# Purification of GST-GFP utilizing the 6x-His Tag and Immobilized Metal Affinity Chromatography.

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Objective:

The main purpose of this project was to optimize the purification protocol of the fusion protein GST-GFP currently used in the Quantitative Biological Methods Laboratory. This protein, 6xHis-GST-TEV-EGFP, is a fusion of enhanced green fluorescent protein (EGFP) and glutathione-S-transferase (GST),with a TEV protease, which can cleave the linker (GST) from the protein of interest (1).

In this project, the main change was replacing GST-affinity chromatography with immobilized metal affinity chromatography (IMAC) and using three different samples and three different elution buffers, each containing an increasing concentration of imidazole. A control was run simultaneously using GST-affinity chromatography and the entire protein purification protocol from the QBM laboratory manual was used for all four samples.

Principle of Methods:

In the current QBM laboratory protocol, the two purification methods used are first, affinity chromatography, followed by ion exchange chromatography (IEX). In the current protocol, the affinity chromatography uses a column with glutathione (GSH) beads, which the GST in the fusion protein has an affinity for. This affinity allows for the GST-tag on the fusion protein to bind and stay bound to the GSH bead, until it is eluted off of the beads by an elution buffer containing enough GSH to compete with the fusion protein for space on the beads, thus forcing the protein off. This elution can be tracked with a graph called a chromatogram, which measures absorbance over time.

For this project, in an to attempt to optimize the purification of GST-GFP, the GST affinity chromatography using a GSH column was replaced with immobilized metal affinity chromatography (IMAC), which is very similar to the affinity chromatography described above. However, as stated earlier, the GST-GFP fusion protein also contains a 6x-His tag, which is a repeat of the amino acid histidine at either the C or N terminus of the protein (1). In IMAC, the column used for the chromatography contains either nickel or cobalt beads (nickel in this project), that the 6x-His tag has an affinity for and thus binds to. Also, instead of using an elution buffer containing GSH, the elution buffer for IMAC contains imidazole, which is an aromatic ring of histidine (1), and just like the GSH in the GST affinity chromatography, competes for space on the nickel beads, eventually forcing the fusion protein off. Also, as with GST-affinity chromatography, the flowthrough and elution are tracked with a chromatogram measuring absorbance at 280nm, and the fractions containing GST-GFP can be visualized further using the Gel Doc and further quantified using the CDNB protein assay.

In this project, in order to determine which concentration of imidazole in the elution buffer would yield the most pure protein sample, three different lysate samples were run using three different elution buffers made with increasing amounts of imidazole. A control was also run alongside, using the GST-affinity chromatography. For IMAC, the wash buffer used also contained imidazole, and the same wash buffer was used for all three samples: 50mM Tris, 150mM NaCl, 20mM imidazole at pH 8, with a total volume of 250ml. There were three lysate samples used for IMAC and one lysate sample for the control, all coming from one filtered lysate sample. The elution buffers for the three different IMAC runs started with the same stock buffer as used for the wash buffer, and the appropriate amount of imidazole was added to each elution buffer to yield the following concentrations of imidazole for a total volume of 25ml: 500mM, 750mM, and 1M imidazole. For the GST-affinity control, the wash buffer was the same stock buffer with no added imidazole, and the elution buffer for the GST-affinity control was the same stock buffer but with a concentration of 50mM GSH.

For the IEX chromatography, and same wash and elution buffers were used for all four samples. The wash buffer used is the same stock buffer of 50mM Tris, 150mM NaCl, pH 8 as used in the affinity chromatography, and the elution buffer was the same stock with a concentration of 1M NaCl, to allow for tracking of conductivity via the chromatogram.

Following both chromatography runs for all four samples, the elution and flowthrough samples for each were normalized and SDS-PAGE was run, each sample being run on their own gel. The gels were run for 25 minutes at 200 volts, and then all four of the gels were visualized side by side in the Gel Doc. Following visualization on the Gel Doc, a Western Transfer was completed (7 minutes) and and the membranes were stored for four days, and then the Western Blot was completed. The primary antibody used for the Western Blot was mouse anti-GFP with a dilution of 1/6500, due to its higher affinity for the GST-GFP protein of interest, and the secondary antibody used was anti-mouse, with a dilution of 1/10000. Each antibody was incubated on the membrane and placed on the shaker for 45 minutes and then washed, with ECL added last to allow for visualization in the Gel Doc.

Additionally, after both chromatography runs were complete, various protein assays were completed to aid in quantifying the purity of the protein of interest. The assays run include CDNB, DC, and Bradford. The Bradford assay was run only on the original, filtered lysate sample before any purification took place, to get a baseline of the starting overall protein concentration. The DC assay was run twice to allow for comparison at the start of the project and again towards the end of the project. All results from the assays can be seen under the Results section below. The CDNB assay was also run on the original lysate sample and the four other samples to allow for quantification of the enzymatic activity of the GST. All tables for these various assays can be seen under the results section.

The quantification values were used in order to normalize the samples so that SDS-PAGE could be run for visualization purposes. Upon completion of the SDS-PAGE, a western transfer was performed to move the protein onto a nitrocellulose membrane in order to perform a western

blot. This method allows for the visualization of proteins on the membrane with the help of antibodies to produce a color-change reaction that can be visualized under the Gel Doc.

Lastly, four separate purification tables were completed to officially quantify the purity of the protein after both affinity and IEX were run, and the calculations were done on the lysate, and then just the elution fractions of each chromatography. The purpose of these tables was to quantitatively determine which method yielded the most pure GST-GFP product and if using the His-tag and immobilized metal affinity chromatography did optimize the purification of GST-GFP. The results of each table can be seen in the results section.

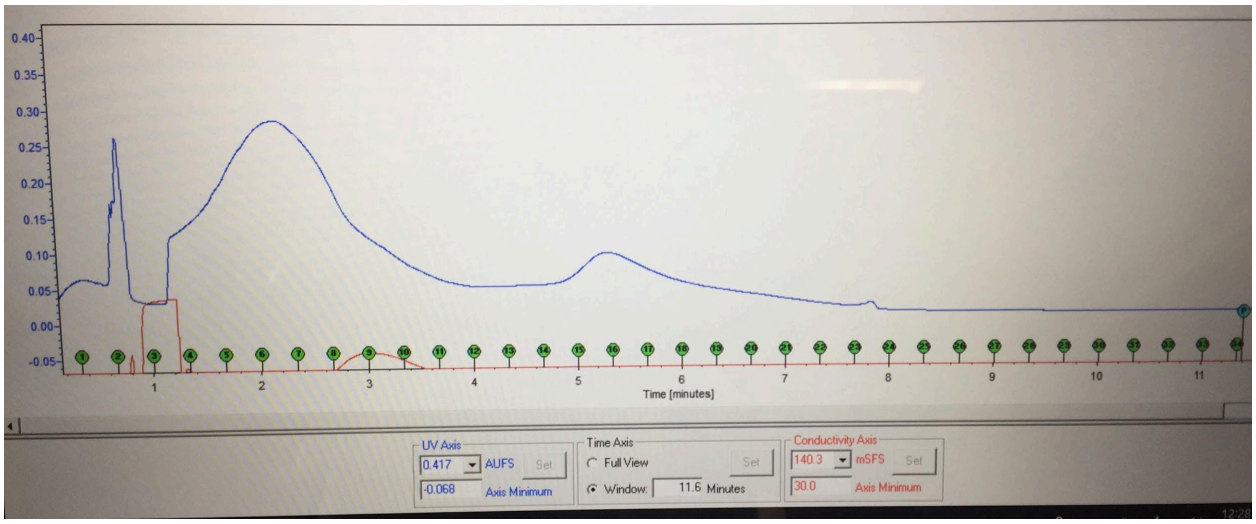
Materials:

- *E. Coli* BL21(DE3) lysate with pET expression vector
- 2 mg/ml bovine serum albumin
- Alkaline copper tartrate
- Sodium dodecyl sulfate (SDS)
- Folin Reagent
- Syringe filter
- glutathione S-transferase protein (GST) affinity column
- BioRad affinity chromatogram
- 10X Reaction Buffer
- 100 mM 1-chloro-2,4-dinitrobenzene (CDNB)
- 100 mM glutathione (GSH)
- Anion exchange column
- IMAC Column

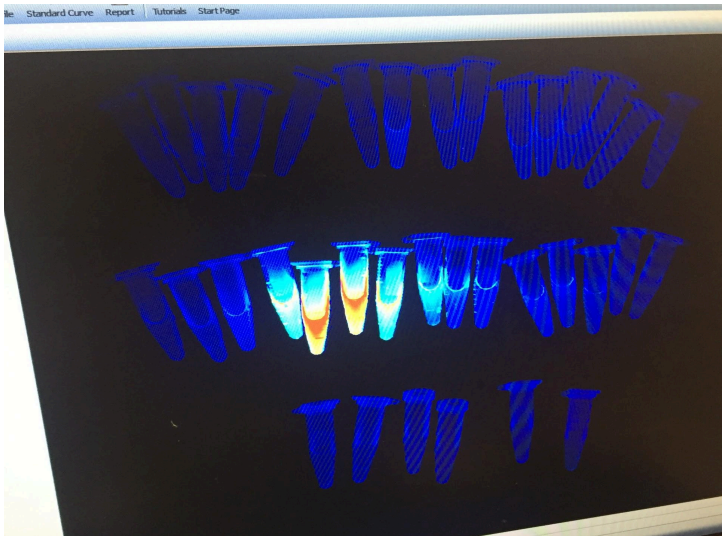
Results:

For this project, Immobilized Metal Affinity Chromatography (IMAC), GST-Affinity Chromatography, and Ion Exchange Chromatography (IEX) were used, with affinity being run first and then followed by IEX for each sample, per the order of the Quantitative Biological Methods Laboratory Manual. A chromatogram and Gel Doc image of the microcentrifuge tubes were obtained for each run (eight runs total), and these images can be seen as follows, starting first the 500mM of imidazole, then 750mM imidazole, then 1M imidazole, and finally the control. In each chromatogram, there are two peaks, each representing important stages in the chromatography process. In each of the chromatograms seen below, the large, leftmost blue peak indicates the flowthrough peak, which is when the wash buffer elutes any loosely bound protein contaminants from the column. The second, smaller blue peak (very subtle in some chromatograms) towards the right indicates the elution peak, which is when the elution buffer competes with the bound protein of interest for space on the beads in the column, thus washing the protein of interest off into various fractions. Additionally, each of the Gel Doc images below show which fractions contain the protein of interest, GST-GFP, via the fluorescence of GFP

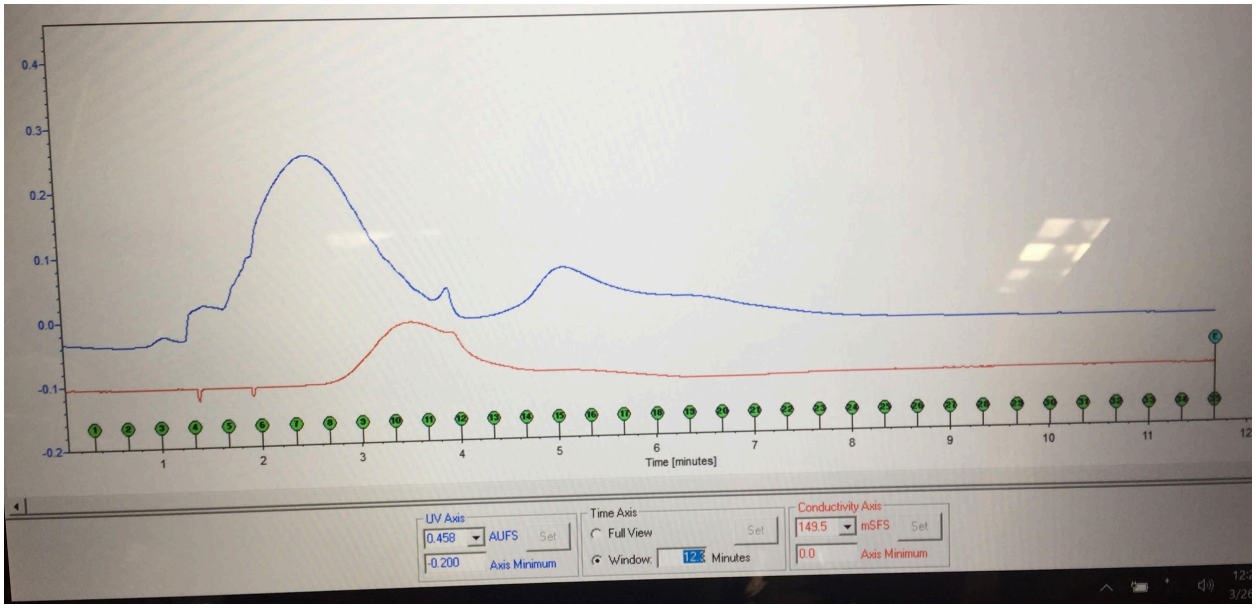
when exposed to very bright light. Tubes that have a bright blue to orange glow indicate the elution fractions and the presence of the protein of interest. These elution fractions are what were collected, aliquoted together and used for further testing. The IMAC and Affinity chromatograms and their respective Gel Doc images can be seen as follows:



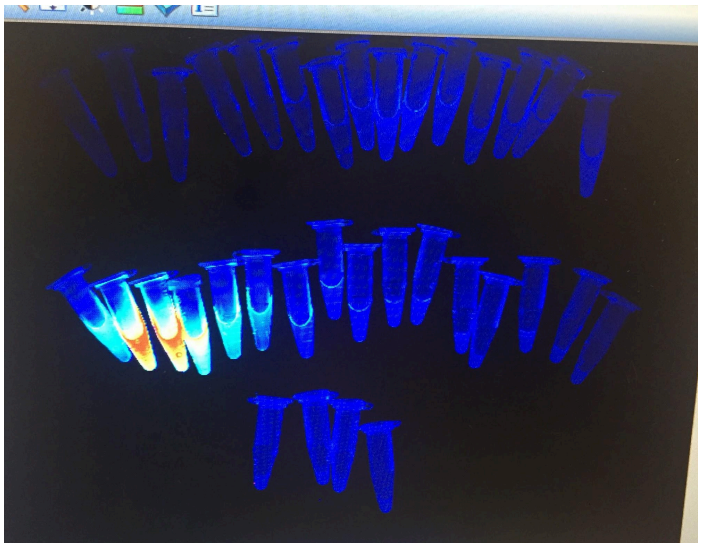

**<u>Image 1.1a:</u> Chromatogram of IMAC of fusion protein GST-GFP using a elution buffer containing <u>500mM of imidazole</u>..**



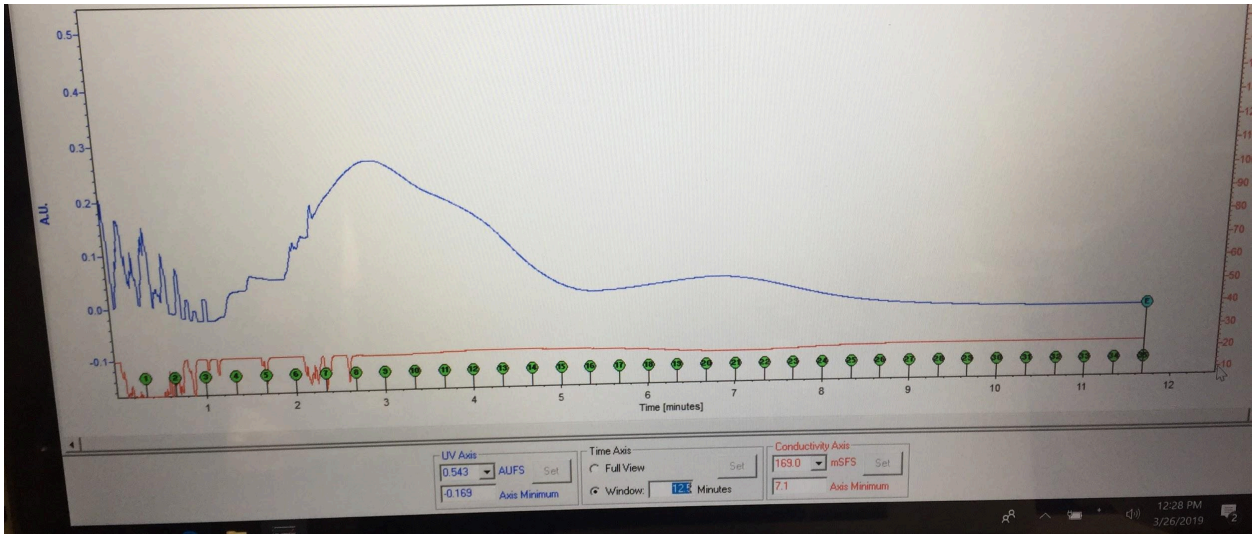

**<u>Image 1.1b:</u> Gel Doc image of fractions following the IMAC chromatography run using elution buffer containing <u>500mM imidazole</u>.**



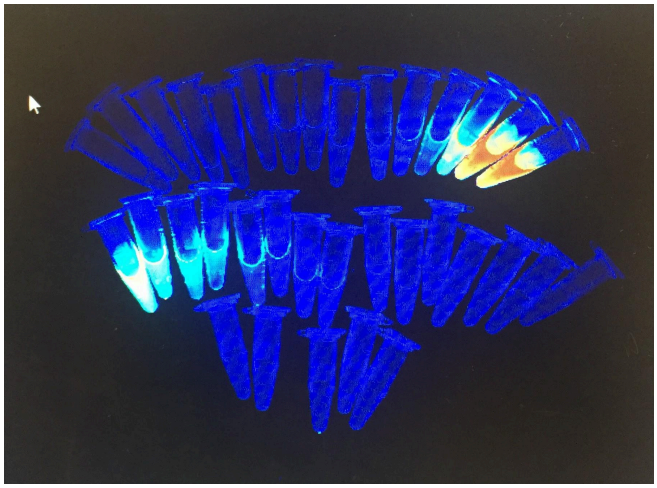

**<u>Image 1.2a:</u> Chromatogram of IMAC of fusion protein GST-GFP using an elution buffer containing <u>750mM of imidazole</u>.**



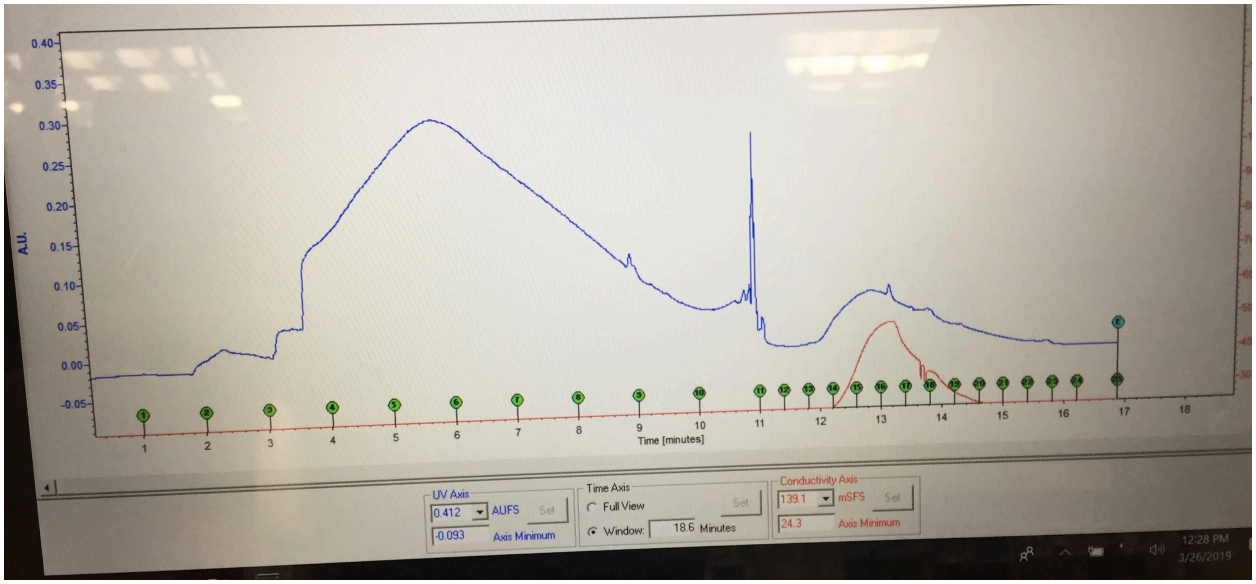
**<u>Image 1.2b:</u> Gel Doc image of fractions following the IMAC chromatography run using elution buffer containing <u>750mM imidazole</u>.**



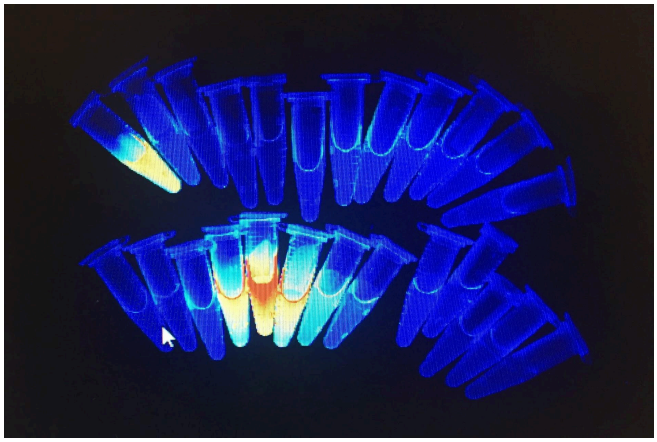

**<u>Image 1.3a:</u> Chromatogram of IMAC of GST-GFP using an elution buffer containing <u>1M of imidazole</u>.**



**<u>Image 1.3b:</u> Gel Doc image of fractions following the IMAC chromatography run using elution buffer containing <u>1M imidazole</u>.**




**<u>Image 1.4a:</u> Chromatogram of GST-GFP affinity <u>control</u> using a GSH affinity column.**



**<u>Image 1.4b:</u> Gel Doc image of fractions following affinity chromatography of <u>control</u> using a GSH affinity column and an elution buffer containing 50mM of GSH. The first fluorescent tube shown in the above image is erroneous.**

As stated in the Principle of Methods, following affinity chromatography, Ion Exchange Chromatography was run on each of the four samples. Very similar to the affinity chromatography, each sample produced a chromatogram, with the only difference being that IEX tracks conductivity through the addition of 1M NaCl to the elution buffer, and can be seen as the red peak on the chromatograms below. The other trends are the same as affinity chromatography for the blue peaks in the chromatogram, as well as the Gel Doc images showing which fractions contain the protein of interest. The chromatograms and Gel Doc images of the four samples following IEX can be seen as follows:

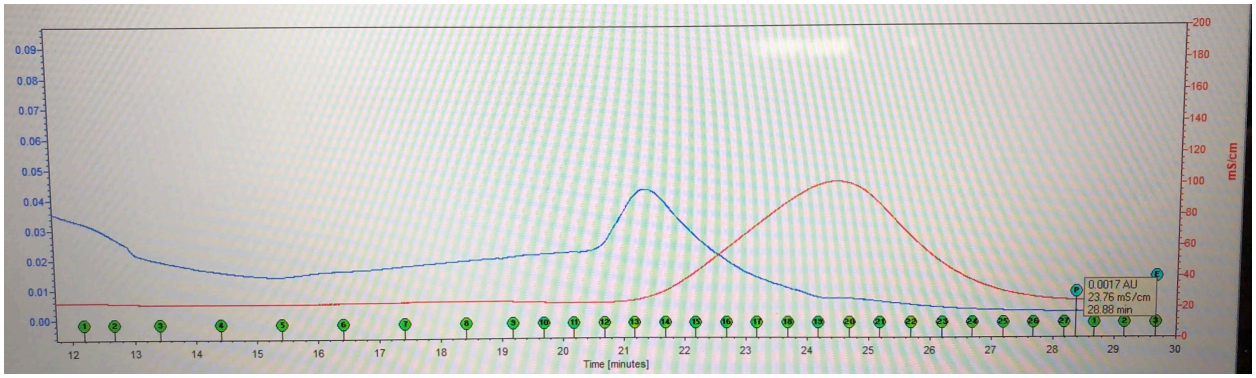



**Image 2.1a: Chromatogram of ion-exchange column on the 500mM sample.**

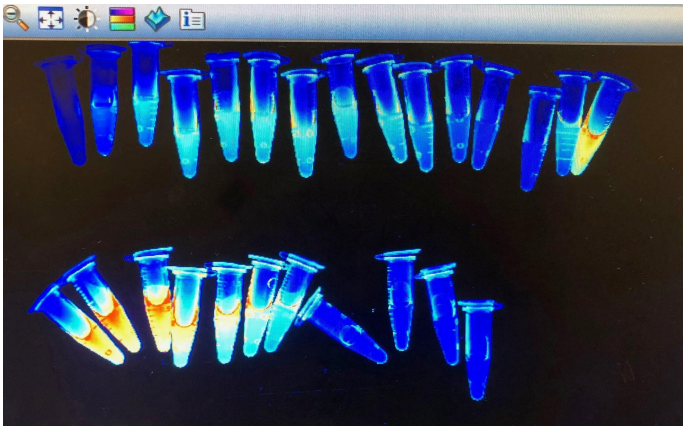


**Image 2.1b: Gel Doc image of the 500mM sample fractions following IEX.**

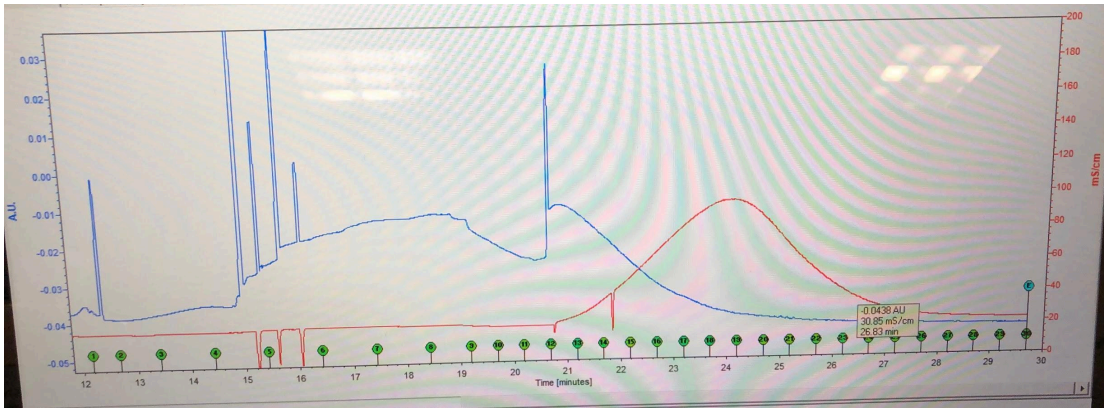



**Image 2.2a: chromatogram of ion-exchange column on the 750mM sample.**

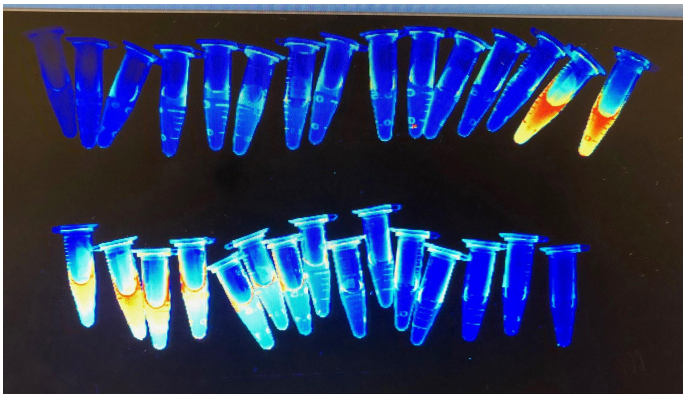


**Image 2.2b: Gel Doc image of the 750mM sample fractions following IEX.**

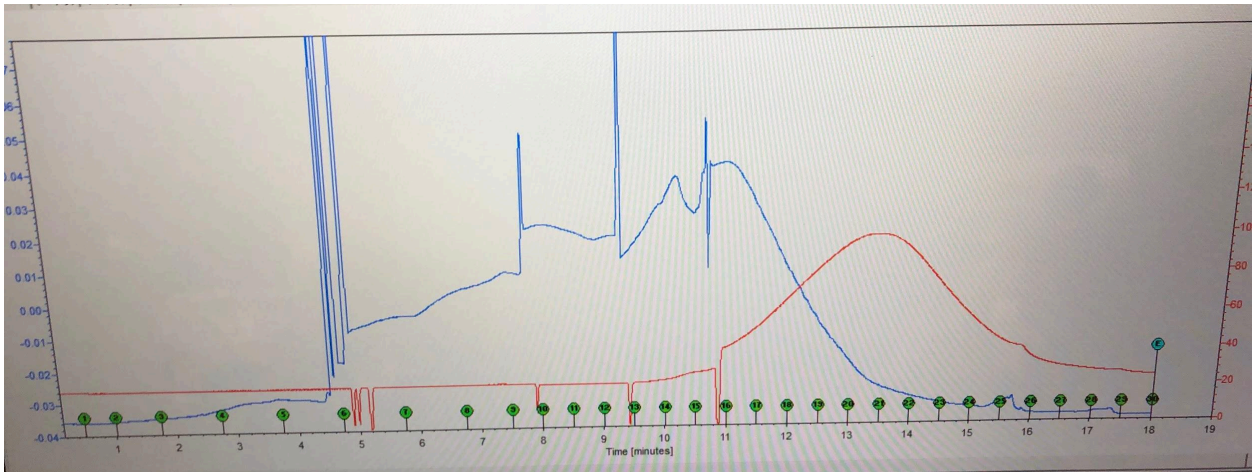



**Image 2.3a: Chromatogram of ion-exchange column on the 1M sample.**

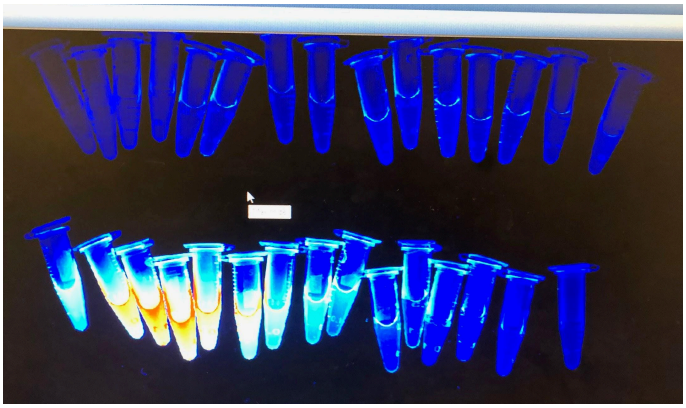
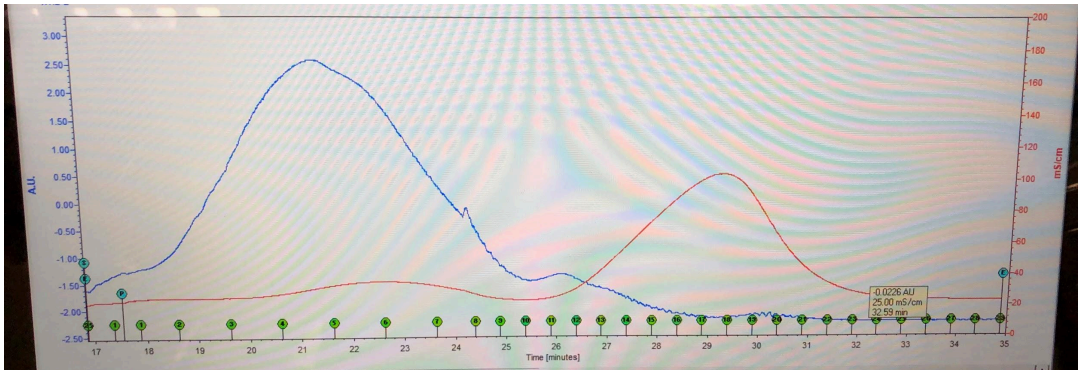
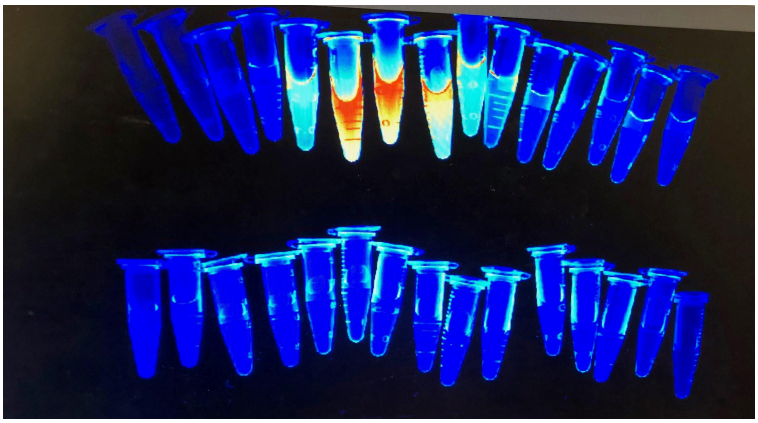


**Image 2.3b: Gel Doc image of the 1M sample fractions following IEX.**

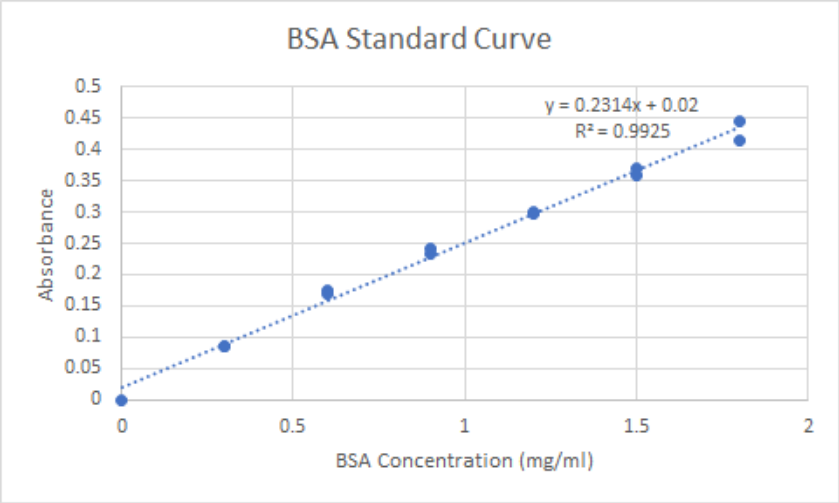



**<u>Image 2.4c:</u> Chromatogram of the ion-exchange column on the GST-GFP <u>control</u>.**



**<u>Image 2.4b:</u> Gel Doc Image of the GST-GFP <u>control</u> following IEX.**

Additionally, various assays were performed on the original lysate, the three samples from the various imidazole concentrations and the control sample to determine protein concentration and the enzymatic activity in each sample. The colorimetric DC protein assay (a detergent compatible variation of the Lowry protein assay) measures absorbance at a wavelength of 750nm and was performed on the original, filtered lysate sample at the beginning of the project before any purification had been done, and then the assay was performed again on a 96-well plate, on the original lysate again, as well as the flowthrough and elution fractions for the affinity and IEX fractions of the 500mM, 750mM, 1M, and control samples. For each DC protein assay run, a standard curve had to be created using BSA (bovine serum albumin) and that standard curve had a line of best first that has an equation (in the form of $y = mx + b$) that is used to calculate the protein concentration of each sample from its absorbance. The following is the standard curve and the absorption values of the first run of the DC protein assay on the original lysate alone, which was performed at the beginning of the project and is used as a comparison for the second, later DC protein assay run:

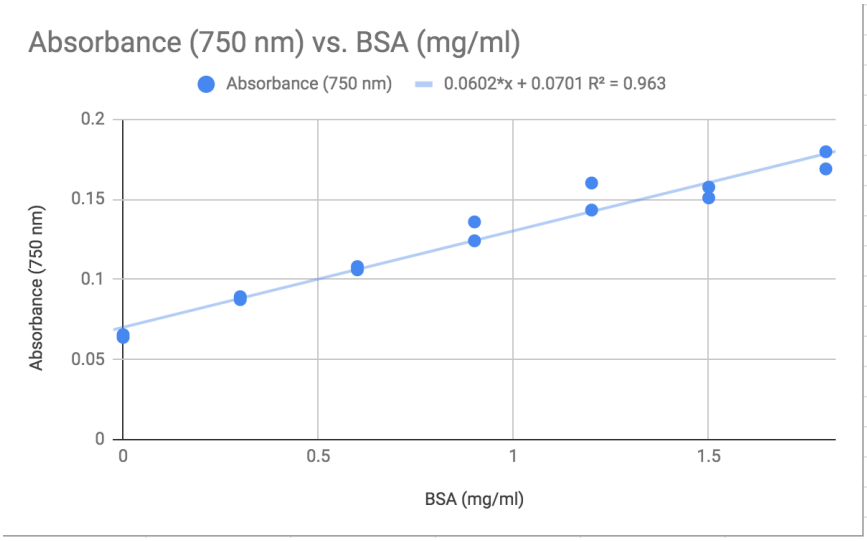



**<u>Graph 1.1:</u> BSA standard curve for first DC Protein Assay run.**
**<u>Equation 1.1:</u> y = 0.2314x + 0.02**

**<u>Table 1.1:</u> DC Protein Assay data for original lysate sample.**

| Sample # | Absorbance (750nm) (Plug in as "y") | Amount (in mg/ml) from BSA standard curve (solve for "x") | Dilution Factor | Protein Concentration (mg/ml) | Average Protein Concentration (mg/ml) |
|---|---|---|---|---|---|
| 13 | 0.039 | 0.082 | 25 | 2.06 | **2.012** |
| 14 | 0.041 | 0.091 | 25 | 2.27 | |
| 15 | 0.124 | 0.449 | 5 | 2.25 | |
| 16 | 0.106 | 0.372 | 5 | 1.86 | |
| 17 | 0.219 | 0.860 | 2.5 | 2.15 | |
| 18 | 0.204 | 0.795 | 2.5 | 1.99 | |
| 19 | 0.280 | 1.12 | 1.67 | 1.88 | |
| 20 | 0.298 | 1.20 | 1.67 | 2.01 | |
| 21 | 0.352 | 1.43 | 1.25 | 1.79 | |
| 22 | 0.365 | 1.49 | 1.25 | 1.86 | **<u>Value 2-2)</u>** |

The following is the data from the second run of the DC Protein Assay on the 96-well plate once both of the chromatography runs for each of the four samples were completed:




**<u>Graph 1.2:</u> BSA standard curve used for second DC protein assay run.**
**<u>Equation 1.2:</u> y = 0.0602x + 0.0701**

The DC Protein Assay data from the run in the 96-well plate is in the following tables and is presented as follows:
**Row A**, columns 3 and 4 are the absorbance values of the original lysate.
**Row B**, starting at column 3, are absorbance values of the aliquoted affinity flowthrough of the 500mM sample (columns 3 & 4), the 750mM sample (columns 5 & 6), the 1M sample (columns 7 & 8) and the control sample (columns 9 & 10) .
**Row C**, starting at column 3, are absorbance values of the aliquoted affinity elution following the same trend of sample order as row B.
**Row D**, starting at column 3, are absorbance values of the aliquoted IEX flowthrough samples, following the same trend of sample order as row B.
**Row E**, starting at column 3, are absorbance values of the aliquoted IEX elution samples, following the same trend of sample order as row B.
The data of each sample is shown as follows, using **equation 1.2** (see above) to calculate for the protein concentration of each:

**<u>Table 1.2:</u> DC Protein Assay absorbance data for the original lysate (row A) and the 500mM sample (starting at row B).**

| Sample # | Absorbance (750 nm) Plug in as "y" | Amount (mg/ml) from BSA standard curve, solve for "x" | Average protein concentration (mg/ml) |
|---|---|---|---|

| | | | |
|---|---|---|---|
| A3 | 0.2843 | 3.56 | **3.26** |
| A4 | 0.2482 | 2.96 | **(Value 6-1)** |
| B3 | 0.1119 | 0.69 | **0.57** |
| B4 | 0.0968 | 0.44 | **(Value 6-2)** |
| C3 | 0.1509 | 1.34 | **1.29** |
| C4 | 0.1445 | 1.24 | **(Value 6-3)** |
| D3 | 0.0926 | 0.37 | **0.28** |
| D4 | 0.0809 | 0.18 | **(Value 6-4)** |
| E3 | 0.0999 | 0.50 | **0.43** |
| E4 | 0.0916 | 0.36 | **(Value 6-5)** |

**Table 1.3: DC Protein Assay absorbance data for 750mM sample.**

| Sample # | Absorbance (750 nm) Plug in as "y" | Amount (mg/ml) from BSA standard curve, solve for "x" | Average protein concentration (mg/ml) |
|---|---|---|---|
| B5 | 0.1248 | 0.908 | **0.904** |
| B6 | 0.1242 | 0.8725 | **(Value 6-2)** |
| C5 | 0.1736 | 1.719 | **1.92** |
| C6 | 0.1978 | 2.121 | **(Value 6-3)** |
| D5 | 0.0881 | 0.299 | **0.298** |
| D6 | 0.0880 | 0.297 | **(Value 6-4)** |
| E5 | 0.0879 | 0.296 | **0.410** |
| E6 | 0.1017 | 0.525 | **(Value 6-5)** |

**Table 1.4: DC Protein Assay absorbance data for 1M sample.**

| Sample # | Absorbance (750 | Amount (mg/ml) | Average protein |
|---|---|---|---|

| | nm)<br>Plug in as “y” | from BSA standard curve, solve for “x” | concentration (mg/ml) |
|---|---|---|---|
| B7 | 0.1246 | 0.905 | **0.802** |
| B8 | 0.1122 | 0.699 | **(Value 6-2)** |
| C7 | 0.1700 | 1.659 | **1.441** |
| C8 | 0.1437 | 1.223 | **(Value 6-3)** |
| D7 | 0.0858 | 0.260 | **0.207** |
| D8 | 0.0793 | 0.153 | **(Value 6-4)** |
| E7 | 0.0973 | 0.452 | **0.548** |
| E8 | 0.1089 | 0.645 | **(Value 6-5)** |

**Table 1.5: DC Protein Assay absorbance data for control sample.**

| Sample # | Absorbance (750 nm)<br>Plug in as “y” | Amount (mg/ml) from BSA standard curve, solve for “x” | Average protein concentration (mg/ml) |
|---|---|---|---|
| B9 | 0.1216 | 0.86 | **0.89** |
| B10 | 0.1246 | 0.91 | **(Value 6-2)** |
| C9 | 0.7497 | 11.28 | **10.17** |
| C10 | 0.6152 | 9.05 | **(Value 6-3)** |
| D9 | 0.1874 | 1.95 | **1.70** |
| D10 | 0.1570 | 1.44 | **(Value 6-4)** |
| E9 | 0.2888 | 3.63 | **3.67** |
| E10 | 0.2931 | 3.70 | **(Value 6-5)** |

In addition to the DC Protein Assay, the CDNB Assay was utilized after both the affinity and IEX chromatography runs to further determine which fractions had the highest enzymatic activity, and measures sample absorbance at a wavelength of 340nm. Since GST (Glutathione S-Transferase) is an enzyme, its activity can be tracked as it acts as a catalyst for the reaction of

GSH (glutathione) and CDNB (1-chloro-2,4-dinitrobenzene) to yield GS-DNB(1). After obtaining absorbance of the 500mM, 750mM, 1M, and control samples after both affinity and IEX, reaction velocity was calculated to determine the enzymatic activity of GST, and the fractions with the highest reaction velocities were aliquoted together to test further. Reaction velocity was calculated using the following equation:

**Equation 1.3:**

$$\textbf{Reaction Velocity/GST Activity} = \frac{(\Delta A_{340})}{(\varepsilon_{GS-DNB\ conjugate})\ (\Delta t)\ (volume)} \textbf{(1)}$$

$\Delta A_{340}$ **= change in absorbance (final - initial)**

$\varepsilon_{CDNB}$ **= extinction coefficient of CDNB conjugate**

$\Delta t$ **= change in time in minutes (final - initial)**

$volume$ **= volume of sample (ml)**

The results of the CDNB Assay and the calculated reaction velocities are as follows, starting with the original lysate, followed by the grouping of results from affinity chromatography and the results of the IEX chromatography for the 500mM, 750mM, 1M, and control samples:

**Table 2.1a: CDNB Assay absorbance data of the original lysate sample. Note that for cuvette 5, the readings stopped once the absorbance reached 1.7.**

| Time (min) | Blank | Cuvette 1 (2ul lysate) | Cuvette 2 (4ul lysate) | Cuvette 3 (6ul lysate) | Cuvette 4 (8ul lysate) | Cuvette 5 (30ul lysate) |
|---|---|---|---|---|---|---|
| 0 | 0 | 0.035 | 0.037 | 0.063 | 0.065 | 0.269 |
| 0.5 | 0.001 | 0.071 | 0.105 | 0.161 | 0.171 | 0.580 |
| 1 | 0.004 | 0.107 | 0.167 | 0.245 | 0.270 | 0.831 |
| 1.5 | 0.008 | 0.140 | 0.225 | 0.330 | 0.367 | 1.089 |
| 2 | 0.011 | 0.176 | 0.281 | 0.412 | 0.455 | 1.333 |
| 2.5 | 0.014 | 0.209 | 0.339 | 0.492 | 0.545 | 1.504 |
| 3 | 0.016 | 0.241 | 0.392 | 0.569 | 0.628 | 1.741 |

**Table 2.1b: Reaction velocity calculations of the original lysate sample.**

| **Value** | **Cuvette #** | **Reaction velocity (units/ml)** | **Average reaction velocity** |
|---|---|---|---|

| | | | |
|---|---|---|---|
| 3-1 | 1 | 3.58 | **2.746** |
| 3-2 | 2 | 3.08 | |
| 3-3 | 3 | 2.93 | |
| 3-4 | 4 | 2.44 | |
| 3-5 | 5 | 1.70 | **(Value 3-6)** |

**Table 2.2a: CDNB Assay absorbance data of the 500mM sample following IMAC.**

| **Time (min)** | **IMAC flowthrough** | **IMAC elution** |
|---|---|---|
| 0 | 0.008 | -0.004 |
| 0.5 | 0.003 | 0.008 |
| 1 | 0.014 | 0.002 |
| 1.5 | 0.026 | 0.004 |
| 2 | 0.036 | 0.006 |
| **Reaction velocity (units/ml)** | **0.229 (Value 4-1)** | **0.042 (Value 4-3)** |

**Table 2.2b: CDNB Assay absorbance data of the 500mM sample following IEX.**

| **Time (min)** | **IEX flowthrough** | **IEX elution** |
|---|---|---|
| 0 | -0.029 | -0.022 |
| 0.5 | -0.035 | -0.007 |
| 1 | -0.028 | 0.009 |
| 1.5 | -0.024 | 0.024 |
| 2 | -0.020 | 0.039 |
| **Reaction velocity (units/ml)** | **0.083 (Value 5-1)** | **0.313 (Value 5-3)** |

**Table 2.3a: CDNB Assay absorbance data of the 750mM sample following IMAC.**

| Time (min) | IMAC flowthrough | IMAC elution |
|---|---|---|
| 0 | 0.043 | -0.004 |
| 0.5 | 0.046 | -0.003 |
| 1 | 0.049 | 0 |
| 1.5 | 0.054 | 0.003 |
| 2 | 0.057 | 0.005 |
| **Reaction velocity (units/ml)** | **0.083 (Value 4-1)** | **0.052 (Value 4-3)** |

**Table 2.3b: CDNB Assay absorbance data of the 750mM sample following IEX.**

| Time (min) | IEX flowthrough | IEX elution |
|---|---|---|
| 0 | -0.002 | -0.007 |
| 0.5 | 0.001 | 0.006 |
| 1 | 0.003 | 0.021 |
| 1.5 | 0.006 | 0.034 |
| 2 | 0.009 | 0.049 |
| **Reaction velocity (units/ml)** | **0.063 (Value 5-1)** | **0.292 (Value 5-3)** |

**Table 2.4a: CDNB Assay absorbance data of the 1M sample following IMAC.**

| Time (min) | IMAC flowthrough | IMAC elution |
|---|---|---|
| 0 | 0.096 | 0.105 |
| 0.5 | 0.099 | 0.120 |
| 1 | 0.103 | 0.137 |
| 1.5 | 0.107 | 0.153 |
| 2 | 0.112 | 0.170 |
| **Reaction velocity (units/ml)** | **0.094 (Value 4-1)** | **0.344 (Value 4-3)** |

**Table 2.4b:** **CDNB Assay absorbance data of the 1M sample following IEX.**

| **Time (min)** | **IEX flowthrough** | **IEX elution** |
|---|---|---|
| 0 | 0.012 | 0.025 |
| 0.5 | 0.014 | 0.046 |
| 1 | 0.017 | 0.068 |
| 1.5 | 0.019 | 0.089 |
| 2 | 0.023 | 0.110 |
| **Reaction velocity (units/ml)** | **0.063 (Value 5-1)** | **0.438 (Value 5-3)** |

**Table 2.5a:** **CDNB Assay absorbance data of the control sample following GST-affinity chromatography.**

| **Time (min)** | **Affinity flowthrough** | **Affinity elution** |
|---|---|---|
| 0 | 0.113 | 0.119 |
| 0.5 | 0.117 | 0.144 |
| 1 | 0.121 | 0.170 |
| 1.5 | 0.126 | 0.194 |
| 2 | 0.131 | 0.219 |
| **Reaction velocity (units/ml)** | **0.104 (Value 4-1)** | **0.510 (Value 4-3)** |

**Table 2.5b:** **CDNB Assay of the control sample following IEX.**

| **Time (min)** | **IEX flowthrough** | **IEX elution** |
|---|---|---|
| 0 | 0.086 | 0.079 |
| 0.5 | 0.089 | 0.094 |
| 1 | 0.092 | 0.109 |
| 1.5 | 0.096 | 0.126 |
| 2 | 0.099 | 0.141 |

| | | |
|---|---|---|
| **Reaction velocity (units/ml)** | **0.073 (Value 5-1)** | **0.333 (Value 5-3)** |

And lastly the Bradford assay was done on just the lysate sample, to help determine how much protein was originally present. The absorbance took place at 595nm, and each cuvette had a different amount of lysate and a corresponding dilution factor to account for when calculating the final concentrations, which were then averaged to give an average final concentration of the lysate sample. This concentration was compared the concentration of the original lysate as determined by the DC protein assay runs. The results of the Bradford Assay are as follows:

**Table 2.6: Bradford Assay data of the original lysate sample.**

| Cuvette # | Bradford reagent (ml) | Lysate (ul) | Absorbance (595nm) | Dilution factor | Final concentration | Average concentration |
|---|---|---|---|---|---|---|
| Blank | 1 | 0 | 0 | -- | -- | -- |
| 1 | 1 | 1 | 0.053 | 20 | 1.06 | **1.22** |
| 2 | 1 | 2 | 0.124 | 10 | 1.24 | |
| 3 | 1 | 4 | 0.269 | 5 | 1.35 | |
| 4 | 1 | 5 | 0.292 | 4 | 1.17 | |
| 5 | 1 | 10 | 0.635 | 2 | 1.27 | **(Value 2-3)** |

After performing the various chromatographies and protein assays, four separate SDS-PAGE gels were run and then had Western Blots performed on them to confirm the presence of the the protein of interest, GST-GFP. Before running the SDS-PAGE gels, the samples had to be normalized in order to ensure that the same amount of protein was being run in each lane to ensure accurate comparisons. For each sample and their respective flowthrough and elution fractions for both affinity and IEX chromatography, the lowest concentration (in $\mu$g/$\mu$l) was multiplied by 50$\mu$l to get total protein. This value was then divided by the average protein concentration of the respective flowthrough or elution fraction, and this gave sample volume to be used. If the volume was less than 50$\mu$l, then the appropriate amount of water was added for the total volume to equal 50$\mu$l. If the sample volume was higher than 50$\mu$l, then just 50$\mu$l of sample was used with no water added. The normalizations for each of the four samples can be seen as follows in tables 3.1 through 3.4.

**Table 3.1: Sample Normalization of the 500mM sample.**

| Sample | Total Protein ($\mu$g) | Sample Volume ($\mu$l) (Col. 3) | dH2O to Add ($\mu$l) (50$\mu$l-Col.3) |
|---|---|---|---|
| Lysate | **21.5 (Value 6-6)** | **6.6 (Value 6-7)** | **43.4** |
| Affinity Flowthrough | **21.5 (Value 6-6)** | **37.7 (Value 6-8)** | **12.3** |
| Affinity Elution | **21.5 (Value 6-6)** | **16.7 (Value 6-9)** | **33.3** |
| IEX Flowthrough | **21.5 (Value 6-6)** | **50 (Value 6-10)** | **0** |
| IEX Elution | **21.5 (Value 6-6)** | **50 (Value 6-11)** | **0** |

**Table 3.2: Sample Normalization of the 750mM sample.**

| Sample | Total Protein ($\mu$g) | Sample Volume ($\mu$l) (Col. 3) | dH2O to Add (50$\mu$l-Col.3) |
|---|---|---|---|
| Lysate | **20.5 (Value 6-6)** | **6.30 (Value 6-7)** | **43.7** |
| Affinity Flowthrough | **20.5 (Value 6-6)** | **22.7 (Value 6-8)** | **27.3** |
| Affinity Elution | **20.5 (Value 6-6)** | **10.7 (Value 6-9)** | **39.3** |
| IEX Flowthrough | **20.5 (Value 6-6)** | **50 (Value 6-10)** | **0** |
| IEX Elution | **20.5 (Value 6-6)** | **50 (Value 6-11)** | **0** |

**Table 3.3: Sample Normalization of the 1M sample.**

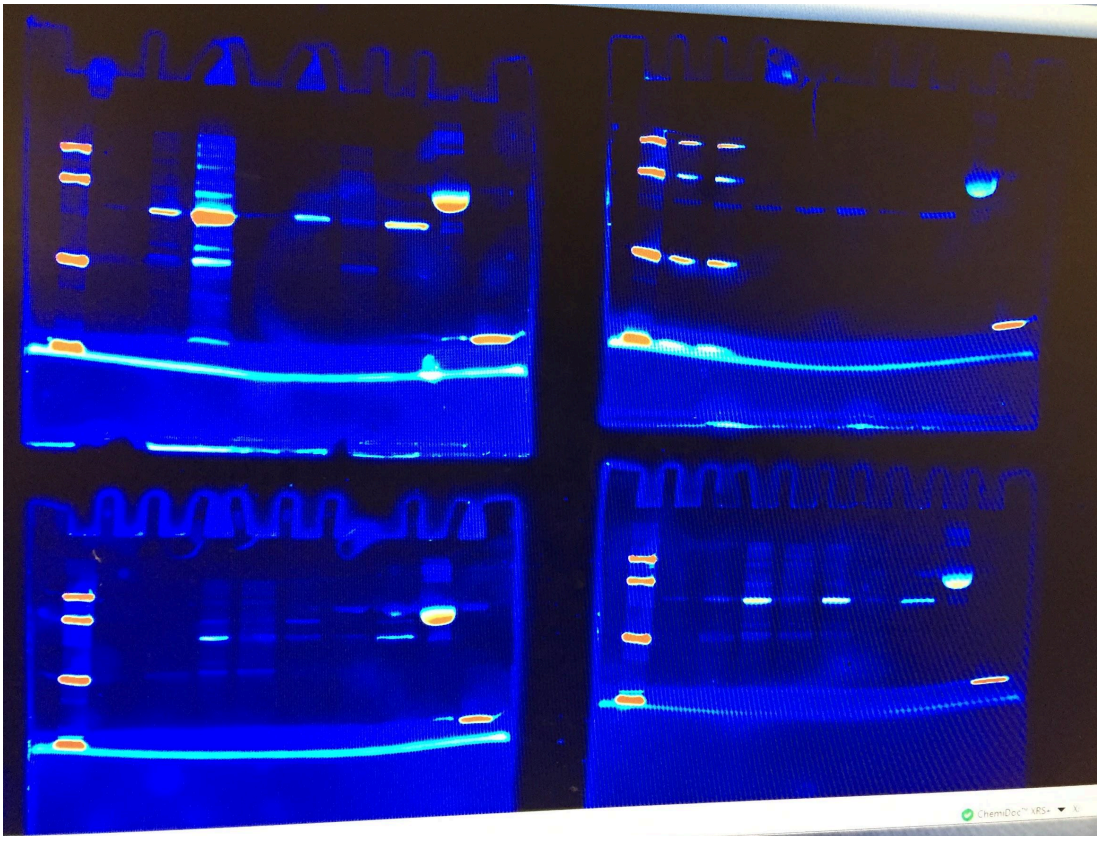
| Sample | Total Protein ($\mu$g) | Sample Volume ($\mu$l) (Col. 3) | dH2O to Add (50$\mu$l-Col.3) |
|---|---|---|---|

| | | | |
|---|---|---|---|
| Lysate | **27.4** **(Value 6-6)** | 8.4 **(Value 6-7)** | **41.6** |
| Affinity Flowthrough | **27.4** **(Value 6-6)** | 34.2 **(Value 6-8)** | **15.8** |
| Affinity Elution | **27.4** **(Value 6-6)** | 19.0 **(Value 6-9)** | **31.0** |
| IEX Flowthrough | **27.4** **(Value 6-6)** | 50 **(Value 6-10)** | **0** |
| IEX Elution | **27.4** **(Value 6-6)** | 50 **(Value 6-11)** | **0** |

**Table 3.4: Sample Normalization of the control sample.**

| **Sample** | **Total Protein ($\mu$g)** | **Sample Volume ($\mu$l) (Col. 3)** | **dH2O to Add (50$\mu$l-Col.3)** |
|---|---|---|---|
| Lysate | **163** **(Value 6-6)** | **50** **(Value 6-7)** | **0** |
| Affinity Flowthrough | **163** **(Value 6-6)** | **50** **(Value 6-8)** | **0** |
| Affinity Elution | **163** **(Value 6-6)** | **16.0** **(Value 6-9)** | **34** |
| IEX Flowthrough | **163** **(Value 6-6)** | **50** **(Value 6-10)** | **0** |
| IEX Elution | **163** **(Value 6-6)** | **44.4** **(Value 6-11)** | **5.6** |

After sample normalization, the 500mM, 750mM, 1M, and control samples were each loaded into separate SDS-PAGE gels and run at 200V for 25 minutes. The fusion protein GST-GFP has a molecular weight of 54kDa, and the results of the four separate SDS-PAGE gels can be seen as follows:

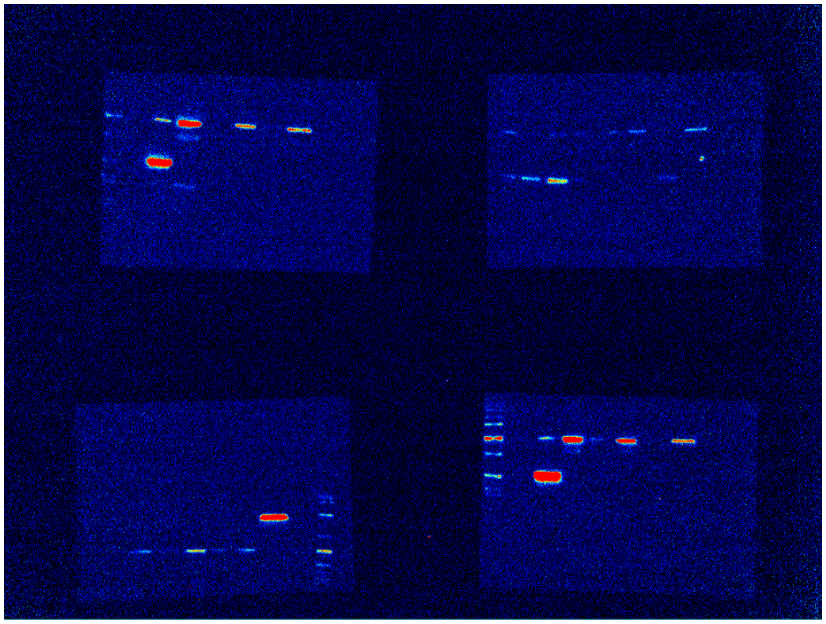


**<u>Image 3.1:</u> SDS-PAGE gels:**
**Top left = control**
**Top right = 500mM sample**
**Bottom left = 750mM sample**
**Bottom right = 1M sample**

The order of the lanes in each gel from left to right are as follows:
Lane 1= Marker (molecular weights from top to bottom: 10kDa, 25kDa, 50kDa, and 75kDa).
Lane 2 = GST control (Mw = 28kDa)
Lane 3 = GFP control (Mw = 27kDa)
Lane 4 = Original lysate
Lane 5 = Affinity flowthrough
Lane 6 = Affinity elution
Lane 7 = IEX flowthrough
Lane 8 = IEX elution
Lane 9 = BSA
Lane 10 = lysozyme

Immediately following visualization of the SDS-PAGE gels under the Gel Doc, a Western Transfer was performed on each of the gels to transfer the proteins from the SDS-PAGE gel onto

a nitrocellulose membrane in order to perform a Western Blot. In order to detect the protein of interest (GST-GFP) via Western Blotting, the primary antibody used was mouse anti-GFP due to its higher affinity for the protein of interest, at a dilution of 1/6500. Next, the secondary antibody used was anti-mouse, with horseradish peroxidase conjugated to it, at a dilution of 1/10000. Visualization took place under the Gel Doc after the addition of ECL (enhanced chemiluminescence), which reacted with horseradish peroxidase to produce luminescence (1). The resulting membranes for the 500mM, 750mM, 1M, and control membranes are as follows:



**<u>Image 4.1:</u> Western Blot membranes of all four samples under the Gel Doc. <u>NOTE</u>: The bottom left gel is upside down and flipped. The order of the membranes is as follows:**
**Top left = control**
**Top right = 500mM**
**Bottom left = 750mM**
**Bottom right = 1M**

The lanes for the Western Blot are the same as the SDS-PAGE gel in image 3.1.

And lastly, to quantitatively determine the GST-GFP protein concentration in each of the four samples to see if immobilized metal affinity chromatography did optimize the purification protocol, four separate purification tables were calculated and compared. Each purification table tracks the concentration of the protein of interest from the original lysate to after ion exchange chromatography. The purification table also tracks reaction velocity, specific activity, percent yield of GST-GFP, and fold purification of GST-GFP. There are various trends expected to be observed in the purification table. It is expected that total protein, total activity, total percent yield, and percent yield of GST-GFP will decrease (columns 3, 5, 7, and 8, respectively), which is expected due to the inevitable loss of protein throughout the purification. Comparatively, it is

expected that specific activity of GST-GFP and fold purification will increase (columns 6 and 9, respectively), due to the loss of contaminants in each sample. The purification tables for the 500mM, 750mM, 1M and control samples are as follows:

**Table 4.1: Purification Table for 500mM sample.**

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Protein Conc. (mg/ml) | Total Volume (ml) | Total Protein (mg) | Reaction Velocity (units/ml) | Total Activity (units) | Specific Activity (units/mg) | Percent Yield (total) | Percent Yield (GST-GFP) | Fold Purif. (GST-GFP) |
| A | Lysate | **3.26** | **3** | **9.78** | **2.746** | **8.238** | **0.842** | **100** | **100** | **1** |
| B | Affinity Elution | **1.29** | **4** | **5.16** | **0.042** | **0.168** | **0.033** | **52.76** | **2.039** | **0.039** |
| C | IEX Elution | **0.43** | **6** | **2.58** | **0.313** | **1.878** | **0.728** | **26.38** | **22.80** | **0.864** |

**Table 4.2: Purification Table for 750mM sample.**

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Protein Conc. (mg/ml) | Total Volume (ml) | Total Protein (mg) | Reaction Velocity (units/ml) | Total Activity (units) | Specific Activity (units/mg) | Percent Yield (total) | Percent Yield (GST-GFP) | Fold Purif. (GST-GFP) |
| A | Lysate | **3.26** | **3** | **9.78** | **2.746** | **8.238** | **0.842** | **100** | **100** | **1** |
| B | Affinity Elution | **1.92** | **5** | **9.60** | **0.052** | **0.26** | **0.027** | **98.16** | **3.16** | **0.032** |
| C | IEX Elution | **0.410** | **6** | **2.46** | **0.292** | **1.75** | **0.711** | **25.15** | **21.24** | **0.844** |

**Table 4.3: Purification Table for 1M.**

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Protein Conc. (mg/ml) | Total Volume (ml) | Total Protein (mg) | Reaction Velocity (units/ml) | Total Activity (units) | Specific Activity (units/mg) | Percent Yield (total) | Percent Yield (GST-GFP) | Fold Purif. (GST-GFP) |
| A | Lysate | **3.26** | **3** | **9.78** | **2.746** | **8.238** | **0.842** | **100** | **100** | **1** |
| B | Affinity Elution | **1.44** | **5** | **7.20** | **0.344** | **1.72** | **0.239** | **73.62** | **20.87** | **0.284** |
| C | IEX Elution | **0.548** | **5** | **2.74** | **0.438** | **2.19** | **0.799** | **28.02** | **26.58** | **0.949** |

**Table 4.4: Purification Table for control sample.**

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Protein Conc. (mg/ml) | Total Volume (ml) | Total Protein (mg) | Reaction Velocity (units/ml) | Total Activity (units) | Specific Activity (units/mg) | Percent Yield (total) | Percent Yield (GST-GFP) | Fold Purif. (GST-GFP) |
| A | Lysate | **3.26** | **3** | **9.78** | **2.746** | **8.238** | **0.842** | **100** | **100** | **1** |
| B | Affinity Elution | **10.17** | **4** | **40.68** | **0.510** | **2.04** | **0.050** | **416** | **24.76** | **0.059** |
| C | IEX Elution | **3.67** | **5** | **18.35** | **0.333** | **1.665** | **0.091** | **188** | **20.21** | **0.108** |

Discussion:

Upon completion of this project, it is difficult to draw conclusions on whether replacing Immobilized Metal Affinity Chromatography (IMAC) did optimize the purification protocol of the fusion protein GST-GFP. That is, if IMAC produced a more pure protein sample than the use of GST-affinity chromatography. Further experiments and calculations would be required to determine this.

Once again, the major change from the current purification protocol used in the QBM laboratory was changing the first step of GST-GFP purification from GST-affinity chromatography to Immobilized Metal Affinity Chromatography (IMAC). There were four lysate samples total, one being a control sample whose purification process had no deviations from the current protocol (that is, used the GST-affinity chromatography as the first step), and then the other three samples were tracked based on the concentration of imidazole used in the elution buffer for IMAC, the three samples being: 500mM, 750mM, and 1M imidazole in the Tris/NaCl elution buffer. As seen in the results section, this is how the samples were kept track of throughout the course the project, being referred to as the 500mM sample, the 750mM sample, 1M sample, and the control sample. Additionally, some of the original, filtered *E. coli* lysate was kept aside for testing in the DC protein assay, the CDNB assay, and the Bradford protein assay, as well as for being run on SDS-PAGE.

The DC protein assay was run on the original lysate twice, once at the beginning of the project and once following the completion of the two chromatography runs. The purpose for running the DC protein assay on the original lysate twice was to allow for comparison of the concentrations, as each DC protein assay run requires the creation of a BSA standard curve and line equation from the line of best fit for that standard curve. The two different runs of the DC protein assay produced different protein concentrations, with the second run being higher, 3.26mg/ml (value 6-1) compared to the first run, which gave a concentration of 2.012 mg/ml (value 2-2). The 3.26mg/ml value was the concentration used for purification table calculations of each of the four samples. Next, the Bradford protein assay is a less-specific assay to determine concentration, and gave a concentration of the original lysate of 1.22mg/ml, which is less than both of the DC assay runs. Additionally, the CDNB assay was performed on the original lysate sample to make sure that the GST retained its enzymatic activity. Reaction velocity was calculated using equation 1.3, to give a value of 2.746 (value 3-6), which was, expectedly, higher than any of the samples following affinity and IEX chromatography.

Additionally, before beginning analysis of each sample, it should be stated that some changes from the current QBM lab procedure were made. An important change was the fact that following both affinity and IEX chromatography, the elution fractions were chosen based only on their fluorescence under the Gel Doc. These fractions were not analyzed via the CDNB assay to test for GST enzymatic activity prior to being aliquoted together, and thus could be the reason for any unexpected or extraneous results.

To begin, for the 500mM sample results, it can be seen in images 1.1a and 1.1b that the IMAC was successful, as the two expected peaks can be observed on the chromatogram, and via the Gel Doc, it is very clear which fractions contain the protein of interest, due to GFPs ability to fluoresce under very bright light. Four of the fluorescent tubes of the IMAC (fractions 19-22) were aliquoted together and one flowthrough fraction was saved, and then this aliquoted 500mM sample was run on Ion Exchange Chromatography, specifically Anion Exchange Chromatography, meaning that the pH of the buffer was above the pI of the protein (1). Very

similar to IMAC, the chromatogram and Gel Doc image for the 500mM sample can be seen in images 2.1a and 2.1b. Once again, the two blue chromatogram peaks are observed as well the red conductivity peak, where the sodium chloride concentration increased to create a gradient from 0 to 100%. Additionally, the Gel Doc image can be seen, and notice that in comparison with the fractions from the IMAC run, the protein seems to be more spread out in the elution fractions and visible in far more numerous tubes. When choosing which tubes to aliquot from the IEX run, a maximum of six tubes were kept, choosing those with the brightest fluorescence (fractions 15-20). And like the IMAC run, one flowthrough fraction from IEX was saved.

Continuing with the 500mM sample, after the two chromatography runs, various assays were performed to determine the reaction velocity and enzymatic activity utilizing GSTs enzymatic ability in the CDNB assay, and testing the concentration of the IMAC and IEX flowthrough and elution samples using the DC protein assay. Beginning with the CDNB assay, equation 1.3 was used to calculate reaction velocity, and it was expected that the elution fractions would have a higher reaction velocity than the flowthrough fractions due to presence of the protein of interest, GST-GFP. However, in table 2.2a, this trend is reversed, as the 500mM IMAC flowthrough ended up having a higher reaction velocity than the 500mM IMAC elution, being 0.229 and 0.042 (values 4-1 and 4-3), respectively. The cause of this is unknown, as the Gel Doc image indicated that no protein was visible in the flowthrough fraction, however it is possible that some residual protein was eluted off prematurely during IMAC, resulting in a higher reaction velocity in the flowthrough fraction. Contrarily, in table 2.2b for the 500mM IEX flowthrough and elution samples, the expected outcome was observed, and the elution fraction had a higher reaction velocity than the flowthrough, being 0.313 and 0.083 (values 5-1 and 5-3), respectively. The other assay performed was the DC protein assay, and the purpose of this assay was to quantitatively determine the concentration of GST-GFP in the 500mM flowthrough and elution fractions of IMAC and IEX. This assay was performed in a 96-well plate, and the results for the 500mM sample can be seen in table 1.2, values 6-2 through 6-5. It was expected that the elution fractions would have a higher protein concentration than the flowthrough fractions, and this trend was observed, with, again, rows B and D being flowthrough, and rows C and E being elution.

To continue, before running the various samples as well as the original lysate on SDS-PAGE, the 500mM flowthrough and elution fractions of IMAC and IEX had to be normalized, as seen in table 3.1. To begin, the lowest average concentration of the 500mM elution samples (from table 1.2), in this case 0.43$\mu$g/$\mu$l, had to be multiplied by 50$\mu$l in order to obtain a total protein amount of 21.5$\mu$g (value 6-6 in table 3.1). Then, this total protein value was divided by each of the average protein concentrations from the flowthrough and elution fractions of IMAC and IEX (values 6-2 through 6-5 in table 1.2). This value gave the sample volume (in $\mu$l) for the 500mM sample. In order to make each sample volume 50$\mu$l, the appropriate amount of distilled water was added by subtracting the sample volumes (values 6-7 through 6-11 in table 3.1) from 50 (last column in tables 3.1-3.4). Additionally, before running on the SDS-PAGE gel,

5.5$\mu$l of 10x Sample Loading Buffer was added to each fraction, which contains multiple substances: Bromophenol blue tracking dye, glycerol, SDS, and □-mercaptoethanol. Bromophenol blue is used for visualization while the samples run and glycerol increases the density of the sample so it stays in the well of the stacking gel. The SDS is a detergent that breaks weak interactions in the protein and coats the protein in a negative charge, and lastly the □-mercaptoethanol breaks stronger interactions within the protein, like disulfide bridges, completely linearizing the protein (1).

The results of the SDS-PAGE for the 500mM sample can be seen in image 3.1, the top right gel. There are multiple problems with this gel. To begin, it can be observed that the marker from lane 1 spilled into lanes 2 and 3, where the GST and GFP controls should be at approximately 27kDa and 28kDa, respectively. Lane 4 is the original lysate, and did not produce a very bright band with lots of various contaminants as expected. The protein of interest, GST-GFP, has a molecular weight of 54kDa, and should be closer to the top of the gel than the GST and GFP controls due to its larger size, and should be slightly above the 50kDa marker 3rd band from the bottom) in lane 1. Lanes 5 and 6 seem to be of similar brightness and intensity, despite the fact that lane 5 is the IMAC flowthrough and expected to be much fainter than the IMAC elution in lane 6. However, for the IEX flowthrough and elution in lanes 7 and 8, the elution band appears to be brighter than the flowthrough band, indicating that IEX was more effective at purifying the protein of interest. Additionally, lanes 9 and 10 were controls, BSA and lysozyme, respectively and used for comparison. After SDS-PAGE and visualization under the Gel Doc, the 500mM gel was transferred to a nitrocellulose membrane via Western Transfer and had a Western Blot performed, with the lane order staying exactly the same as it was in SDS-PAGE. A Western Blot is a method to confirm that the bands observed on the SDS-PAGE gel belong to that of the protein of interest, GST-GFP. The primary antibody was mouse-anti-GFP due its higher affinity than GST, and it was at a dilution of 1/6500. The secondary antibody used was anti-mouse, and was conjugated with horseradish peroxidase, which reacted with Enhanced Chemiluminescence (ECL) to produce luminescence for visualization (1). The 500mM sample Western Blot membrane can be seen in the top right of image 4.1. This Western Blot makes it clear that a concentration of 500mM imidazole in the IMAC elution buffer did not yield very pure protein. As observed in the image, the bands that align with the elution fractions of IMAC and IEX are very faint, and there appears to be binding taking place in lane 1, 2, 3, and 7. This would indicate that it is possible that the GST and GFP got cleaved throughout the purification process, and that the primary antibody bound to free GFP, thus causing it to fluoresce.

Lastly, for the 500mM sample, a purification table exactly like the one currently used in the QBM laboratory was completed. Table 4.1 is the purification table for the 500mM sample, and tracks nine total values for the original lysate sample, the IMAC elution, and the IEX elution. To reiterate, columns 3, 5, 7, and 8 should have decreasing values, while columns 6 and 9 should have increasing values. Column 1 is the protein concentration, with row A being value

6-1, row B being value 6-3, and row C being value 6-5. Column 2 is total volume, with row A being the volume of original lysate (3ml) and rows B and C being the volume of the IMAC and IEX elution fractions that were saved and aliquoted together, being 4ml and 6ml, respectively. Moving to column 3, we observe the expected trend of a decrease in total protein, which is expected due to the inevitable loss of protein throughout the purification process. Next is column 4, which does not have an observable trend and whose values depend directly on volume. More volume likely would contribute to a higher reaction velocity due to more GST-GFP present compared to a smaller volume. As seen in table 4.1, the IEX elution has a higher reaction velocity compared to the IMAC elution, likely due to previously stated reason. Moving to column 5, total activity should decrease, once again, due to the inevitable loss of protein throughout purification. However, the total activity dropped significantly from the lysate to the IMAC elution, but then rose again for the IEX elution. The cause of this could be contributed to contaminants present in the IEX elution sample, or again, due to a larger volume than the IMAC elution. Next is column 6, which should have increasing values due to the loss of contaminants and the increasing purity of GST-GFP. However, both the IMAC and IEX elution specific activity decreased from that of the lysate, with the IMAC being lower than the IEX elution. This could suggest that GST degraded over time through the various thawing and freezing cycles and thus losing its enzymatic activity, or it could suggest that 500mM of imidazole in the elution buffer was not enough to make IMAC an optimized purification technique. Moving to column 7, which is total percent yield. This column should show a decrease in percentage because of, once again, the inevitable loss of protein throughout purification. This decrease is observed in table 4.1, with the total percent yield of the IMAC being more than that of the IEX elution, which was expected. Column 8 should also show a decrease for the same reason as column 7, this time being percent yield of GST-GFP. As seen in table 4.1, the IMAC elution GST-GFP percent yield was significantly lower than that of the lysate and the IEX elution. The rise of percent yield from IMAC elution to IEX elution again suggests that 500mM of imidazole was not an effective elution buffer. And lastly, column 9 gives the values for fold purification, which should increase as the purity of GST-GFP increases. As seen in table 4.1, this increasing trend is not observed, as the fold purification decreased from that of the original lysate. However, the fold purification increased substantially for the IEX elution to give a value of 0.864, which is significant.
The results of the 500mM sample can be compared to that of the 750mM, 1M, and control (see below and tables 4.2-4.4) for further analysis.

The purification tables for the 750 mM imidazole buffer and 1 M imidazole buffer showed similar trends to the purification table of the 500 mM sample. The percent yield in both of these tables is expected to decrease going from the lysate to the affinity elution sample and again from the affinity elution to the ion-exchange results. However, in both tables, the percent-yield goes down from the original lysate sample to the IMAC sample, but then the percent yield rises again. This is not expected.

Another unexpected trend that was also noticed in all of the tables was found in the purification fold column. While this value should increase going down the column, the purification tables obtained showed a decrease in the purification fold from the original lysate to the IMAC elution samples, but it increases again in both tables from the IMAC elution to the ion-exchange. Still, the fold purification for the 500 mM, 750 mM, and 1 M samples were all under 1. In order to have a successful purification scheme, this value should be greater than 1. This may occur due to loss of protein or protein denaturation, as our experiments were performed over several weeks and for many hours at a time in the lab. At this time, our samples are not in the freezer, allowing for protein denaturation.

Lastly, we have the control sample. The control was a lysate sample that was purified using the protocol currently used in the QBM lab. That is, GST-affinity chromatography was not replaced by IMAC, and the rest of the purification protocol was followed. Just like the other three samples, GST-affinity chromatography was the first purification step, and images 1.4a and 1.4b show the chromatogram and respective Gel Doc image. The chromatogram in image 1.4a shows the two expected blue peaks, again, the first, larger one being the flowthrough peak and the second, smaller one being the elution peak. Image 1.4b shows the Gel Doc image of the control fractions following GST-affinity chromatography, and the elution fractions can be observed due to their bright fluorescence. However, it can be seen that fraction 1 is fluorescent, which is unexpected due to it being a flowthrough fraction. The cause of this erroneous fraction is not known with certainty. The observed fluorescent tubes (fractions 16-19) were collected and aliquoted together and a flowthrough fractions was saved, and then the aliquoted elution samples were run on Ion Exchange Chromatography (IEX), again, specifically being Anion Exchange Chromatography. Just like in the affinity chromatography, the chromatogram and respective Gel Doc image can be seen in images 2.4a and 2.4b. Again, image 2.4a shows the two expected blue peaks corresponding to the flowthrough and elution, but the chromatogram also shows a red line, which tracks conductivity, where once again there is a sodium chloride gradient from 0 to 100%. And just like the IMAC, the Gel Doc image (image 2.4b) for the IEX control clearly shows which fractions contain the protein of interest. But once again, the IEX elution fractions are much more spread out compared to the affinity elution fractions, possibly due to a slower elution of the protein of interest off of the column, resulting in more fractions containing the protein of interest. For the IEX elution, fractions 5-9 were kept and aliquoted together and one flowthrough fraction was saved.

Continuing with the control sample, just like the three other samples, the DC protein assay and the CDNB assay were run on the various elution and flowthrough samples obtained from the two chromatography runs. Starting with the results of the CDNB assay, which again is used to determine the enzymatic activity of GST. The results of the CDNB assay for the control sample following affinity chromatography and IEX can be seen in tables 2.5a and 2.5b,

respectively. Just like with the other three samples, it is expected that the flowthrough from both affinity and IEX will be lower than the elution as there should be no protein of interest in the flowthrough fraction. As seen in table 2.5a, for the flowthrough and elution samples (values 4-1 and 4-3, respectively) following affinity chromatography, the elution had a significantly higher reaction velocity, indicating the presence of GST-GFP in the elution fractions . This same trend can be observed in table 2.5b, which show the results of the CDNB assay following IEX. Again, the elution fraction had a much higher reaction velocity than the flowthrough fraction (values 5-1 and 5-3, respectively), once again indicating that the protein of interest is only present in the elution fractions, and also suggests that both the GST-affinity and IEX chromatography were successful in purifying the protein of interest. Next, the DC protein assay was run to determine the protein concentration of each flowthrough and elution fraction for the control sample. The results of the DC protein assay for the control can be seen in table 1.5, values 6-2 through 6-5. It is expected that the elution fractions will have a higher protein concentration than that of the flowthrough fractions. This trend is observed, again with rows B and D being flowthrough, and rows C and E being elution. However, there was an extraneous value for the affinity elution (value 6-3), with a very high protein concentration of 10.17mg/ml. This value is significantly higher than the original lysate concentration, 3.26mg/ml (value 6-1) and the IEX elution of the control, 3.67mg/ml (value 6-5). The cause of this is not entirely known, but the most likely cause is other protein contaminants.

This very high concentration for the affinity elution proved to be a challenge when doing to appropriate calculations to normalize the control samples before running SDS-PAGE. Again, the purpose of sample normalization is to ensure that an equal amount of protein is present in each sample to allow for accurate comparisons within lanes in the gel and comparison with other gels. When normalizing the various control samples (table 3.4), the original lysate ended up being the lowest concentration, 3.26$\mu$g/$\mu$l. This value was multiplied by 50$\mu$l to determine the total protein amount. This value, 163$\mu$g (value 6-6 in table 3.4), was then divided by values 6-2 through 6-5 from table 1.5 to give sample volume (in $\mu$l), which are values 6-7 through 6-11 in table 3.4. In order for each sample volume to equal 50$\mu$l, the appropriate amount of distilled water was added by subtracting values 6-7 through 6-11 by 50. If any of the 6-7 through 6-11 values were above 50$\mu$l, then 50$\mu$l of sample was used with no water added. Additionally, before running on SDS-PAGE, 5.5$\mu$l of 10x Sample Loading Buffer was pipetted into each fraction, which as stated earlier, contains components that allow for the visualization of the protein, increasing its density, and linearization and even negative charge distribution on the protein.

Once all the control samples were normalized, they were loaded into the stacking gel and the SDS-PAGE gel was run for 25 minutes at 200 volts. The result of the control SDS-PAGE can be observed in image 3.1, and is the top left gel. Lane 1 is the marker, and from bottom to top, the bands are 10kDa, 25kDa, 50kDa, and 75kDa. The protein of interest, GST-GFP is 54kDa, and its band will be slightly above the 50kDa marker. Lanes 2 and 3 are the GST and GFP controls, having molecular weights of 28kDa and 27kDa, respectively. As seen in image 3.1 in

the control gel, lane 2 has a very light and nearly indistinguishable GST control band. Lane 3 has the similar problem, with the GFP marker band being almost invisible. Also in lane 3, a bright band around where the protein of interest would be (~54kDa) is visible. The cause of this is likely due to spilling of the contents of lane 4, which is the original lysate, into lane 3. As stated, lane 4 is the original lysate, and a very bright band can be seen at ~54kDa, indicating the presence of the protein of interest. Also note in lane 4 there are many other fainter bands, indicating other protein contaminants within the original lysate. Next is lane 5, which is the affinity flowthrough of the control sample. There is a very faint band visible, which is most likely due to GST-GFP eluting early, but as a whole, the flowthrough contains very little to no protein of interest, as expected. Lane 6 is the affinity elution, and as expected, contains a semi-bright band around 54kDa, likely indicating the presence of the protein of interest. On to lane 6, which is the IEX flowthrough of the control sample. Like the affinity flowthrough, there is a very faint band at 54kDa, and this lane contains an extra band at ~27-28kDa, possibly indicating free-floating GFP which got cleaved from the GST at some point in the purification and eluted out in the flowthrough, along with a minimal amount of GST-GFP. Next is lane 8, which is the IEX elution. This lane shows a fairly bright band around 54kDa, which again, indicates that this is most likely the protein of interest. Lanes 9 and 10 are control lanes of BSA and lysozyme, respectively, and are used for comparison, as BSA has a molecular weight of 66kDa and lysozyme 14.4kDa.

Following SDS-PAGE, the gels were transferred to a nitrocellulose membrane via a Western Transfer, with the the lane order remaining the same as it was during SDS-PAGE. Following the transfer, a Western Blot was performed on the membrane to confirm that the bands on the SDS-PAGE gel are in fact the protein of interest, GST-GFP. The primary antibody was mouse anti-GFP, and the secondary antibody was conjugated anti-mouse, with horseradish peroxidase as the conjugate, which reacted with Enhanced Chemiluminescence (ECL) to produce luminescence needed for visualization. The Western Blot membrane of the control sample can be viewed in image 4.1, the top left gel. As observed in the image, There was some residual free-floating GFP in lane 3, which the primary antibody bound to. Additionally, note that luminescence only occured in the elution lanes (lanes 6 and 8), and not in the flowthrough lanes (lanes 5 and 7), indicating that both chromatography runs were successful in purifying the protein of interest. Also, in lane 4, GST-GFP can be seen as a very bright band in the original lysate. Also, notice in lane 3 the very bright band at ~27kDa, which is the lane containing the GFP control, but no band in the GST control due to the antibody not being specific for GST.

Lastly, to quantitatively compare protein purification, a purification table like the one currently used in the QBM lab was calculated and can be seen as table 4.4. Once again, lanes 3, 5, 7 and 8 should all have decreasing values, while lanes 6 and 9 should have increasing values. Row A is the original lysate, row B is affinity elution, and row C is IEX elution. Looking at table 4.1, column 1 is protein concentration, and row A is value 6-1, row B is value 6-3, and row C is value 6-5. Column 2 is total volume of each sample, with row A being the total volume of lysate

(3ml), and rows B and C being final elution volumes of affinity and IEX, 4ml and 5ml, respectively. Next is column 3, showing total protein. Again, the values in this column should decrease due to the inevitable loss of protein through the purification procedure. However, in table 4.4, a sharp increase from the lysate to affinity elution can be seen and then a decrease again to the IEX elution protein concentration. These unreasonable values correlate with the very high concentrations seen in the DC protein assay and sample normalization (see tables 1.5 and 3.4). The cause of this steep increase in protein concentration from the lysate to both the affinity and IEX elution samples could be due to protein contaminants, however it is not entirely clear what caused such high protein concentration readings. On to column 4, which is reaction velocity, there is no trend expected in this column, and the values are largely based on volume. Next is column 5, which is total activity. It is expected that the values will decreases, and this trend is observed in table 4.4, again, due to to inevitable loss of protein through purification. Next is column 6, which is specific activity, which should have increasing values due to the increased purity of the protein of interest. However, the opposite can be observed in table 4.4, with the affinity elution having an even lower value than the IEX elution. It is likely that these very low numbers were caused by the degradation of the GST, where the GST degraded over time due to multiple thawing and freezing cycles, and become no longer enzymatically active. Next is column 7, which is total percent yield, and should have decreasing values, again due to the inevitable loss of protein throughout the purification. However, seen in table 4.4, there was a significant increase in total percent yield for both the affinity and IEX elutions, being 416% and 188%, respectively. These values are of course not feasible, and these values again correlate to the large protein concentration observed in the DC protein assay (table 1.5). The cause of this sharp increase in total percent yield is not fully known, and the only feasible explanation is protein contaminants in the affinity and IEX elution samples. On to column 8, which is percent yield of the GST-GFP. This column should decrease due to the protein loss throughout the purification, which is observed in table 4.4. Also, this column gave much more reasonable and expected percent yield values compared to column 7. Lastly is column 9, which tracked the fold purification of GST-GFP. This column should have increasing values due to the expected increased purity of the samples following affinity and IEX chromatography.

However, as seen in table 4.4, this was not what was observed, as it can be seen that the fold purification decreased for the affinity and IEX elution samples compared to the lysate. Why this occurred is not fully known, however it could be due to degradation of the protein over time, because all of the samples underwent multiple thawing and freezing cycles over the course of 3-4 months, which could result in degradation. However, this does not line up with the fact that a very high protein concentration was observed via the DC protein assay and the Western Blot image showing that GST-GFP was present at the end of both the affinity and IEX chromatography runs.

Upon completion of this project, there are some concerns that have to be taken into account before continuing this research. One concern is the decrease in fold purification (column

9 of purification tables 4.1-4.4). The cause of this is not fully known, but it is important that the calculations for each purification table be revisited and re-calculated before continuing research and drawing complete conclusions. These large discrepancies were not represented in the visualization steps, suggesting that the error occurred in the calculations and not the protocol itself.

A second concern is what was observed with the IEX fractions upon visualization using the Gel Doc. It can be seen in images 2.1b-2.4b that the elution fractions were very spread out for all four samples, compared to the localization of elution fractions as observed for affinity elution fractions in images 1.1b-1.4b. However, this problem was most extreme in the three samples that first ran through the IMAC problem. This spread of protein across several fractions during purification may be suggestive of the column being old or improper settings for the machine itself. This concern should be addressed in further experimentation. This may also be the cause of the purification fold decrease observed in all of the purification tables, as protein was lost in this process, making it difficult to concentrate.

References:

1.) Borgon Ph.D, Robert, Nicole Verity. *Quantitative Biological Methods.* 7th ed., Hayden-McNeil, 2018.