

University of Nevada, Reno

Genentech: Purification of a Monoclonal Antibody

*A thesis submitted in partial fulfillment of the
requirements for the degree of*

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By

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We recommend that the thesis
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Abstract

Lucentis, a monoclonal antibody fragment produced in *Escherichia coli*, is used to treat wet age-related macular degeneration, one of the leading causes of legal blindness. The objective of this thesis is to analyze the process of antibody fragment separation and to purify a solution of “Lucentis like” protein provided by Genentech to a 99% purity. This paper focuses on the chromatography aspect of the process and investigates the multiple types of chromatography used in both the base case and alternative case presented with a focus on cation exchange chromatography.

The project was completed over an eight-month time period spanning two semesters. The team studied various theories, design models, performance equations,, chromatography techniques, literature reviews, and economic analyses. The results obtained from experimental analysis, and after consulting with various teams, instructors, and Genentech employees, highly suggested that the “Lucentis like” protein used was denature and no longer had the properties of the desired protein.

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Introduction

Ranibizumab, more commonly known as Lucentis, is a drug developed by Genentech in South San Francisco, California to treat wet age-related macular degeneration. Wet age-related macular degeneration is one of the leading causes of legal blindness. Lucentis is a monoclonal antibody fragment produced in *Escherichia coli* using standard recombinant DNA technology. This protein is targeted to bind to a vascular endothelial growth factor A (VEGF-A) which prohibits VEGF-A binding to its receptors. By stopping VEGF-A from binding to its receptors, Lucentis is able to reduce the progression of wet age-related macular degeneration.

The Chemical Engineering Seniors at the University of Nevada, Reno have been given the task to analyze and design the process of antibody fragment separation. The students will study processes involving a homogenizer, anion and cation chromatography, membrane separation, and centrifugation. These processes will be combined in a way that will most efficiently purify the target antibody on a commercial scale. The desired final product will be an aqueous solution of the pure antibody in solution.

The objective of this design project is to purify a solution of “Lucentis like” protein provided by Genentech to a 99% purity. Lucentis is known to have a molecular weight of approximately 48 kDa. Lucentis’ light chains and heavy chains have molecular weights of approximately 23 kDa and 25 kDa respectively. This unit operation directly follows the tangential flow filter, which lyses the cells into their component proteins, one of which is the “Lucentis like” protein. The challenge is to remove to undesired waste proteins from the “Lucentis like” to produce a useable product.

The overall goal of this project is to optimize purity and step yield for each step in the process and for each student to have a full understanding of the mechanism designed and how this goal is achieved. The students will also conduct an economic analysis on their process and provide operational and raw material costs for the mechanism proposed.

In order to achieve the most efficient mechanism, and to understand each process step as thoroughly as possible, the process was divided into three main sections, bioreactor, tangential flow filtration, and chromatography, with a group of 4-6 students working on each section. This paper focuses on the chromatography aspect of the process and investigates the multiple types of chromatography used in both the base case and alternative case presented with a focus on cation exchange chromatography.

Timeline

This project was completed over a two-semester timeline beginning in the Fall of 2016 in CHE 450 and ending in the Spring of 2017 in CHE 482. The Gantt chart in Figure 1 was created at the beginning of the first semester and provides an overall timeline for the project with anticipated deadlines and important events. It was followed very closely throughout the whole project. The Gantt Chart helped to keep the team on track and organized throughout both semesters and helped to ensure that all due dates were met.

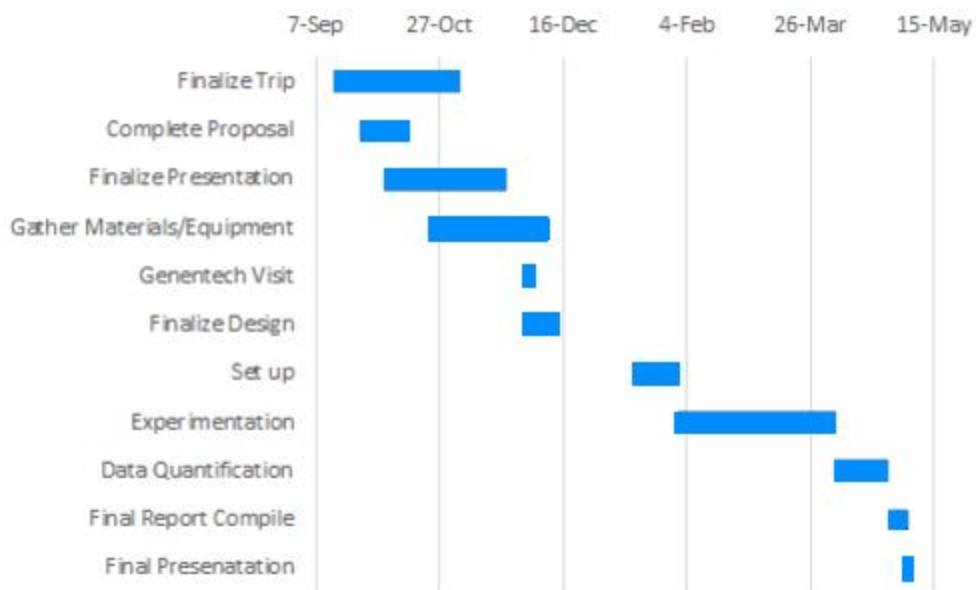


Figure 1: Gantt Chart for the chromatography team for the Genentech Project conducted by the Chemical Engineering Seniors between September 2016 and May 2017.

The first semester was mostly dedicated to studying engineering economics and determining a plan of action for the project while the second semester was completely spent designing, analyzing, and testing different models in order to achieve sufficient results.

The economics learned in the first semester helped students complete a full economic analysis on the completed process design as well as assisted with a full understand of scale-up economics for the process studied. The rest of the semester focused on learning as much about the process as possible before testing began. This included developing design ideas and finalizing a design, gathering necessary materials and equipment, preparing a proposal presentation for the Genentech staff, and visiting the

Genentech site in South San Francisco.

The second semester focused on experiment design, process modeling, and testing and was split up into four quarters. The first quarter was spent doing extensive literature reviews on the types of chromatography used in the base and alternative cases. Through literature reviews and consulting with multiple faculty members, the team decided at this stage to focus on chromatography techniques of: anion exchange, cation exchange, and hydrophobic interaction. After looking into what type of resins, buffers, and columns that would be needed, the team was able to prepare a list of all materials and track down all appropriate materials and equipment. During the week of February 6th, team members began working on mathematical models for each of the chromatography experiments the team decided on and looked into various scale up designs as well as an experimental design for the project. In order to begin experiments, a standard operating procedure for the team to follow was created and approved by the lab instructor. During the week of February 13th, the team began packing their first column for an anion exchange experiment.

During the second quarter of the semester, modeling equations and calculations were finalized and were able to provide the team with insight into how the chromatography columns would perform. The team also continued to look into scale up calculations and considerations for the proposed mechanism. During the week of March 13th, the team received new 20 cm x 2.5 cm columns, and began to pack one of them with anion exchange resin. The team's biggest progress during this quarter was has been obtaining a Masterflex pump to assist in packing and experimental testing.

The third and fourth quarter were spent running the protein through the column and testing the protein concentration of the effluent in order to determine the level of purity obtained. This quarter was also spent preparing the final presentation for the Genentech staff and compiling results with the other two teams, bioreactor and tangential flow filter.

Theory

The reason chromatography is used to separate a Lucentis like protein from the rest of the lysed bacteria. Different types of chromatography work with different kinds of affinities to separate various mixtures of fluids. In our case, we will be using Cation, Anion, and hydrophobic chromatography methods.

Chromatography principles

In Cation exchange the resin inside of the column is positively charged. This means that any negatively charged ions will get caught inside the column while the positive ions will go through the column. How well the negative ions bind to the resin is called ionic capacity and it is a measurement of how many ions can attach to the resin per volume. Now, in order to elute the column, a buffer must be run through the column. The buffer has a strength measured in ions per volume and it is called ionic strength, but it also has a specific PH. The ionic strength of the buffer and its PH can remove the bound proteins and ions from the resin. This can work by either removing the bound things by having a stronger ionic strength than the resin, or by changing the PH of the

molecules and changing their charges. Say for instance, the protein we are collecting has a negative charge at PH 6, but becomes neutral at 9. You could remove the protein from the positive resin by change its PH to 9 stopping the ionic interaction. An Anion exchange would bind positive proteins to its resin, and a hydrophobic resin would attract lipids. Using these principles of chromatography it is possible to individually separate the cell waste from the desired proteins.

Literature Review

The concept of Ion-exchange chromatography is based on the interaction between charged molecules on the support material and the charged molecules and ions in the mobile phase. In anion-exchange chromatography, negatively charged molecules compete for the positive sites on the support material. These anionic groups are usually tertiary ammonium moieties such as diethyl amino ethyl. In cation-exchange chromatography, positively charged molecules compete for the negative sites on the support material. These cationic groups are often sulfonic acid moieties such as sulfo-propyl or carboxymethyl.

The isoelectric point (pI) is important in determining what type of ion-exchange chromatography is needed. If the protein is more stable above its pI, then an anion exchanger is used. On the other hand, if the protein is more stable below its pI, then a cation exchanger is used. Cation-exchange chromatography is most suitable for protein

purification due to their stability in an acidic environment.¹

Other forms of chromatography that will be employed in the alternative case include multimodal and monoclonal chromatography. Multimodal chromatography combines multiple types of chromatography such as ion exchange, hydrophobic interaction, affinity, and size exclusion chromatography to improve the selectivity of certain protein purification. It effectively combines complementary methods and reduces the total number of columns needed in the system. Hydroxyapatite and hydrophobic ion exchange ligands are popular types of mixed-mode media available for use in multimodal chromatography.²

Monoclonal chromatography often includes at least one ion exchange chromatography step. This helps to remove impurities such as viral particles, host cell proteins, and residual DNA. Monoclonal chromatography can use both flow through, which removes impurities from a product that flows through the column, and bind-and-elute, which elutes out impurities while the product remains in the column.³

Base Case

The base case incorporates three types of chromatography, cation ion exchange chromatography, anion ion exchange chromatography, and hydrophobic interaction chromatography. As can be seen in Figure 2 which outlines the process, cation exchange chromatography occurs directly after the protein leaves the centrifuge and is followed by anion exchange chromatography. After the protein leaves the anion chromatography column it passes through a tangential flow filter before entering the hydrophobic

interaction chromatography column.

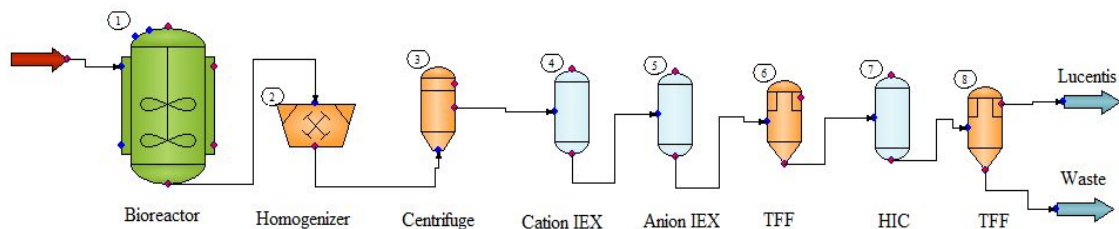


Figure 2: Base Case for Purification Process.

Ion Exchange Chromatography

Ion exchange chromatography (IEX) is a powerful technique for protein purification, and is frequently used in industry because of the high binding capacity of the column and the unlikelihood of denaturing processed proteins^{5,6}. Cation exchange chromatography uses a negatively charged ion exchange resin to remove particles in the liquid mobile phase with a positive charge. Anion exchange chromatography uses a positively charged ion exchange resin to remove particles in the liquid mobile phase with a negative charge

Principles of Operation

Ion Exchange chromatography separates charged molecules in a mobile phase with charged molecules in a stationary resin³. Proteins in particular are captured by manipulating the charge of the protein of interest. Proteins consist of many individually positive or negative amino acids, and so can have a positive charge, a negative charge, or

no charge, depending on the surrounding pH. The pH at which a molecule has no net charge is called its isoelectric point (pI). By raising the pH, the molecule adopts a negative charge, and by lowering the pH, the molecule adopts a positive charge. By surrounding the protein in a buffer of the appropriate pH, the protein can be given a charge and forced to bind with an appropriate counter-ion in the IEX resin ⁵. Table 1, below, clarifies the circumstances of anion IEX and cation IEX.

Table 1: Ion Exchange Differences

Ion Exchange type	Net protein charge	Resin charge	Running pH
Cation IEX	+	-	Below pI
Anion IEX	-	+	Above pI

Under normal operation, a protein is run through the ion exchange column in a buffer solution until the protein of interest is entirely captured by binding to the resin, while some contaminants which cannot bind exit in the outlet. Afterwards, the protein and perhaps other bonded contaminants are released from the ion exchange resin. This is normally done by increasing the salt concentration in the column (to a maximum of 1 molar salt), which in turn increases the ionic strength of the solution. The counter-ions

supplied by the salt eject the protein of interest as well as other contaminants by binding to the resin in their place. This process is called elution, and if the salt concentration is increased carefully, the protein of interest will separate from all other contaminants, resulting in a purified protein⁸. It should be noted that anion IEX is generally preferable for protein purification due to general trends in protein stability³.

Other strategies for protein purification using ion exchange chromatography do exist, namely a flow-through elution strategy (as opposed to the traditional bind-and-elute method) and chromatofocusing. In the flow through method, the product flows through while impurities and contaminants are instead captured by the IEX resin. However, this method is not as consistent in its results as binding-and-eluting, and does not do much to concentrate the protein of interest, which is also a goal for this project⁵. Chromatofocusing is the process of carefully changing the pH of the IEX column until it approaches the pI of the protein of interest. This will cause the protein of interest to elute as it no longer holds a charge, meaning it will no longer bind to the resin^{5,8}. Although this method provides similar resolution to elution with a changing salt gradient, it is often difficult to implement.

Effects on Design

The material safety data sheet for Lucentis provides an isoelectric point of 7.1. As Lucentis should be similar to the Lucentis-like-protein (LLP) being studied, it can be assumed that the LLP has a similar isoelectric point. Industry standard is to operate IEX at roughly 1 pH above or below the isoelectric point of the protein of interest⁵. As such, a

Tris-HCl buffer with a pH of 8.1 was decided upon for anion IEX, and a Sodium Phosphate buffer with a pH of 6.1 was chosen for cation IEX. Both IEX columns will be eluted with a stepwise gradient of NaCl. These choices for buffers and counterions in the salt are appropriate to recommendations in the literature ⁴. It is worth noting that an MES buffer would better suit elution at a pH of 6.1 for the cation IEX, but MES is expensive and dangerous.

Additionally, both chromatofocusing and a flow-through elution strategy were decided against for individual reasons. Chromatofocusing was deemed too difficult to effectively use in the given laboratory setting. A flow-through elution strategy would not really increase protein concentration, and so it was also deemed unsuitable for the given objectives.

Hydrophilic Interaction Chromatography

Hydrophilic interaction chromatography, or hydrophobic interaction liquid chromatography (HILIC) is a type of chromatography which is primarily used to separate biomolecules based on polarity. Specifically, by utilizing a polar stationary phase and a partially aqueous eluent, polar substances are able to be separated ⁶. HILIC has grown significantly more popular since its introduction, and is well-suited to the separation and purification of proteins. Hemstrum and Irgum fully detail hydrophobic interaction liquid chromatography (HILIC) in their paper, including information regarding usage, application, and common issues in troubleshooting operation of HILIC columns. Although the team is primarily focused on IEX at the current moment, it will be

necessary to consider implementation of HILIC in a laboratory setting in the near future.

Alternate Cases and Readings

Multimodal Chromatography

Multimodal or ‘Mixed Mode’ chromatography is a type of chromatography, which combines several chromatographic techniques together to improve the selectivity protein purification. By combining various methods, the strengths of different separation techniques are able to be combined into one work-step, which simultaneously reduces the number of columns required and associated costs. Multimodal chromatography is optimized by varying the parameters relevant to each mode, or type of separation, included in the process. Unfortunately, some parameters may not be unique to a particular mode, and so improving the ability of one mode to purify may simultaneously reduce the ability of another mode to purify.¹⁰

All the same, multimodal chromatography is still a very effective purification process, and can be especially useful as a final polishing step in which trace impurities and product variants are removed¹¹. Additionally, multimodal chromatography can be used with both the bind-and-elute as well as the flow-through method of elution. In fact, one of the advantages of multimodal chromatography is the added efficiency of the flow-through method being a generally good choice. For these reasons and more, Multimodal chromatography is included in the alternate case as a semi-final purification step, as shown in figure 3, below.

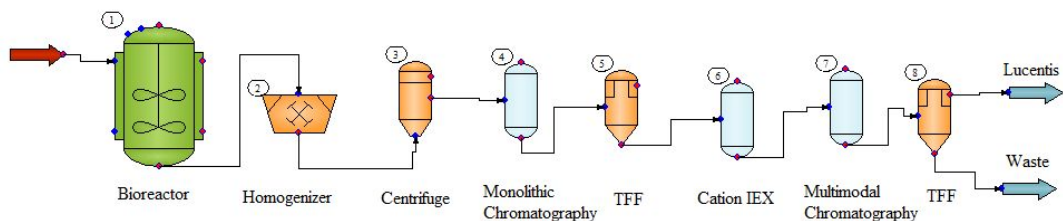


Figure 3: The alternate case for purification of a Lucentis-like protein.

The proposed multimodal resin is GE Cpto adhere ImpRes, which is a strong anion multimodal exchange resin, designed specifically for the high-resolution polishing of monoclonal antibodies¹². An anion resin was chosen to remove contaminants, which may have passed by the cation IEX preceding the multimodal chromatography. Conveniently, the same Tris-HCl buffer used in the base case can be used for the multimodal chromatography step. Additionally, the adhere ImpRes resin features a high dynamic binding capacity (85 mg/mL), fast mass transfer between the stationary and mobile phases, and high resolution with small column volumes¹¹. As a multimodal resin designed for the large-scale purification and polishing of monoclonal antibodies, this seems perfect for the objectives of this project.

Monoclonal Antibody Purification

Ranibizumab is an antibody fragment (Fab) from a monoclonal antibody (mAB). As Ranibizumab is the primary “active ingredient” in lucentis, and the team is observing

a lucentis-like protein, it can be assumed that purification techniques for a mAB can be applied to the LLP. Most mAB purification processes include an ion exchange chromatography¹, which reinforces the previously made decision to include IEX in the base process. Moreover, Liu et al. also explain the efficacy of both the flow-through and bind-and-elute elution strategies for purifying mAB's. However, this is with respect to several contaminants like viral particles, host cell proteins, residual DNA, and more¹. As none of these contaminants are present in the LLP, the flow-through elution strategy will be discarded as previously discussed.

Monolithic Chromatography

Monolithic chromatography is a type of chromatography, which involves a singular silica-based monolith as the stationary medium rather than a columnar packed bed. High contact between the stationary phase and the mobile phase is provided by the incredibly high porosity and internal surface area of the monolithic column⁹. Additionally, both the size of the through pores of the column and the size of the column's internal structures can be selected separately, whereas in traditional media, both properties are linked⁹. Other benefits include a relatively high efficiency, with a 10 cm monolithic column having 11,200 theoretical plates⁹.

The proposed industrial monolithic chromatography column is a 'BIA Separations CIMmultus™ SO3-8000 Advanced Composite Column', shown in figure 4. The chosen column is a monolithic strong cation exchange column of the largest commercially available size, designed to concentrate the product, remove large impurities, reduce

backpressure, and quickly isolate products. As the lucentis-like protein is particularly stable at a slightly acidic pH, a cation IEX column was deemed most appropriate. The same sodium phosphate buffer used in the base case for cation IEX is also used in this alternate case.



Figure 4: CIMmultus™ SO3-8000 Advanced Composite Column for monolithic chromatography. This column is designed for flow-through operation, providing a contrast to other cases.

The design parameters and manufacturer-suggested heuristics for the monolithic column are shown in table 2, below. Note that 40 Column volumes of preparing and cleaning the column is associated with each run of 1000 L (which would be 125 column volumes by itself). Additionally, note that processing the full 1000 L takes just over two hours. As such, it is possible that up to eleven monolithic chromatography columns could be run in series to more drastically purify and concentrate the LLP. This would

necessitate a re-ordering of the existing process diagram to make the monolithic chromatography the final purification step, but this may be worthwhile.

Table 2: Design Parameters for Monolithic Chromatography.

Design Parameter	Parameter Value
Column Volume	8 L
Flowrate	10 L/min
Pressure	1.4 MPa
pH	8.1
Temperature	4 – 40 °C
Column Volumes per Batch	165
Time for total operation	2.2 hours

Modeling

Velocity Modeling

Due to the presence of solid material in the column, the mobile phase can only take up a certain amount of the column volume. The fraction of the column volume occupied by the mobile phase is denoted by ϵ_u . When modeling flow through a chromatography column, it is important to distinguish between the different types of fluid velocity. Superficial velocity, u_s , is the flow per unit area, given in equation 1.

$$u_s = \frac{F}{A} \quad (1)$$

The superficial velocity is insignificant when modeling flow through a packed column, but can be used to calculate the mobile phase velocity, u_m .¹ The mobile phase velocity is given in equation 2.

$$u_m = \frac{u_s}{\epsilon_u} \quad (2)$$

Solving first for the superficial velocity using equation 1, and then plugging that into equation 2 gives the mobile phase velocity. The diameter of the column and volumetric flow rate are given in the heuristics. The fraction of the column volume occupied by the mobile phase is assumed to be 0.25. The calculations for the velocity are shown below.

$$A = \pi r^2 = \pi(15)^2 = 706.86\text{cm}^2$$

$$F = 220\text{cm/hr} * 706.86\text{cm}^2 = 155,508.84\text{cm}^3/\text{hr}$$

$$u_s = \frac{155,508\text{cm}^3/\text{hr}}{706.86\text{cm}^2} = 220.0\text{cm/hr}$$

$$u_m = \frac{220.0\text{cm/hr}}{0.25} = 880\text{cm/hr}$$

$$880\text{cm/hr} = 14.67\text{cm/min}$$

Flow and Pressure Drop Modeling

Flow in a chromatography column is nearly always laminar. When flow is laminar it can be modeled using Darcy's law. Darcy's law equation is given in equation 3 where B is the specific permeability of the particles in the column and η is the dynamic viscosity.¹

$$u_m = -\frac{B}{\eta\epsilon_u} \frac{dp}{dz} \quad (3)$$

In liquid chromatography, the pressure drop, dp/dz , is constant along the length of the column. The Darcy equation can then be simplified to the form shown in equation 4.

$$\Delta p = \frac{\epsilon_u \eta L u_m}{B} \quad (4)$$

For a packed column, the specific permeability, B , can be found using the Carman-Kozeny equation given in equation 5.¹

$$B = \frac{d_p^2}{180\psi^2} \frac{\epsilon_u^3}{(1-\epsilon_u)} \quad (5)$$

The specific shape factor, ψ^2 , is 1.7 for porous non-spherical particles. The diameter of the particles, d_p , is assumed to be 0.005cm. The dynamic viscosity is assumed to be 0.00012Ns/cm² and the length of the column is given in the heuristics. The calculations for the pressure drop along the column are given below.

$$B = \frac{(0.005)^2}{180*1.7^2} * \frac{0.25^3}{(1-0.25)^2} = 1.34x10^{-9}$$

$$\Delta p = \frac{(0.25)(0.00012)(20)}{1.34x10^{-9}} = 448\text{Pa}$$

$$108Pa = 0.004\text{atm}$$

Adsorption Modeling

The binding of ions to a charged resin can be modeled by the Langmuir isotherm as shown in equation 6².

$$q_i = (q_i)_m * \frac{K_i * c_i}{1 + \sum K_j c_j} \quad (6)$$

Where $(q_i)_m$ and all of the K_i 's are experimentally determined constants, q_i is the amount of solute adsorbed, and c_i is the concentration of solute in solution. Equation 7 can be used to model the concentration and adsorption of a solute in ion exchange chromatography².

$$K_{A,B} = \frac{\left(\frac{Q}{Q_A}\right)^{n-1} y_A (1-x_A)^n}{x_A (1-y_A)^n} \quad (7)$$

Where A is the ion being exchanged with the ion bonded to the resin, which is B, C and Q are the total equivalent concentrations in the liquid and ion exchanger phases,

respectively, and x_A and y_A are the equivalent fractions of A in the liquid and ion exchanger phases, respectively. Once q_i is determined from equation 6, equation 7 can then be used to predict x_A once the number of equivalents of the solute is determined.

Laboratory Scale Equipment Specifications

The equipment utilized for this experiment consists of the following:

- Two 2.5 cm x 20 cm Econo-Column Chromatography Columns
- POROS 50 HS Resin
- Capto Q ImpRes Resin

The specified columns were chosen to obtain a larger elution flowrate, which would enable the possibility for further experiment trials. However, a larger column results in a larger volume necessary to reach a bed height of 15 cm. Below is the following volume calculation:

Inner column diameter, $D = 2.5\text{cm}$; Bed height, $h = 15\text{ cm}$. Finding Volume (V_1)

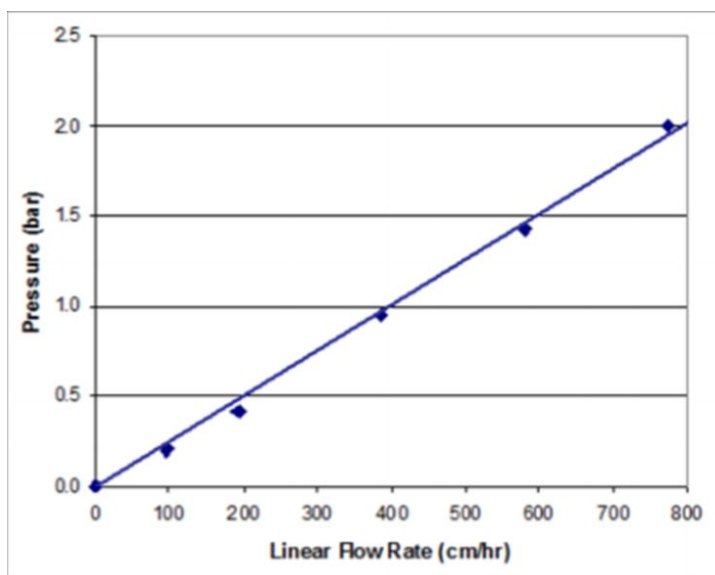
$$V_1 = \frac{\pi h D^2}{4} = \frac{(2.5^2)(15\text{cm})\pi}{4} = 73.6\text{ mL}$$

Thus, a total resin volume of 73.6 mL is necessary for each column. Therefore, the team needs a minimum of 150 mL of each resin to complete a full packing, as well as having a reservoir in case the packings needs to be redone. In the table below, the costs for all materials are listed.

Table 3: List of materials and prices^{3,4}.

Equipment	Amount	Price
2.5 cm x 20 cm Econo-Column	2 Columns	\$84
POROS 50 HS Resin	150 mL	\$400
Capto Q ImpRes Resin	150 mL	\$564
Total Price		\$1048

To determine the theoretical flow rates of the ordered columns, the following figures were utilized:

Figure 5: Linear flow rate plot provided by the manufacturer for the POROS 50 HS resin. ⁷

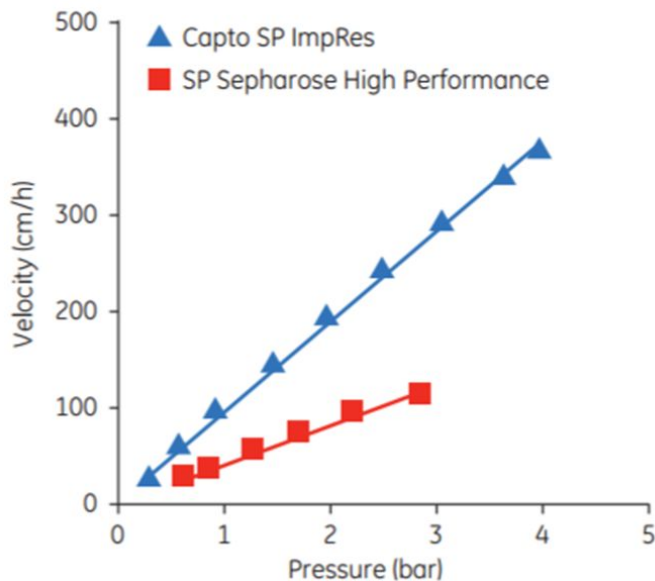


Figure 6: Linear flow rate plot provided by the manufacturer for the Capto Q ImpRes resin.⁶

Using Figures 5 and 6, the following calculations were performed to calculate the theoretical flow rates of the columns.

For the POROS 50 HS resin, the following would be the resulting flow rate:

Assumptions

Area of the Column: 4.906 cm^2

Pressure at Pump: 1 bar

Thus, the linear flow rate is about: $v = 400 \text{ cm/hr} = 6.667 \text{ cm/min}$ from Figure 5.

$$F_1 = A_1 * v = (4.906 \text{ cm}^2)(6.667 \frac{\text{cm}}{\text{min}}) = 32.71 \frac{\text{cm}^3}{\text{min}}$$

For the Capto Q ImpRes resin, the following would be the resulting flow rate:

Assumptions

Area of the Column: 4.906 cm²

Pressure at Pump: 1 bar

Thus, the linear flow rate is about: $v = 100 \text{ cm/hr} = 1.667 \text{ cm/min}$ from Figure 6.

$$F_1 = A_1 * v = (4.906 \text{ cm}^2)(1.667 \frac{\text{cm}}{\text{min}}) = 8.18 \frac{\text{cm}^3}{\text{min}}$$

Ultimately, these new columns will yield flow rates of 32.71 mL/min and 8.18 mL/min for each respective resin. These flow rates are completely viable for the laboratory scale experiment.

Equipment Specifications for a Scaled-Up Process

The scaled-up process utilizes the same resins listed previously, however an industry scale column is necessary, as well as a much larger supply of each resin. Below are the specifications for an industrial column that could be utilized.



GE BPG 300/500 Glass Chromatography Column

- Ideal for process development and biopharmaceutical manufacture
- Column Diameter: 29.6 cm
- Cross-Sectional Area: 688 cm²
- Bed Height Range: 0 cm to 26 cm
- Max Operating Pressure: 4 bar

Figure 7: Specifications for the GE BPG 300/500 Glass Chromatography Column.³

The chosen column requires a large volume to reach a bed height of 15 cm and the necessary volume calculations below:

Column Diameter = D = 29.6 cm, Bed Height = h = 15 cm

$$V = \frac{\pi h D^2}{4} = \frac{(29.6^2 \text{ cm}^2)(15 \text{ cm})\pi}{4} = 10322 \text{ mL}$$

Thus, volume of 10.322 L is necessary for each resin. The table below provides the total costs for the whole system.

Table 4: List of materials and prices^{3,4,5}.

Equipment	Amount	Price
GE BPG 300/500 Glass Chromatography Column	1 Columns	\$5000
POROS 50 HS Resin	11 Liters	\$26000
Capto Q ImpRes Resin	11 Liters	\$21000
Total Price		\$52,000

To determine the theoretical flow rates of the industrial column, the Figures 5 and 6 are utilized.

For the POROS 50 HS resin, the following would be the resulting flow rate:

Assumptions

Area of the Column: 688 cm²

Pressure at Pump: 2 bar

Thus, the linear flow rate is about: $v = 800$ cm/hr from Figure 5.

$$F = A * v = (688 \text{ cm}^2)(800 \frac{\text{cm}}{\text{hr}}) = 5.50 * 10^5 \frac{\text{cm}^3}{\text{hr}} = 550.4 \frac{\text{L}}{\text{hr}}$$

For the Capto Q ImpRes resin, the following would be the resulting flow rate:

Assumptions

Area of the Column: 688 cm²

Pressure at Pump: 3 bar

Thus, the linear flow rate is about: $v = 280$ cm/hr from Figure 6.

$$F = A * v = (688 \text{ cm}^2)(280 \frac{\text{cm}}{\text{hr}}) = 1.926 * 10^5 \frac{\text{cm}^3}{\text{hr}} = 192.64 \frac{\text{L}}{\text{hr}}$$

Therefore, the expected flow rates for each respective resin is 550.4 L/hr and 192.64 L/hr

for the industrial column, which is completely reasonable for such a large-scale process.

Table 5: Scale-Up Parameters and Estimated Cost.

Equipment or Material	Quantity	Unit Price	Cost (year basis)
Chromatography Columns	9 Columns	\$29,731.00	\$267,579
Capto Q ImPres	488 L	\$3063 per 1L	\$1,494,744
POROS HS 50 resin	310 L	\$2480 per 1L	\$768,800
DI water	12,825 L/day	\$21.50 per 1000 gallons	\$26,587
Sodium Phosphate (buffer)	4,988 L/day	\$1.1 per kg	\$16,449
Tris (buffer)	7,838 L/day	\$1.33 per kg	\$31,191
NaCl	312.29 kg/day	\$200 per metric ton	\$22,797
Total			\$2,628,147

Experimental Procedure

Due to time, budget, and supply constraints, only the cation exchange chromatography column was utilized and only one run was conducted. Using the Masterflex pump to speed up the elution process, protein was added to the 2.5 cm diameter chromatography column filled with POROS 50 HS strong cation resin which had a bed height of about 11 cm. Figure 8 shows the setup of the chromatography column and pump.



Figure 8: Cation chromatography column and pump setup.

The buffer used was a 50mM sodium phosphate buffer at a pH of 6.1. To elute the protein, buffers of 0.4, 0.6, 0.8, and 1 M of Sodium chloride concentrations in sodium phosphate buffer were gradually added. Seventy-two three milliliter samples were collected of the effluent. Six samples were randomly chosen to run gel electrophoresis in order to determine if the process worked and the protein was purified. A BCA assay was also run on the 72 samples in order to determine the protein concentration. Figure 9 shows the curve for the BCA Assay which helped determine the protein concentration.

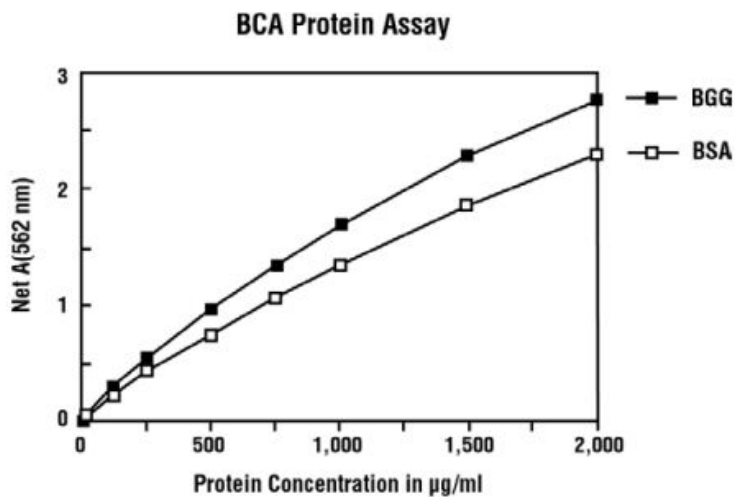


Figure 9: BCA Assay Curve

The following steps were taken in order to conduct the BCA Assay using the spectrophotometer:

1. Set the absorbance to the reference 562 nm wavelength
2. Subtract the blank absorbance at 562 nm from the sample and standard absorbances to find the Net A (net absorbance).
3. Plot the Standards Net A to create the BCA Standard Curve (figure to the left)
4. Utilize the calculated Net A for each unknown sample on the curve to find the concentration.
5. Example: Net A = 1, thus the concentration is 750 $\mu\text{g/mL}$

Experimental Results

Gel Electrophoresis

Out of the six samples analyzed in the gel electrophoresis, only two samples showed results. The two samples that showed protein were the Lucentis-like protein in buffer, and the first sample of the effluent. The results of the gel electrophoresis can be seen in Figure 10. The right sample refers to the Lucentis-like protein in buffer and the left is the first elution sample.



Figure 10: Results of Gel Electrophoresis

From the ladder shown in figure 10 the samples correlate to 180 kDa. From these results, it can be assumed that the protein conglomerated and passed directly through the column without being purified.

BCA Assay Results

The BCA Assay results showed that the initial “Lucentis like” protein in buffer and first elution sample were the only samples reading anything. All of the other samples

gave a zero reading. Figures 11 and 12 show the results of the BCA Assay. The results agree with the findings of the gel electrophoresis and will be discussed in the next section.

Concentration $\mu\text{g/mL}$	Trial	564 nm Abs	564 nm Abs Retested	Trial	564 nm Abs	Concentration of Lucentis $\mu\text{g/mL}$
2000	A			Lucentis	-1.561	680.8
1500	B	-0.103	-0.065			657.8
1000	C	-2.297	-2.297			
750	D	-0.117	-0.071			
500	E	-1.958	-1.909			
250	F	-2.418	-2.394			

Figure 11: Results of the BCA Assay

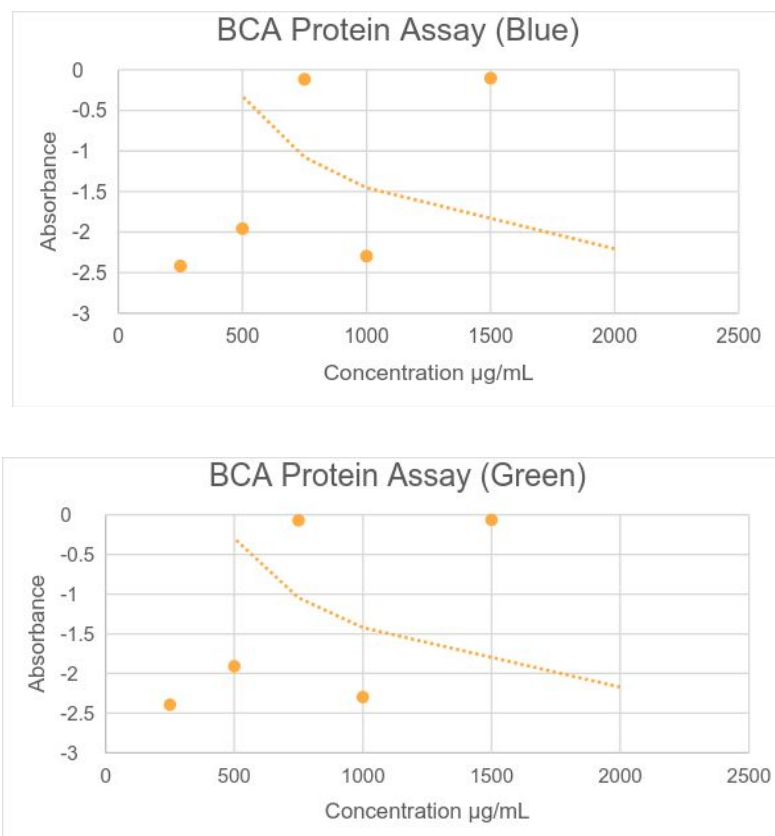


Figure 12: BCA Assay Results Curve

Discussion of Results and Conclusions

There are a few possibilities to consider regarding the results from the cation exchange chromatography run. The first possibility, which is believed to be the most accurate, is that the protein has become denatured and thus no longer exhibits characteristics of a protein. This would explain the results obtained from the gel electrophoresis and BCA Assay. The protein is 11 years old, so this is a believable assumption. After consulting with the bioreactor and tangential flow filtration groups, it was revealed that their results correlated to denatured protein as well, which would further support this belief. However, last year the the chemical engineering seniors were able to obtain results from the same protein and it is unlikely, but not impossible, for the protein to denature in the past year after being fine for 10 years.

Another possibility to consider is the amount of protein loaded into the column. With a column diameter of 2.5 cm and a bed height of 11 cm, the bed volume is about 54 cm³. Only about 5 milliliters of protein were loaded into the column. It is possible that the protein was not denatured and instead the concentration of the protein was so minute it was unable to show up on the gel electrophoresis and BCA Assay.

Lastly, there could have been an error with the operation of the gel electrophoresis. Before loading the samples into the gel electrophoresis, they need to be heated up which will break them down and allow the gel electrophoresis to run them. The samples were only heated to 50 degrees celsius prior to being loaded into the gel electrophoresis, which was possibly not hot enough to allow them to be broken down

sufficiently.

For next years chemical engineering seniors, it is advised to test the protein prior to beginning experimentation in order to determine it is not denatured. It is also advised to begin experimentation in the first semester. Time constraints were a huge issue and lead to the team only being able to conduct one trial on one time of chromatography. It would have been wonderful if the team had the ability to run multiple types of chromatography, or at the very least both the cation and anion in order to compare results.

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Appendix A

Chromatography Heuristics

Resin Height	Conventional resin height is approximately 15-20 cm--never above 30 height.
Diameter	Approximately 2 meters - an increase in diameter of columns will di correlate with an increase the resin volume.
Number of Theoretical Plates	Approximately 1,400 to 10,000 theoretical plates. The number o theoretical plates depends on the resin (many small beads).
HETP	Should be the same as the diameter of the individual resin l approximately 50 μm . This is common for almost all columns.
Pressure Drop	A consistent column height and a larger diameter will prevent an increa the pressure drop and degradation of the resin.
Number of Columns	To minimize maintenance costs, smaller columns are run in parallel. F designs may investigate having fewer larger columns.
Column Material	Stainless steel is preferred, but acrylic is cheaper and can handle the amount of pressure and design specs. However, acrylic is harder to sterili.

Appendix B

Standard Operating Procedure:

Cation and Anion IEX

Standard Operating Procedure: Cation and Anion IEX

Safety:

<u>Safety Equipment</u>	<u>Hazard</u>	<u>Consequences</u>	<u>First Aid/Solutions</u>
Safety glasses/goggles	Chemical splash back	Eye irritant	Flush eyes for 15 minutes
Protective Clothing/Nitrile Gloves	Chemical contact or splash back	Skin irritant	Wash with soap and water
Waste Vessel	Chemical spills/Sewage contamination	Slipping, chemical contact/splash back	Disposal into vessel and wipe up any spills
Labels	Utilization of incorrect chemical	Unwanted reactions	Label all unidentifiable material

**Refer to the chemical specific MSDS document listed in the References to further review hazards and first aid.*

Table A1: Outline of safety equipment, hazards, consequences, and first aid/solutions for the Cation and Anion IEX process.

Materials:

40 cm Glass Column	100 mL Beaker (5)	POROS CEX Resin	Stirrer
Stand w/ Rubber clamps	Scoopula	DEAE Resin	Pipettes (4)
Test tubes (10)	Tris Base	Hydrochloric Acid	Sodium Phosphate
Sodium Chloride	Graduated Cylinder	pH Strips	Sodium Hydroxide

Table A2: List of materials needed for the Cation and Anion IEX

Procedure:

250 mL Tris Buffer Preparation for Anion IEX

1. Transfer 210 mL of deionized water into a beaker and add the appropriate amount of Tris buffer to the water.
2. Mix the solution until the solution is clear and transparent.
3. Take a pH reading and adjust the pH by adding drops of 10mM HCl or 10mM of NaOH to reach a pH reading of 8 to accommodate for the sample's isoelectric point.
4. Add additional deionized water to reach a volume of 250 mL, and take a pH reading to make sure it remains at the desired point. Adjust the pH if it has changed.
5. Label the buffer in order to prevent confusion when utilizing the buffer.

250 mL Sodium Phosphate Preparation for Cation IEX

1. Transfer 210 mL of deionized water into a beaker and add the appropriate amount of the Sodium Phosphate buffer to the water.
2. Mix the solution until the solution is clear and transparent.
3. Take a pH reading and adjust the pH by adding drops of 10mM HCl or 10mM of NaOH to reach a pH reading of 6 to accommodate for the sample's isoelectric point.
4. Add additional deionized water to reach a volume of 250 mL, and take a pH reading to make sure it remains at the desired point. Adjust the pH if it has changed.
5. Label the buffer in order to prevent confusion when utilizing the buffer.

Chromatography Column Operation

1. Attach 40 cm glass column on rubber clamps and fasten the clamps. Make sure the valve is in the closed position.
2. Mix 85 cm³ of the appropriate resin with water in a beaker. Stir the resin to form a slurry. Utilize a POROS CEX resin for cation exchange, and a Diethylaminoethyl (DEAE) Resin for anion exchange.
3. Allow the resin to settle and then decant the excess liquid from the slurry. Add deionized water to the slurry, and repeat the decanting process until the excess liquid is clear colored. Once this point is reached, leave the resin suspended in water to prevent it from drying.
4. Stir the resin once again to achieve a mobile slurry, and pour into the column.

Additional deionized water is to be added to the resin to allow more resin into the column in order to reach a resin height of 20 cm.

5. Once the resin has settled, open the release valve and drain the water into a beaker until the excess liquid level is at the level of the resin. This is to prevent the resin from drying up.

6. Wash the resin with a buffer solution in 25 cm³ portions. Add the first 5 cm³ using a pipette by running the buffer solution down the side of the column to avoid disturbing the resin layer, and carefully pour the remaining buffer solution down the side of the column. If the resin gets disturbed, allow the resin to settle and gently tap the sides of the column in order to keep the top resin layer even.

7. Drain the buffer solution into the beaker until the liquid level reaches the top layer of the resin. Continue washing the resin with two to three 25cm³ buffer portions in order to prepare the column for the loading of the sample.

8. Load the 5 cm³ Lucentis-like sample by running it gently down the side of the column with a pipette.

9. Elute the sample by draining the liquid into a beaker until the liquid level reaches the top layer of the resin.

10. Add 5 cm³ of the buffer solution by running it down the side of column with a pipette. Elute the buffer until the liquid level reaches the top layer of the resin. This is to ensure that the loaded sample has traveled down the resin before adding a large portion of

buffer solution.

11. Add a 50 cm³ portion of buffer solution by running down the first 5 cm³ down the side of the column with a pipette, and pouring the rest down the side of the column. Be careful to not disturb the top layer of the resin.

12. Line up a rack of 10 test tubes and elute 5cm³ samples into the test tubes, until all the buffer has been eluted down the column.

13. These samples are then tested by electrophoresis to determine whether the refined Lucentis-like sample had been successfully eluted into some of the test tubes.

Appendix C

Governors Cup Entry

University of Nevada, Reno

**ChromaClean Industries
Business Plan**

By:

Arielle Salmon

Advisor: Dr. Alan Fuchs

Abstract

A business plan for a proposed company specializing in gas chromatographs designed to lower pollution in the air is presented. Before deciding on the final topic of gas chromatography multiple chemical engineering concepts and ideas were considered for their practicality in entrepreneurship. The proposed business of ChromaClean Industries is defined, with detailed information regarding organizational structure of management, an in depth analysis of the market, potential products to be offered, services to be provided, a comprehensive listing of necessary start-up funding, and projections of prospective financial data. Through this thorough report, ChromaClean Industries hopes to receive the necessary funding for start-up.

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Introduction:

Nevada's collegiate students have a unique opportunity to test their entrepreneurial skills through the Governor's Cup. Through the Governor's cup, students are challenged to develop business plans and are able to publically share them with entrepreneurial networks and investors where they have to possibility of one day becoming a reality. Engineering and entrepreneurship go hand in hand, with a plethora of potential business ideas involving engineering design and concepts. When given the funding and resources necessary to propose a business idea and possibly begin a business, there are endless possibilities for engineers who are interested in entrepreneurship. Through this competition, students incorporate their acquired skill set and imagination to go above and beyond what has been asked of them.

The competition is judged based on the business plan proposed by each team. Only those chosen to be semi-finalists will have the opportunity to present their business proposals to the judges. Almost \$400,000 in cash prizes is awarded to the top competitors to promote the start-up of their business. The proposed business discussed in this business proposal, entitled ChromaClean Industries, seeks a cleaner future and hopes to achieve that by providing a machine to lower the amount of carbon dioxide in polluted air. Through an extremely thorough and competent description of the business, its goals, and its plans to achieve those goals, ChromaClean Industries hopes to receive the necessary funding for start-up. The mentor for the proposed business ChromaClean Industries, Dr. Alan Fuchs, a professor in chemical and materials engineering, helped to guide the team through this process.

Executive Summary

Carbon dioxide is the main pollutant in the air that is causing global warming. Carbon dioxide levels in the air are higher now than they've ever been before and pollution in the air is only getting worse over time. It's become more and more essential to combat carbon dioxide levels in the air in order to prevent detrimental damage to the atmosphere.

Gas chromatography, a separation technique that is commonly used to purify a particular substance, can be used to clean polluted air by removing the carbon dioxide from it. By commercializing and popularizing a carbon dioxide gas chromatograph that can be mounted in high-polluting areas, the levels of carbon dioxide in the air will be greatly diminished, slowing down the effects of global warming.

Carbon dioxide is predominantly released by cars, airplanes, factories and power plants, basically anything that incorporates the burning of fossil fuels and natural gas. ChromaClean Industries' target buyers will be big name companies with un-popularly large carbon footprints, such as those with large polluting factories and power plants, as well as government organizations.

ChromaClean Industries will develop an open-floor organizational structure for its first few years in business keeping everything very intertwined while the business is getting started. ChromaClean Industries will be completely directed by its founder, who will then delegate specific branches of the business to several managers with teams of their own. Further expansion will result in multiple locations and possibly dozens of additional employees.

There are a few scale-up risks associated with ChromaClean Industries as it becomes a larger business. In order to combat these risks and deal with them in the early stages, ChromaClean Industries is requesting an initial investment of \$400,000. This investment will supply the required funds and resources for construction of preliminary chromatographs. This investment will allow ChromaClean Industries to become a top carbon dioxide combater in the nation, supplying hundreds of companies with carbon dioxide gas chromatography and reducing the carbon footprints of the most polluting businesses in the country.

Company Description

ChromaClean Industries seeks a world with clean air, air with little to no pollution. Air that is healthy to breath and enjoyable to live in; air that doesn't hurt the environment or the living things in it. ChromaClean Industries is taking action against pollution by combatting it directly. By reaching out to huge polluters, such as factories and power plants, ChromaClean Industries hopes to clean the air one liter at a time in order to lessen the effect of these businesses on the environment and to make them more EPA friendly.

ChromaClean Industries products will be relatively affordable for the businesses they are appealing to. The products produced by the company will reduce each respective business's carbon footprint both making the business look better in the eyes of the population and the EPA all while lessening their direct effect on the environment. One ChromaClean air chromatograph will have the ability to remove 60 percent of carbon

dioxide per liter of air, while filtering up to 25 liters per hour, 600 liters per day, drastically reducing the amount of carbon dioxide in the air around high-polluting facilities.

Market Analysis

As of March 2017, no gas chromatographs with the aim to clean polluted air are being mass-produced and sold for an affordable price for large companies leaving behind an indiscernible carbon footprint. This would give ChromaClean a huge market advantage due to the lack of competitors in the industry. ChromaClean will quickly gain traction in the community by raising awareness of the environment and protecting it from further harm by large factories and power plants.

The target market for ChromaClean will be high-polluting facilities such as large businesses with power plants and factories as well as government agencies that seek to reduce the amount of pollution in the air. In order to comply with the Clean Air Act (CAA), companies need to regulate their air emissions in order to attempt to achieve National Ambient Air Quality Standards in place to protect the health and welfare of the public. ChromaClean chromatographs can be used to ensure these standards are achieved.

Gas chromatographs can range in price anywhere from a couple hundred dollars to a couple thousand dollars depending on the size and flow rate. ChromaClean's products will range from \$2,000 to \$5,000. The smallest chromatograph, with the slowest flow rate, will be \$2,000 while the largest will be \$5,000. These prices are fairly low and completely reasonable for the multi-million dollar companies they are targeted for.

Organization & Management

ChromaClean Industries will follow an open floor structure of organizations, maintaining a very intertwined structure during its first few years. The founder will take on the role of Chief Executive and will appoint and oversee the five main branches: Chief of Operations, Chief of Engineering, Chief of Marketing, Chief of Financials, and Chief of Logistics. The owner, Arielle Salmon, will appoint the heads of each of the above branches of organization and will oversee all executive decisions.

The Chief Executive will be the head of the executive branch and will be responsible for determining the company's goals and future endeavors. A small group of people will be a part of the Chief Executive's team and will assist with implementation and execution of established goals and endeavors.

The Chief of Operations will be responsible for the production of the chromatographs on a daily basis. The Chief of Operations will have a relatively large team in order to ensure the production process is operating at peak efficiency at all times.

The Chief of Engineering will be responsible for overseeing the research and development of the process and will focus on improvements to streamline the production line. The engineering team will also assist with scale-up of production. During the early years of the business, the engineering team will be relatively large compared to other teams in order to achieve a cost efficient and highly effective process. After this has been accomplished the engineering team will be downsized.

The Chief of Marketing will oversee all product advertising and customer service

associated with sales. The marketing branch will be composed of two main teams, each of which will be run directly by a manager appointed by the Chief of Marketing. The two main teams will be sales and advertising. The sales team will deal with direct sales and any customer inquiries that may be presented. The advertising team will work with promoting the products and popularizing ChromaClean Industries' name.

The Chief of Financials will be responsible for securing future business partners and obtaining grants for research. This team will aim to improve the profitability of ChromaClean in all aspects. The team itself will be composed of only a few people who are well interconnected throughout the entire business.

The Chief of Logistics will be responsible for determining which branch, if any, needs assistance in any area and will help in determining a strategic plan for improvement of that branch. The team will consist of a few highly skilled and innovative individuals who will be responsible for implementing and overseeing these changes. Overall, this team will ensure that the company is running at high efficiency in all aspects.

Gas Chromatography Mechanism and Design

Gas chromatography is a separation technique that is commonly used to purify a particular substance. In terms of ChromaClean Industries, it can be used to clean polluted air by removing the excess carbon dioxide. Gas chromatography uses a gas mobile phase, instead of a solid phase used in liquid chromatography, to separate the desired components. In typical laboratory chromatographs, the mixture in question enters the

chromatograph manually through a syringe where it is heated and instantly vaporized. An eluant is then added to assist with the movement of the gases through the column. As the mixture moves along the length of the column it is separated into its components through adsorption.

ChromaClean's chromatographs will use hydrogen gas as an eluant. Each machine will obtain hydrogen gas directly from the air using a dehumidifier. Figure 1 shows the process by which a dehumidifier is able to obtain water directly from air.

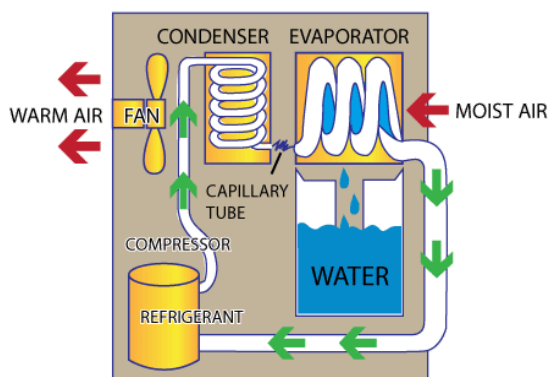


Figure C1: Dehumidifier: Process by which water will be collected from the air. A dehumidifier cools air causing it to condensate. The condensate is then collected in a pool which will be transported via pipe to the electrolysis chamber.

A feedback control system will be employed to ensure the water chamber will not be over or under filled with water at any point. The water obtained will be collected and transferred via pipe to the electrolysis chamber, where it will be broken down into hydrogen and oxygen gas. The oxygen gas will be released into the atmosphere while the hydrogen gas will continue on to the chromatography column where it will assist with the movement of the polluted air through the column. Figure 2 outlines the process occurring

within the electrolysis chamber.

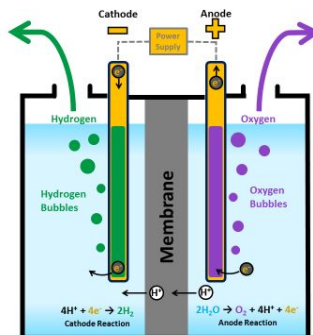


Figure C2: Outline of process occurring in the electrolysis chamber. Using an electric current, water can be broken up into its two elements, hydrogen and oxygen. Hydrogen continues on to the gas chromatograph column where it will be used as the eluent and oxygen is released back into the atmosphere.

Using the above mechanism to obtain the eluent will allow ChromaClean's chromatographs to be sold for a much lower price as compared to competitors as it will eliminate the need to purchase additional eluent and for the manual replacement of it in the machine.

The addition of the dehumidifier and electrolysis in order to obtain the hydrogen eluent directly from the air as well as the following design modifications will allow ChromaClean's chromatographs to function automatically and independently of operator assistance. The first modification will involve the incorporation of three additional pumps used to pump polluted air into the chromatograph and clean air out. Pump size will vary for each type of chromatograph offered, depending on the desired flow rate for each product. In addition to the pumps, there will also be a waste compartment to collect the separated carbon dioxide once the air has passed through the column. The size of the waste compartment will also vary depending on the size of the chromatograph. The

chromatographs will also be constructed out of weather-resistant material, as they will be mounted outside. Figure 1 shows a preliminary P&ID of a standard ChromaClean chromatograph machine.

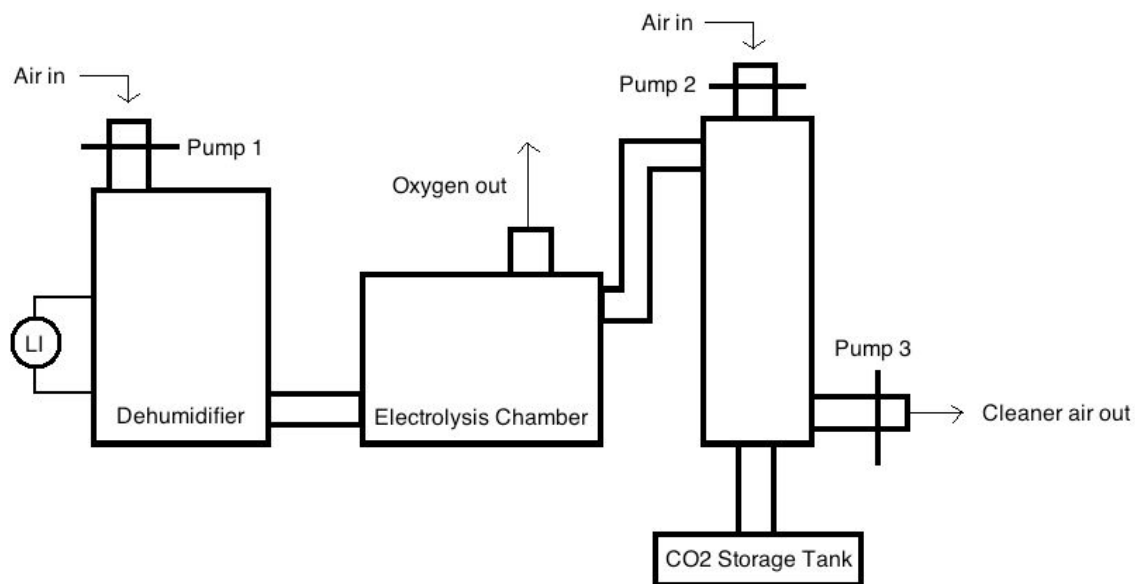


Figure C3: Piping and Instrumentation diagram of ChromaClean's product: includes dehumidifier, electrolysis chamber, gas chromatograph column, and carbon dioxide chamber.

Product Line and Services

Initially, ChromaClean Industries will offer chromatographs with three different flow rates: 5 liter per hour, 10 liters per hour, and 25 liters per hour. The flow rate will have no effect on the amount of carbon dioxide filtered from the air; for each machine roughly 60 percent of the carbon dioxide in the air will be removed from the air and kept in the waste compartment. The small machine, that runs at 5 liters per hour, will sell for approximately \$2,000, the medium machine, 10 liters per hour, will sell for approximately \$3,000, and the large machine, 25 liters per hour, will sell for

approximately \$5,000. As ChromaClean Industries expands larger and smaller machines will be added to the product line in order to appeal to a larger client base.

As ChromaClean Industries expands, research and development on improving upon each machine and of introducing new machines will take place. ChromaClean aspires to supply a complete line of chromatographs that can be used for any purpose. The initial products, as mentioned throughout this proposal, will be aimed toward large business. ChromaClean aspires to one-day supply personal chromatographs for household use. These chromatographs will likely be much smaller than the industrial ones, and hopefully much less expensive. The expansion of the product line will be conducted by a team within the engineering branch. The Chief of Engineering will appoint a manager to directly oversee this team.

The purchase of a ChromaClean product includes a lifetime warranty with each machine as well as a lifelong check-up service provided free of charge. This will include a checkup every six months beginning immediately after purchase of a ChromaClean product. The main goal of this check-up is to ensure the machine is running correctly and at peak efficiency. This check-up will also include a detailed report of the effectiveness of the machine. Any parts working incorrectly or under peak efficiency will also be replaced at this time. During the check-up, the waste compartment containing the filtered carbon dioxide will be emptied and cleaned, the carbon dioxide collected will be safely disposed of, and the refrigerant in the dehumidifier will be replaced. All other components of the machine will also be cleaned at this time. The check-up service will be conducted by a small team within the logistics branch of organization and will be

overseen by a sub-manager appointed by the Chief of Logistics. Each check-up will be conducted by no more than two employees at a time.

Marketing & Sales

Marketing and sales will be conducted by the marketing branch and will be overseen by the Chief of Marketing. The marketing branch will be composed of two main, rather large, teams: advertising and sales. The advertising team will promote the products, which will be predominantly done by direct communication with viable clients in the company's first few years after start-up. Once a large client base has been established, and the company has a solid turnover rate, advertising will expand to include billboards and radio advertisements. Billboards will be placed in highly populated industrial areas in order to reach as many potential clients as possible.

The sales team will keep constant communication with clients, ensuring their needs are being met and that ChromaClean's products are working as advertised. The sales team will also be responsible for reaching out to new potential clients, via phone or personal visit. A sub team within sales will be responsible for all customer service. The entire sales team will be competent in every aspect of ChromaClean Industries and will be able to answer any and all questions that may be thrown their way. The sales team provides the main communication between ChromaClean Industries and its clients, and for this reason the sales team will be polite, knowledgeable, calm, and eager to help in order to maintain a positive relationship with clients.

Four Year Cash Flow Analysis

Year One

ChromaClean Industries will require an initial investment of \$400,000 in order to purchase all necessary resources, hire all necessary employees, and to establish itself as powerful business. This diagram details the cash transactions over ChromaClean Industries' first 12 months. A substantial amount of the requested investment will be used to obtain the materials needed to construct the the gas chromatograph machines. This includes materials for the gas chromatograph machines themselves as well as machines to streamline the construction process. In addition to this, a large amount of the investment will be used to hire engineers and assistants. Engineers are needed to oversee the construction, to get the process up and running, and to ensure that everything is working smoothly and at peak efficiently at all times. Assistants will be required to operate the construction line and help with any additional tasks that may arise throughout the plant.

Year Two

The second year at ChromaClean Industries will see a huge expansion in research and development. Engineers will look to enhance the production rate and efficiency of construction of products, looking to cut back on production costs in order to increase revenue. By the second year, at least two new products will be introduced to the product line, increasing the potential client base. Although more money will be spent on materials, research, and development in this year, a larger income will be seen.

Year Three

By year three, ChromaClean Industries will be a top carbon dioxide combater in the nation. The company will also see huge growth in this year. Research and development will continue in this year, with the goal of introducing a household-sized chromatograph prototype. As ChromaClean Industries continues to collect more revenue in this year as compared to previous years, the cash flow will start to show the hard work the team has contributed to this project. The surplus in revenue will allow for the expansion of the company, including additional employees. At the end of year three, ChromaClean will begin to be profitable.

Year Four

The company will be the most profitable it has been in year four. ChromaClean Industries will already have established itself as a power player in the pollution combater industry. A large client base will have been established, and chromatography machine prices will be lowered in order to thank clients and to gain new clients while making the products more affordable. At the end of year four, ChromaClean Industries will be ready to go public. This will pave the road for future expansions including adding multiple locations across the country and, one day, around the world. ChromaClean will open the door for a cleaner future. As the company grows, research and development will grow and ChromaClean's products will only become better and more efficient. Larger flow rates will be achieved and a higher percentage of air will be cleansed.

Funding Request

ChromaClean Industries is seeking the maximum amount of funding offered for the Governor's Cup Competition. The team understand that this will not be nearly the amount requested for the initial investment. In order to gain the entire investment asked for, the company will be competing in numerous start-up competitions, as well as seeking additional sponsors and investors. ChromaClean Industries will also continue to compete in competitions throughout the first few years after start-up in order to obtain the necessary funds. The company will be looking mainly at business competitions, but will also compete in Green competitions and engineering competitions in order to maximize the possible amount of funds. ChromaClean Industries will find a way to grow, however an investment of any amount would only help the company achieve its goals faster.

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