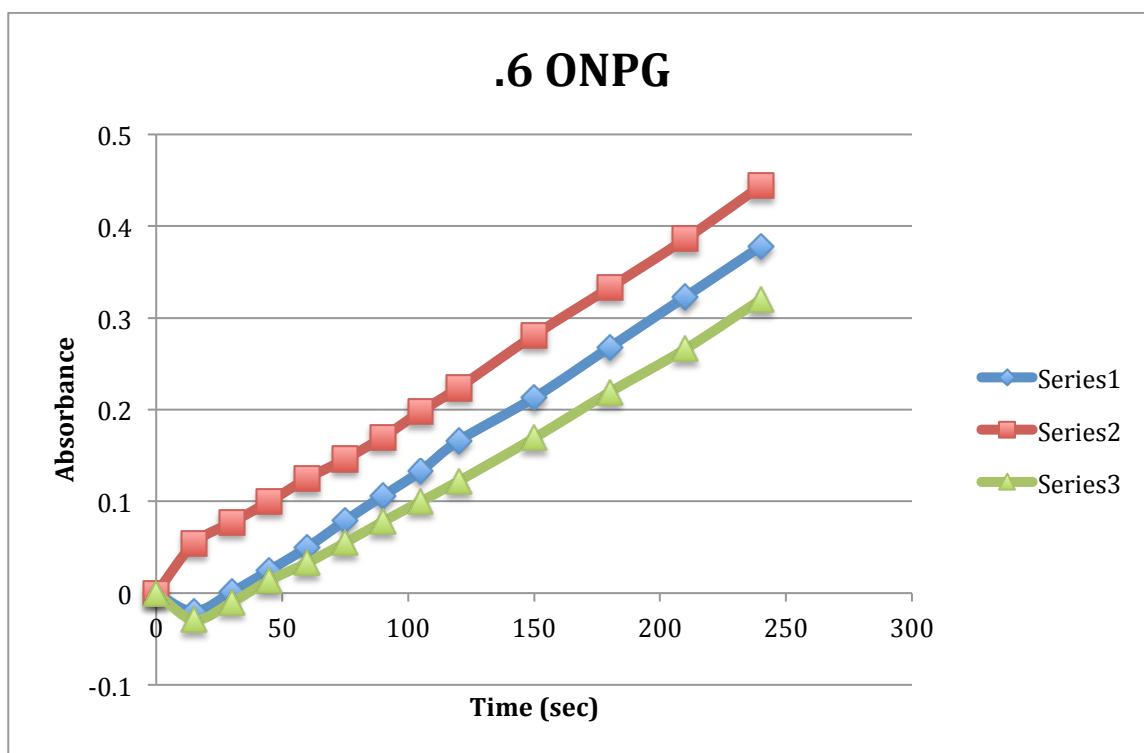
**Graph 3 - Ave for ENZ 10<sup>-1</sup>**

The graph above shows the average absorbance over a 4-minute period of the fastest dilution series (ENZ10<sup>-1</sup>).



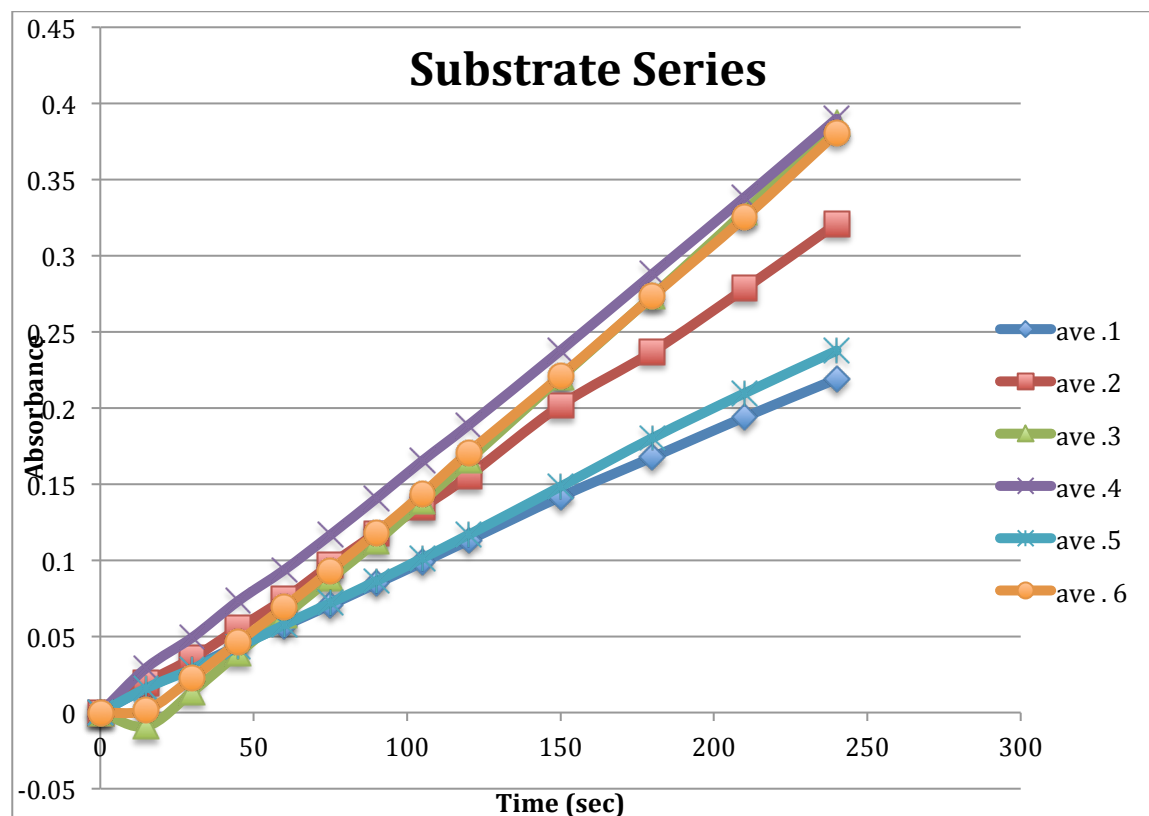
Graph 4 - .3 ONPG sub series

This graph shows the activity of the enzyme in a solution with .3 ONPG.



Graph 5 - .6 ONPG sub series

The graph above shows the activity of .6 ONPG in a solution with the enzyme.



**Graph 6 – Average of all OPNG variations**

The graph above shows the activity of a standardized enzyme mix with solution set a 2.7ml but a different amount of ONPG each time.

Results for Nanodrop Spectrophotometer:

The A280 for the Beta-Gal is .045 and the 260/280 ratios is .33 meaning that our Beta-Gal is not very concentrated but extremely pure.

## Discussion:

The uncut plasmid of DNA shows where the protein is compared to the ladder. Using Figure 10 one can see which enzyme can be used and when they should be used to cut out the Beta-Gal protein.

The results for our colonies of cells were as expected. The colonies in plasmid had the ability to survive in the ampicillin whereas the cells without the plasmid could not survive. Both cells survived and thrived in the gel without ampicillin.

Our cell digests order was Ladder, Control, SacI, XhoI, EcoRV, EcoRV and XhoI, SacI and XhoI, EcoRV and SacI. Clearly something went wrong in the digest of our enzymes. A couple factors that can be brought into the failed experiment could be. The amount of enzyme that made it in to the digest was not present. This is because the pipet is only measurable at 2.0 microliters and we had to put .5 microliters in. If one were to come to a conclusion on the relationship between mass and migration look at an APE map of the digest the more enzymes that cut the DNA the farther it moves. So the single digests only move so far because they have a lot of mass while the double digests move a bit more because they have less mass.

One can see that as the process of purification goes on more and more junk gets taken out and even with all the problems encountered during these last couple labs, for instance the solute not re-suspending multiple times, the protein was still able to be purified. By look at the gel it confirms that the sample as a purified protein in the beta-gal sample and also the purified protein sample. This protein that was confirmed will be used in later labs.

Using the standard curve graph above and plugging in the absorbance of the unknown concentration (-.008) the standard equation gives the unknown a concentration of 1.18 micrograms/ml. This fits with the SDS-Page gel, which confirmed the protein was present and purified. A problem ran in to during this process was negative absorbance numbers for samples that had a concentration. This flawed our standard curve because the values were off. A better way to find the equation would be to increase the know concentration so they are not as small.

Much can be learned from both the dilution series and the substrate series. During the dilution series it can be seen that the more diluted the enzyme the slower the reaction. The highest concentration of the enzyme reacts much faster than the 2<sup>nd</sup> or 3<sup>rd</sup> dilutions. Looking at the substrate series one can come to the conclusion that the lower dilutions start at fast but

slow down whereas the higher concentrations start fast and continue to stay at a faster rate. The .6 solution of ONPG although stay very similar to the .3 dilution series.

### **Problems:**

A couple of problems happened during this experiment. The first problem was that the bacteria that was suppose to be used did not reproduce so Dr. Lease's E. Coli had to be used. The other problem encounter was something happen with the digest that made them not run like they should have. Fortunately, a gel using APE was created to mimic what is should have looked like. This gel shows the relationship between mass and how far the gel plasmid will run.

Updates to Lab protocol:

Although the lab protocol was very helpful and informative it could use some improvement. One improvement to the protocol could be that the procedure be written more clearly so it can be understood by non-biology people who want to preform this experiment. The procedure was very thick in was hard to follow without Ryan Bell's help on explaining what to do. Also a little more background information should have been given in the protocol itself so not as much outside reading and research is needed to understand key aspects of the protocol. These updates would only help student understand the lab better and would allow a non-biology student complete the lab without and an instructor's help.

### **Conclusion:**

Enzyme purification and ingesting it into other DNA can revolutionize modern medicine, farming, and life in general. Learning about how certain enzyme effect protein production can be done using many methods. The objective of these labs was to learn about the preparation and plasmid transformation of E. coli, how to express and produce the enzyme Beta-galactosidase, how to purify protein by the use of purification methods and the analyses of enzyme activity. Through all these labs one can learn how to produce proteins and enzymes and how enzymes catalyze reactions.

### **Citation:**

Banki, Mahmoud, Liang Feng, and David Wood. "Simple bioseparations using self-cleaving elastin-like polypeptide tags." (2005): n. page. Print.

Birnboim, H.C., and J Doly. "A

rapidalkalineextractionprocedureforscreeningrecombinantplasmidDNA." *Nucleic Research*. France: 1975.

Diwan, Joyce. *Signal Transduction Cascades*. Diss. Rensselaer Polytechnic Institute , 2008. Print.

<<http://www.rpi.edu/dept/bcbp/molbiochem/MBWeb/mb1/part2/signals.htm>>.

Simon, Eric, Jean Dickey, and Jane Reece. *Essential Biology with Physiology*. Fourth. London:

Dorling Kinderley Limited, 2013. Print.